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Fonctions et modes d'action des Bcl-x et Bax dans la réponse cellulaire aux agents anticancéreux

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L'apoptose est un processus de mort cellulaire contrôlé génétiquement qui joue un rôle fondamental dans la maintenance de l'homéostasie cellulaire. Des dérégulations de l'apoptose sont impliquées dans la tumorigénèse et la progression des cellules tumorales. Dans le traitement des cancers, la plupart des drogues utilisées en chimiothérapie induisent la mort des cellules tumorales par apoptose. Par conséquent, les gènes impliqués dans le contrôle de l'apoptose peuvent déterminer la réponse et la sensibilité des cellules tumorales aux drogues utilisées en chimiothérapie. Un contrôle défectif de l'apoptose représente une nouvelle forme de résistance aux traitements.

Les gènes de la famille des *ced9/egl-1/bcl* jouent un rôle déterminant dans la phase d'engagement de l'apoptose. Parmi les produits de ces gènes, certains inhibent et d'autres stimulent l'apoptose et de nombreux travaux suggèrent que le niveau d'expression des protéines Ced-9/Egl-1/Bcl modulent la sensibilité des cellules tumorales aux agents cytotoxiques utilisés en chimiothérapie.

Dans le but d'étudier la fonction et le mode d'action de certains membres de la famille des Ced-9/Egl-1/Bcl, nous avons exprimé dans les cellules tumorales humaines Namalwa et U937, les protéines Bcl-xL et Bax-α ainsi que deux nouvelles isoformes, Bcl-xES et Bax-σ, que nous avons identifiées et clonées. Les cinétiques de fragmentation de l'ADN associée à l'apoptose, dans les cellules témoins et transfectées, montrent que Bcl-xL et Bcl-xES protègent les cellules face à l'apoptose induite par différentes drogues anticancéreuses telles que la camptothécine, l'étoposide, la vinblastine, le cisplatine et le taxol. En revanche, Bax-α ainsi que Bax-σ, sensibilisent les cellules à l'effet cytotoxique de certaines de ces drogues. La quantification des complexes de clivage induits par la camptothécine ainsi que les mesures du taux d'inhibition de la synthèse de l'ADN dans les cellules transfectées et les cellules témoins, indiquent que Bcl-xL et Bax-α n'interfèrent pas avec le mécanisme d'action primaire de la drogue.

Les mitochondries jouent un rôle central dans l'activation de l'apoptose. Suite à un dommage à l'ADN, la libération du cytochrome c par les mitochondries conduit à l'activation des caspases impliquées dans l'exécution du processus de mort cellulaire. L'utilisation d'un système acellulaire, la mesure des activités des caspases à l'aide de substrats fluorescents spécifiques ainsi que des analyses par buvardage Western, nous

ont permis de démontrer que Bcl-xL et Bcl-xES retardent la libération du cytochrome c et l'activation subséquente des caspases alors que Bax-α et Bax-σ exercent l'effet opposé.

Nous montrons également que l'expression de la protéine Bcl-xL dans les cellules Namalwa modifie la réponse cellulaire face aux dommages induits par la camptothécine d'une part, en inhibant l'activation du programme apoptotique et d'autre part en permettant la restauration d'un point-contrôle du cycle cellulaire à la transition G2/M. Des expériences de co-immunoprécipitations *in vivo* montrent que Bcl-xL interagit avec les complexes cdc2/cdk1-cycline A/B1 impliqués dans la progression S/G2 et la régulation de la transition G2/M du cycle cellulaire. De plus, la protéine recombinante Bcl-xL(ΔTM) inhibe de façon dose-dépendante l'activité kinase de cdc2/cdk1 *in vitro*. Des expériences de compétition *in vitro* avec des peptides correspondant à des séquences spécifiques de Bcl-xL, ont permis de déterminer un domaine d'interaction de Bcl-xL avec les complexes cdk1/cdc2-cycline A/B1, localisé dans la région flexible de Bcl-xL. Ces résultats suggèrent, qu'en plus de son activité anti-apoptotique au niveau des mitochondries, Bcl-xL participe à un point-contrôle du cycle cellulaire en réponse à un dommage à l'ADN.

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Ac-DEVD-CHO: acétyl-Asp-Glu-Val-Asp-aldéhyde
Ac-DEVD-AMC: acétyl-Asp-Glu-Val-Asp-7-amino-4-methylcoumarin
Ac-LEHD-AFC: acétyl-Leu-Glu-His-Asp-7-amino-4- trifluoromethylcoumarin
Ac-YVAD-CHO: acétyl-Tyr-Val-Ala-Asp-aldéhyde
ADN: acide désoxyribonucléique
ADNc: ADN complémentaire
ADP: adénosine di-phosphate
AIF: apoptosis inducing factor
ANT: adenine nucleotide translocator
Apaf: apoptotis protease activating factor
ARN: acide ribonucléique
ATP: adenosine tri-phosphate
bFGF: basic fibroblast growth factor
BH: Bcl-2 homology domain
CAD: caspase-activated DNase
CARD: caspase recruitment domain
Cdk: cyclin dependent kinase
Ced: cell death abnormal
Ci: curie
CisPT: cis-platinum(II) diammine dichloride
Cpan : caspase-activated nuclease
CPT: 20-S-camptothecin lactone
DABCYL-YVADAPV-EDANS: 4-(4-dimethyl-aminophenylazo)benzoyl-Tyr-Val-Asp-Ala-Pro-Val-5-((2-aminoethyl)amino)-naphthalene-1sulfonic acid
dATP: deoxy adenosine-tri-phosphate
DD: death domain
DED: death effector domain
DEVDase: Asp-Glu-Val-Asp-protease
DFF: DNA fragmentation factor
DISC: death inducing signaling complex
$\Delta\psi m$: mitochondrial inner transmembrane potential
DNA: deoxyribonucleic acid
DRD: death effector recruting domain
Egl: egg laying defective

FADD: Fas-associated death domain
FasL: Fas ligand
G_1: gap phase 1
G_2: gap phase 2
G_0: gap phase 0
G-CSF: granulocyte colony-stimulating factor
GM-CSF: granulocyte macrophage colony-stimulating factor
h: hour
HA-tag: hemagglutinin epitope tag sequences
ICAD: inhibitor of caspase-activated DNase
ICE: interleukin-1β-converting enzyme
IL-3: interleukine-3
kDa: kilodalton
kpb: kilo paire de bases
MORT-1: mediator of receptor-induced toxicity 1
MPT: mitochondrial permeability transition
MPTP: mitochondrial permeability transition pore
PARP: poly(ADP-ribose) polymérase
pb: paire de bases
PFD: pore forming domain
RT-PCR: reverse transcriptase-polymerase chain reaction
SIDA: syndrome d'immunodéficience acquise
SCID: severe combined immunodeficiency
Taxol: paclitaxel
Thr: thréonine
TNF: tumor necrosis factor
TNFR: tumor necrosis factor receptor
Top1: topoisomérase de type 1
Top2: topoisomérase de type 2
TPCK: N-tosyl -L-phenylalanyl chloromethyl ketone
TRADD: TNFR-associated death domain
TRAIL: TNF-related apoptosis-inducing ligand
Tyr: tyrosine
U: unit
VDAC: voltage dependent anion channel
VINB: vinblastine

VEGF: vascular endothelial growth factor

VP-16: etoposide

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CHAPITRE 1. INTRODUCTION

1.1 L'apoptose ou mort cellulaire programmée

L'apoptose, ou mort cellulaire programmée, est un processus de mort cellulaire physiologique qui permet à l'organisme d'éliminer les cellules non désirées ou endommagées et potentiellement dangereuses. Elle joue un rôle crucial dans le développement des organismes pluricellulaires et le maintien de l'homéostasie cellulaire qui résulte d'un équilibre contrôlé entre la prolifération et la mort cellulaire (1-4). L'apoptose est un processus contrôlé génétiquement qui est activé par des stimuli physiologiques normaux, intra ou extracellulaires mais également par des stimuli pathologiques qui portent atteinte au bon fonctionnement de la cellule tels que, par exemple, une infection virale, un choc thermique, un stress oxydatif ou des dommages induits à l'ADN. Des dérégulations de l'apoptose, qui aboutissent à une mort cellulaire excessive ou insuffisante, sont impliquées dans certaines pathologies humaines telles que les maladies neurodégénératives, le SIDA, les maladies autoimmunes ou encore le cancer (5-8).

1.1.1 Les caractéristiques de l'apoptose

La mort cellulaire par apoptose est caractérisée par des altérations morphologiques spécifiques. Parmi ces altérations on peut observer une réduction du volume cytoplasmique, la condensation de la chromatine au niveau de la membrane nucléaire, une perte d'asymétrie de la membrane plasmique et la fragmentation de l'ADN. La cellule entière finit par être démentelée sous forme de vésicules ou corps apoptotiques qui contiennent le cytoplasme, les organelles cellulaires ainsi que la chromatine fragmentée. Contrairement aux cellules nécrotiques qui déversent leur contenu dans le milieu extracellulaire, les cellules en apoptose sont rapidement reconnues et phagocytées par les cellules avoisinnantes sans susciter de réaction inflammatoire (1, 9-11). La fragmentation de l'ADN, qui résulte de l'activation d'endonucléases spécifiques, semble se faire en deux étapes. Dans un premier temps, l'ADN est clivé en fragments de haut poids moléculaire d'environ 300 et/ou 50 kpb (12-15). Cette digestion est suivie d'une fragmentation internucléosomique qui génère des fragments mono- et oligonucléosomiques qui peuvent être visualisés sous la forme caractéristique d'échelle après électrophorèse en gel d'agarose (16). Dans certains modèles de cellules en apoptose, la fragmentation internucléosomique de l'ADN n'est

pas observée (17, 18). L'apoptose est également caractérisée par des altérations biochimiques majeures. Parmi ces altérations on peut observer une perte des fonctions mitochondriales, la libération de facteurs pro-apoptotiques mitochondriaux tels que le cytochrome c ou l'AIF *(apoptosis inducing factor)* et enfin, l'activation des caspases et la protéolyse de substrats cellulaires spécifiques (19, 20).

1.1.2 La mort cellulaire programmée est un processus conservé au cours de l'évolution

Des études génétiques réalisées chez le nématode *Caenorhabditis elegans* ont permis d'identifier quatre gènes centraux impliqués dans la régulation de l'apoptose au cours du développement du nématode. Parmi ces gènes, *ced-4* et *ced-3 (cell death abnormal)* sont requis pour l'initiation et l'exécution de l'apoptose. Le gène *ced-9* agit en amont des gènes *ced-4* et *ced-3* et inhibe leurs activités pro-apoptotiques. Récemment, le gène *egl-1 (egg-laying defective)* a été identifié. *Egl-1* agit en amont des gènes *ced-9*, *ced-4* et *ced-3* et régule négativement l'activité de *ced-9* (21-25). Des analyses fonctionelles et des études d'interaction des produits de ces gènes ont permis de définir un modèle d'activation de l'apoptose chez *C. elegans*. Dans ce modèle, la protéine Ced-3 est la protéine effectrice de l'apoptose. La protéine Ced-4 interagit avec Ced-3 et permet l'activation de Ced-3 et l'initiation de l'apoptose. L'activation de Ced-3 par Ced-4 est inhibée par la protéine Ced-9 qui séquestre Ced-4 et/ou le complexe Ced-4/Ced-3 au niveau des membranes intracellulaires. Suite à un stimulus de mort cellulaire, la protéine Egl-1 interagit avec Ced-9, permettant ainsi l'activation de la protéase Ced-3 par Ced-4 dans le cytoplasme (25-30) (Figure 1).

Les homologues des gènes *ced-3*, *ced-4*, *ced-9* et *egl-1* ont été identifiés chez les mammifères. La protéine Ced-3 appartient à la famille des protéases à cystéine, ou caspases, dont on connaît actuellement 14 membres. Ced-9 et Egl-1 présentent des homologies avec le proto-oncongène Bcl-2 qui est le premier membre identifié d'une grande famille de protéines régulatrices de l'apoptose et enfin, Ced-4 est l'homologue de Apaf-1 *(apoptosis activating factor)*, Flash et Nod-1/Card-4, identifiés récemment (24, 31-35). Des études récentes ont révélé que ces familles de protéines identifiées chez les mammifères, présentent également des homologies fonctionelles avec les protéines régulatrices de l'apoptose du nématode, et suggèrent que certains des mécanismes fondamentaux de la mort cellulaire programmée sont conservés au cours de l'évolution. En effet, dans les cellules de mammifères, les Ced-3/ICE/caspases sont

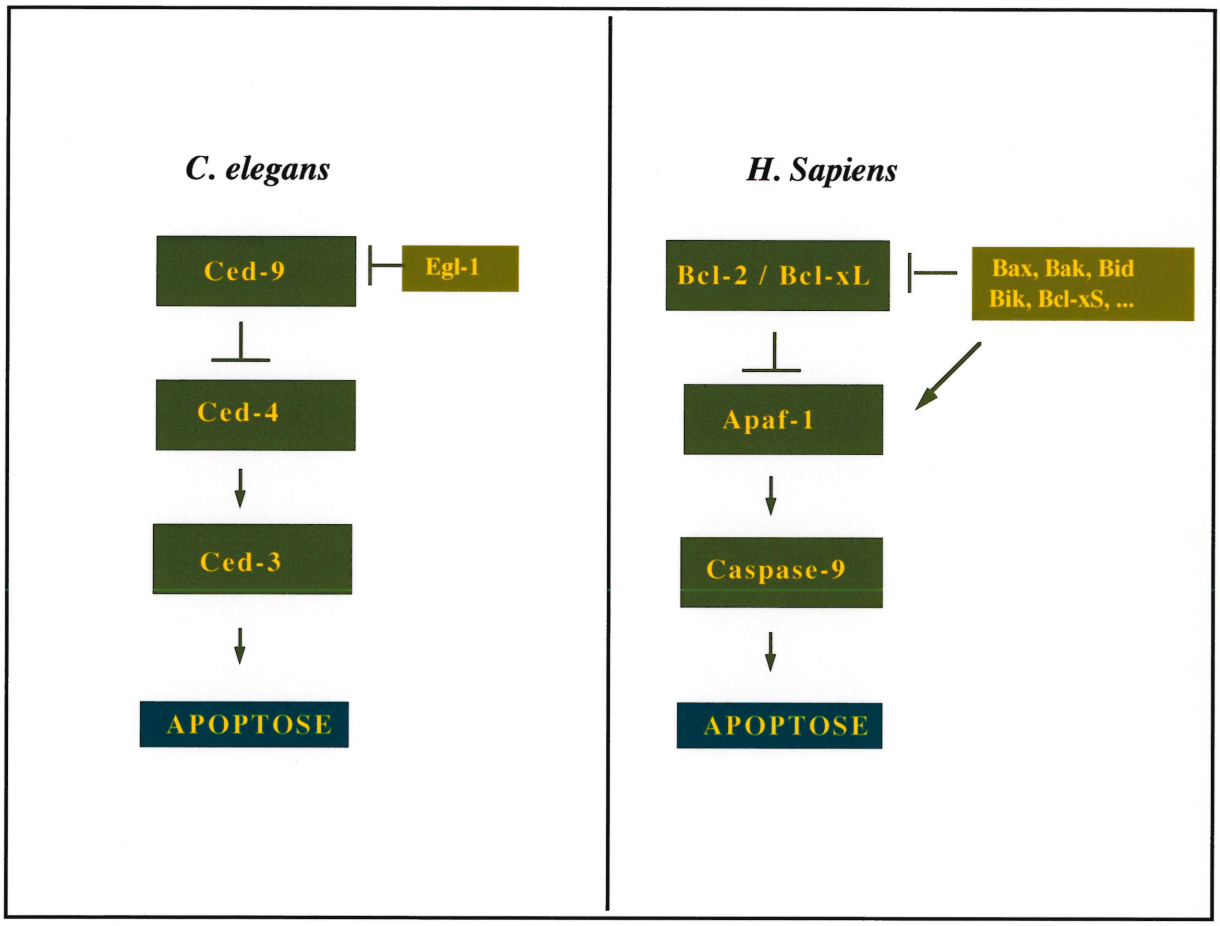

Figure 1: Les mécanismes d'activation de l'apoptose sont conservés au cours de l'évolution

les protéines effectrices de l'apoptose alors que les Ced-9/Egl-1/Bcl agissent en amont et régulent l'activation des caspases. Apaf-1 et Nod-1/Card-4, homologues humains de Ced-4, interagissent avec le précurseur de la caspases-9 et l'activent. L'activation de la caspase-9 par Apaf-1 est régulée négativement ou positivement par les différents membres de la famille des Ced-9/Egl-1/Bcl (26, 36-40) (Figure 1). Si le schéma général d'activation de l'apoptose est conservé entre nématodes et mammifères, la multiplicité des gènes impliqués dans l'apoptose chez les mammifères augmente le niveau de complexité des mécanismes de régulation de la mort cellulaire programmée chez les organismes supérieurs.

1.2 Les protéines de la famille des Ced-9/Egl-1/Bcl

Le gène *bcl-2 (B-cell lymphoma/leukemia-2 gene)* a été identifié en 1985 au niveau de la translocation chromosomique t(14;18) impliquée dans un grand nombre de lymphomes B humains (41). En 1988, l'équipe de D.L. Vaux montre que la surexpression du gène *bcl-2* protège les cellules hématopoiétiques de la mort induite par la suppression de l'interleukine-3 (IL-3) (42). Peu de temps après, en 1990, D.M. Hockenbery et ses collaborateurs démontrent l'implication de Bcl-2 dans la mort cellulaire programmée (43). L'activité anti-apoptotique de Bcl-2 est alors démontrée par plusieurs groupes (44-47) mais ce n'est qu'en 1994, que M.O. Hengartner et H.R. Horvitz mettent en évidence l'homologie de séquence, de structure et de fonction entre les protéines Bcl-2 et Ced-9 (31).

Aujourd'hui plus de 18 membres de la famille des Ced-9/Egl-1/Bcl ont été identifiés chez les mammifères. Certains exercent une fonction anti-apoptotique analogue à Ced-9 mais d'autres ont une activité pro-apoptotique. Ces protéines présentent toutes des homologies avec Bcl-2 au niveau de régions conservées appelées domaines BH *(Bcl-2 Homology domain)*. Les protéines anti-apoptotiques Bcl-xL (48), Bcl-w (49), Mcl-1(50), A-1/Bfl-1(51, 52), et Diva/Boo (53, 54) présentent le plus d'homologies avec Bcl-2 et une combinaison variable de domaines BH. Actuellement la fonction anti-apoptotique de la protéine Diva/Boo est encore incertaine (53, 54). Les protéines pro-apoptotiques sont divisées en deux sous-groupes. Le premier groupe comprend Bax (55), Bcl-xS (48), Bak (56-58) et Bok/Mtd (59, 60) qui possèdent au-moins deux domaines BH. Le second groupe comprend les protéines Bad (61), Bik/Nbk/mBlk (62-64), Bid (65), Hrk/Dp5 (66, 67), Bim/Bod (68, 69), Nip1, Nip3 (70) et les homologues Bnip3L/Bnip3α/Nix (71-73). Ces protéines possèdent un

domaine d'homologie unique, le domaine BH3 et sont les homologues fonctionnels de Egl-1 (Figure 2).

En plus de la diversité des gènes de la famille des *ced-9/egl-1/bcl*, un même gène peut générer plusieurs isoformes de fonction et de localisation cellulaire variées qui peuvent être exprimées de façon tissu-spécifique. Un épissage alternatif du gène *bcl-2* génère les isoformes Bcl-2α et Bcl-2β qui ont respectivement une localisation membranaire et cytosolique (74). Le gène *bcl-x* code pour les isoformes Bcl-xL *(long)*, Bcl-xS *(short)* et Bcl-xβ. Bcl-xL et Bcl-xS exercent une fonction opposée. Une autre isoforme appellée Bcl-xγ a été identifiée chez la souris et semble être exprimée spécifiquement dans les cellules T (48, 75, 76). Six isoformes de Bax (Bax-α Bax-β, Bax-γ, Bax-δ, Bax-ω et Bax-ε), trois isoformes de Bim/Bod (BimL, BimEL et BimS) ainsi que quatre isoformes de BNip1 (BNip1, Bnip1a, Bnip1b et Bnip1c) ont également été décrites (55, 77-81). Au laboratoire nous avons identifié une nouvelle isoforme de Bax appelée Bax-σ ainsi qu'une nouvelle isoforme de Bcl-x appelée Bcl-xES *(extra short)*. L'existence de plusieurs isoformes de caspases et de Apaf-1 a également été rapportée, suggérant que l'épissage différentiel des gènes joue un rôle important dans la régulation de l'apoptose (82-84)

1.2.1 Localisation cellulaire

La majorité des membres de la famille des Ced-9/Egl-1/Bcl possèdent une extrémité C-terminale hydrophobe qui leur confère une localisation membranaire. Bcl-2 et Bcl-xL sont essentiellement localisés au niveau de la membrane mitochondriale, la membrane nucléaire et au niveau du réticulum endoplasmique alors que Bax-α a surtout une localisation cytosolique (85-87). Des expériences de délétion des domaines C-terminaux de Bax-α et Bcl-2 suggèrent que la localisation de ces protéines au niveau de sites stratégiques dans la cellule contribue à leur fonction (88, 89). Des études récentes mettent en évidence une redistribution de la protéine Bax-α du cytoplasme vers les mitochondries suite à un stimulus de mort cellulaire. L'insertion de Bax-α dans la membrane mitochondriale est nécessaire à son activité pro-apoptotique et entraîne la libération du cytochrome c et l'activation des caspases (87, 89, 90). Cette translocation implique une homodimérisation et/ou des changements conformationnels de la protéine et semble être favorisée par un facteur cytoplasmique inhibé par Bcl-2 (91-93).

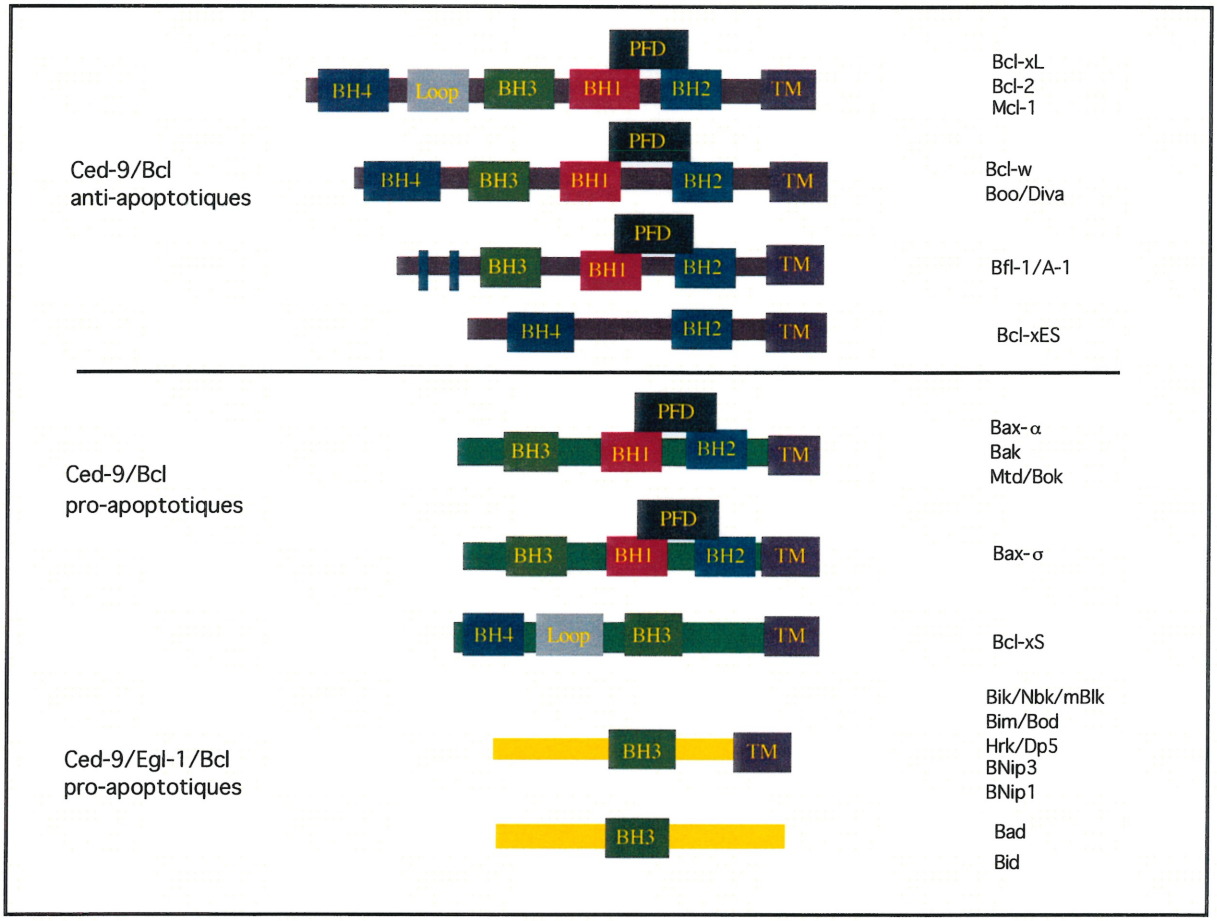

Figure 2: Les protéines de la famille des Ced-9/Egl-1/Bcl

L'activation de la protéine pro-apoptotique Bid au niveau du récepteur de mort cellulaire CD95/Apo-1/Fas entraîne également une redistribution de Bid du cytoplasme vers les mitochondries où elle exerce sa fonction (39, 40). La protéine Bad exerce une activité pro-apoptotique en interagissant avec Bcl-xL au niveau de la mitochondrie. En réponse à l'IL-3, des signaux de survie sont transmis dans la cellule. Ces signaux entraînent la phosphorylation de Bad et la translocation de la protéine de la mitochondrie vers le cytoplasme où elle est séquestrée par la protéine 14-3-3 (94). L'ensemble de ces données suggère que le déplacement des protéines Ced-9/Egl1/Bcl d'un compartiment cellulaire à un autre, joue un rôle essentiel dans leur fonction et permet la transduction de signaux de mort cellulaire du cytoplasme vers les sites d'initiation de l'apoptose tels que la mitochondrie.

1.2.2 La formation d'homo- et d'hétérodimères

Les membres de la famille des Ced-9/Egl-1/Bcl peuvent former des homodimères, ou des hétérodimères avec des partenaires spécifiques de fonction opposée (55, 95). Les domaines BH-1 et BH-2 des protéines anti-apoptotiques et le domaine BH-3 des protéines pro-apoptotiques sont impliqués dans la formation d'hétérodimères. D'après l'analyse de la structure de Bcl-xL et les homologies de séquences entre Bcl-xL et les autres membres de la famille, les domaines BH-1, -2 et -3 des Ced-9/Bcl anti-apoptotiques forment une région hydrophobe en forme de clé qui constitue le site d'interaction des Ced-9/Egl-1/Bcl pro-apoptotiques (96). Des expériences de mutagénèse dirigée au niveau des domaines BH-1 et BH-2 de Bcl-2 et Bcl-xL, ainsi que des expériences de délétion du domaine BH-3 de Bak ou Bad, montrent une corrélation entre l'activité anti- ou pro-apoptotique de ces protéines et leur capacité de former des hétérodimères (95, 97-99). Lorsque la protéine Bax-α est surexprimée, les cellules sont davantage sensibles à un stimulus de mort cellulaire. Quand les cellules surexpriment à la fois Bax-α et Bcl-2 ou Bcl-xL, l'effet pro-apoptotique de Bax-α est neutralisé par la formation d'hétérodimères Bcl-2-Bax-α ou Bcl-xL-Bax-α. La protéine Bad, quant à elle, favorise l'apoptose induite par Bax-α en interagissant avec Bcl-xL et en restaurant ainsi l'activité de Bax-α. Ces résultats ont suggéré un modèle selon lequel le niveau d'expression des protéines Ced-9/Egl-1/Bcl pro-apoptotiques par rapport aux protéines Ced-9/Bcl anti-apoptotiques détermine la sensibilité des cellules à un stimulus de mort cellulaire (55, 61, 100). Bien que les interactions entre les protéines de la famille des Ced-9/Egl-1/Bcl jouent un rôle important dans leur fonction, d'autres études de mutations ou délétions des domaines

BH, ainsi que des études génétiques, montrent que Bcl-xL, Bcl-2, Bak et Bax-α, peuvent réguler l'apoptose de façon indépendante (101-104).

1.2.3 Protéines adaptatrices et protéines d'ancrage

Plusieurs interactions directes ou indirectes entre certaines protéines de la famille des Ced-9/Bcl et des protéines non homologues ont été mises en évidence, mais la signification biologique de ces interactions est souvent peu ou pas connue. L'interaction de Bcl-xL avec Apaf-1, l'équivalent humain de Ced-4, joue un rôle central dans la régulation de l'apoptose. Suite à un stimulus de mort cellulaire, la formation du complexe Apaf-1/procaspase-9 au niveau de la mitochondrie induit l'activation de la procaspase-9 et l'initiation de la cascade apoptotique. La protéine anti-apoptotique Bcl-xL peut interagir avec le complexe Apaf-1/procaspase-9. Cette interaction entraîne la séquestration du complexe au niveau de la membrane mitochondriale et inhibe l'activation de la procaspase-9 (28, 36-38).

D'autres études mettent en évidence une interaction entre Bcl-2 et la sérine/thréonine kinase Raf-1 ou les GTPases R-, H-, N-, et K-Ras. Ces interactions provoquent le recrutement de Raf-1 et des différentes formes de Ras au niveau de la mitochondrie (105-107). Bcl-2 interagit également avec la protéine Bag-1, la protéine phosphatase calcineurine, et p53-Bp2, une protéine de liaison à p53 (108). Au niveau du réticulum endoplasmique, Bcl-2 ainsi que Bcl-xL interagissent avec la protéine p28 Bap31 (109). Récemment, une interaction directe entre la protéine pro-apoptotique Bax-α et les protéines ANT *(adenine nucleotide translocator)* et VDAC *(voltage dependent anion channel)* qui composent les mégacanaux mitochondriaux a été montrée. Ces interactions semblent favoriser l'ouverture des mégacanaux et la libération du cytochrome c, un événement précoce dans l'initiation de l'apoptose (110-113). Au contraire, la liaison des protéines anti-apoptotiques Bcl-xL et Bcl-2 à VDAC semble favoriser la fermeture des mégacanaux et empêcher la libération du cytochrome c (112, 114).

1.2.4 Les canaux ioniques

L'analyse tridimensionelle de Bcl-xL révéla une analogie de structure avec les domaines de formation de canaux ioniques de certaines toxines bactériennes telles que la colicine A et E1 et la toxine diphthérique. Le domaine d'insertion dans les membranes de Bcl-xL est appelé domaine PFD *(pore forming domain)* et est

essentiellement constitué de deux hélices-α hydrophobes situées entre les domaines BH-1 et BH-2 de la protéine (96). Par la suite, une série d'études électrophysiologiques démontrèrent la capacité de Bcl-xL, Bcl-2, et Bax-α de former des canaux ioniques dans des membranes artificielles. Ces canaux sont sensibles au potentiel membranaire ainsi qu'au pH et démontrent des caractéristiques dynamiques différentes. Les canaux formés par les protéines anti-apoptotiques semblent plus perméables aux cations alors que ceux formés par les protéines pro-apoptotiques sont plus perméables aux anions (115-118). Bien que l'activité de canal de Bcl-2, Bcl-xL et Bax-α n'ait pas été démontrée *in vivo*, des études de mutation de leurs domaines PFD respectifs suggèrent qu'elle participe à leur fonction (119, 120). La localisation membranaire des protéines Bax-α, Bcl-2 et Bcl-xL et leur activité potentielle de canal, permettent d'envisager l'hypothèse que ces protéines contrôlent l'apoptose en régulant les efflux d'ions au niveau des membranes intracellulaires. Cette hypothèse est supportée par un certain nombre de données. Les protéines anti-apoptotiques Bcl-2 et Bcl-xL inhibent l'apoptose en stabilisant le potentiel transmembranaire des mitochondries ($\Delta\Psi m$), alors que la protéine pro-apoptotique Bax-α exerce l'effet opposé et favorise la chute de $\Delta\Psi m$ (121-123). D'autres études mettent également en évidence une corrélation entre l'inhibition de l'apoptose par Bcl-2 et la régulation des flux intracellulaires de calcium (124-126) ainsi que la redistribution cytosol:noyau du glutathione (127).

1.2.5 Régulation des Ced-9/Egl-1/Bcl

L'expression des membres de la famille des Ced-9/Egl-1/Bcl peut être régulée par des signaux de survie cellulaire, des stimuli de différentiation ainsi que par des stimuli de mort cellulaire. La régulation transcriptionelle de cette famille de gènes est cependant encore peu connue. La survie de la plupart des cellules dépend de la présence de cytokines ou facteurs de croissance dans le milieu extracellulaire. Ces cytokines régulent la prolifération et la différenciation cellulaire mais exercent également une fonction anti-apoptotique. La surexpression des protéines Bcl-2 ou Bcl-xL supprime la dépendance des cellules vis-à-vis de l'IL-3 alors que la surexpression de Bax-α exerce l'effet opposé (48, 55, 128). D'autre part, dans certains modèles cellulaires, la présence de cytokines telle que bFGF *(basic fibroblast growth factor)*, IGF-I *(insulin-like growth factor I)* ou VEGF *(vascular endothelial growth factor)* protège les cellules de l'apoptose induite par les radiations ionisantes et divers agents cytotoxiques, suggérant que les cytokines modulent l'expression ou l'activité de gènes

régulateurs de l'apoptose (129-131). Plusieurs cytokines induisent ou inhibent l'expression des *ced-9/bcl* mais les voies de signalisation liées à l'expression des *ced-9/bcl* sont peu connues. L'IL-3 stimule l'expression du gène *bcl-2* dans les cellules érythroleucémiques humaines TF-1 (132). VEGF augmente l'expression de *mcl-1* dans les cellules hématopoiétiques CMK86 (131) alors que l'estrogène induit l'expression de *bcl-2* dans les cellules de cancer du sein estrogène-dépendantes (133). Une étude récente montre que l'accumulation de neutrophiles dans certains désordres inflammatoires est associée à une diminution de l'expression du gène *bax* dans les cellules BAL et la survie prolongée des cellules dans un contexte d'inflammation. Les cytokines G-CSF *(granulocyte colony-stimulating factor)* et GM-CSF *(granulocyte macrophage colony-stimulating factor)* surexprimées par les cellules BAL semblent responsables de la régulation négative de *bax* (134). La transactivation du gène *bax* et la répression du gène *bcl-2* par le facteur de transcription p53 a été démontrée mais le rôle de p53 dans la régulation de ces gènes semble tissu-spécifique (135, 136). Bien que plusieurs sites potentiels de liaison de divers facteurs de transcription aient été identifiés dans la région 5' non transcrite du gène *bcl-x*, la régulation transcriptionelle du gène est peu connue (137). Des études très récentes démontrent que le gène *bcl-x* est transactivé directement par le facteur de transcription Stat5 en réponse à la stimulation du récepteur à l'érythropoiétine dans les cellules érythroides progénitrices (138, 139). Le facteur de transcription Stat5 induit également l'expression de Bcl-xL dans les cellules hématopoiétiques Ba/F3, en réponse à l'IL-3 (140). Une autre étude suggère que les gènes *bcl-2* et *bcl-x* sont transactivés par le facteur de transcription NFκB suite à la stimulation des récepteurs du TNF-α (*tumor necrosis factor α*). L'activation du gène *bfl-1/a-1* par NFκB a également été démontrée (141, 142).

L'activité de certaines protéines de la famille des Ced-9/Egl-1/Bcl est régulée par la phosphorylation de résidus spécifiques. En réponse à un signal de survie cellulaire, la protéine Bad est phosphorylée au niveau de deux résidus sérines par les protéines kinases AKT et PKA. Ces phosphorylations inhibent l'activité pro-apoptotique de Bad au niveau de la mitochondrie en favorisant sa séquestration dans le cytoplasme par la protéine 14-3-3 (143-145). La phosphorylation des protéines Bcl-2 et Bcl-xL en réponse à des drogues qui altèrent la structure des microtubules, telles que le taxol, la vinblastine ou la vincristine, a également été mise en évidence. Dans le cas de Bcl-2, cette modification post-traductionelle diminue en général son activité anti-apoptotique et est associé à l'accumulation des cellules en phase M du cycle cellulaire (146-149). Les protéines Bcl-2, Bcl-xL et Mcl-1 possèdent une région non conservée

flexible (LOOP), située entre les domaines BH-4 et BH-3. Cette région contient plusieurs sites potentiels de phosphorylation et sa structure ouverte constitue un site de reconnaissance potentiel pour les protéases. Bien que la fonction de ce domaine flexible ne soit pas encore déterminée, certaines études suggèrent qu'il régule négativement l'activité anti-apoptotique de Bcl-2 et Bcl-xL (148, 150).

1.3 Les protéines de la famille de Ced-4/Apaf-1

Les homologues de Ced-4 chez les mammifères constituent une famille de protéines adaptatrices se liant aux Ced-3/ICE/caspases, qui comprend Apaf-1, Card-4/Nod-1 et Flash (32-35). Ces protéines ont en commun un domaine d'homologie à Ced-4 contenant un motif Walker A et B permettant la liaison du dATP, à l'exception de la protéine Flash qui possède un motif Walker tronqué non-fonctionnel (Figure 3). Apaf-1 et Card-4/Nod-1 contiennent un domaine de liaison CARD *(caspase recrutment domain)* et des domaines répétitifs WD et LRR, respectivement, alors que Flash contient un domaine DRD *(death effector recruiting domain)*. Les protéines de la famille des Ced-4/Apaf-1 agissent au niveau des complexes d'initiation de l'apoptose et permettent le recrutement et l'activation des caspases initiatrices de la cascade protéolytique impliquée dans la phase d'exécution de l'apoptose, tel que mentionner dans la section 1.4.

1.4 Les protéines de la famille des Ced-3/ICE/caspases

A ce jour 14 caspases (cysteinyl aspartate-specific proteinase) ont été identifiées chez les mammifères. Les caspases peuvent être divisées en deux familles selon leur degré d'homologie avec ICE/caspase-1 *(interleukin-1 converting enzyme)* ou Ced-3/caspase-3 (Figure 4). La première famille qui comprend caspase-1, -4, -5, -11, -12, -13, et -14 semble principalement impliquée dans le processus de maturation des cytokines à l'exception de la caspase-12. Les caspases possédant une plus grande homologie avec Ced-3, c'est à dire caspase-2, -3, -6, -7, -8, -9, et -10, jouent un rôle central dans l'apoptose (20, 151). Les caspases sont des protéases à cystéine et possèdent une séquence QACxG conservée qui contient la cystéine catalytique. Elles sont synthétisées dans les cellules sous forme de précurseurs inactifs communément appelés procaspases. Les procaspases sont activées par un clivage protéolytique au niveau d'au moins deux résidus Asp conservés. Cette protéolyse libère la grande et la petite sous-unité de l'enzyme et permet la formation d'un hétérodimère catalytiquement

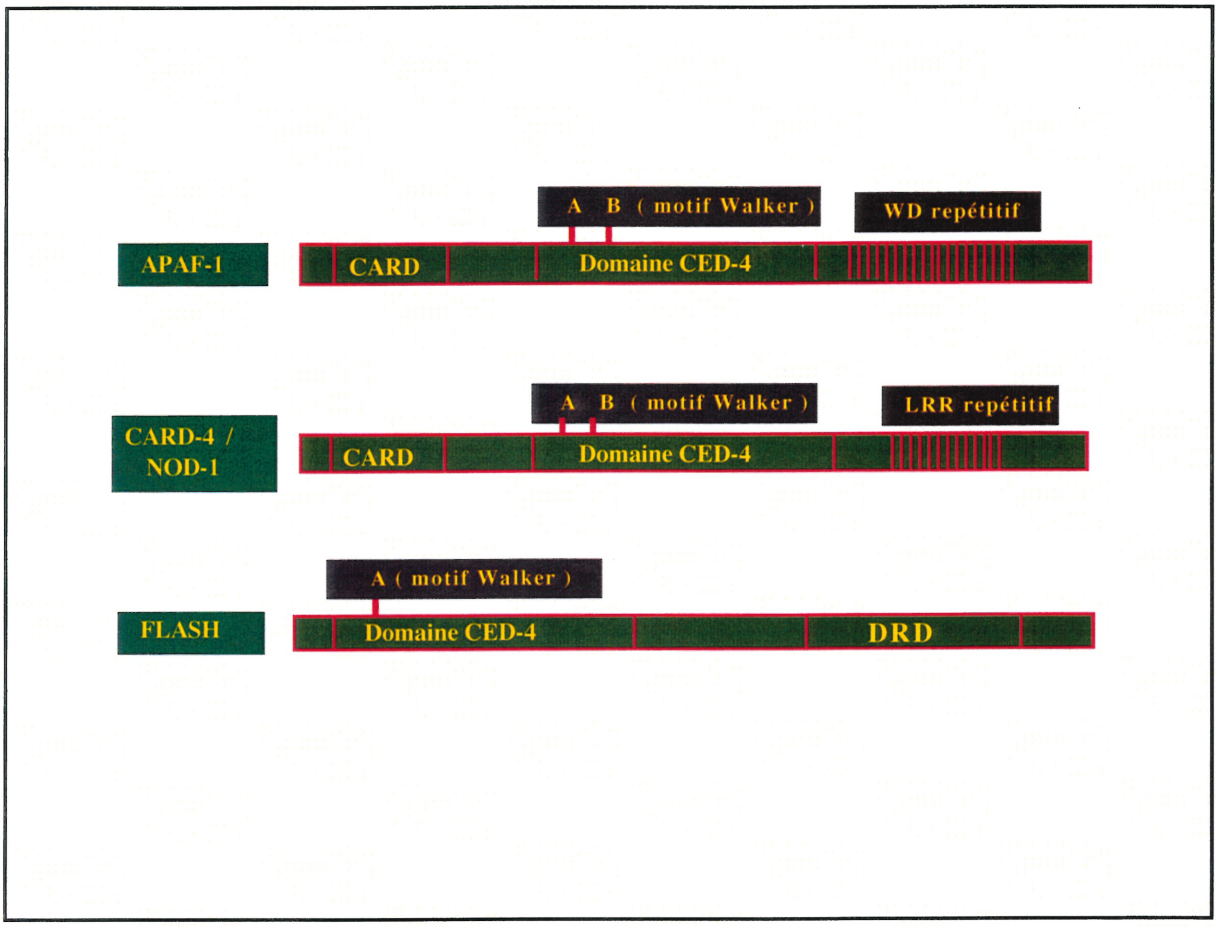

Figure 3: Les protéines de la famille des Ced-4/ Apaf-1

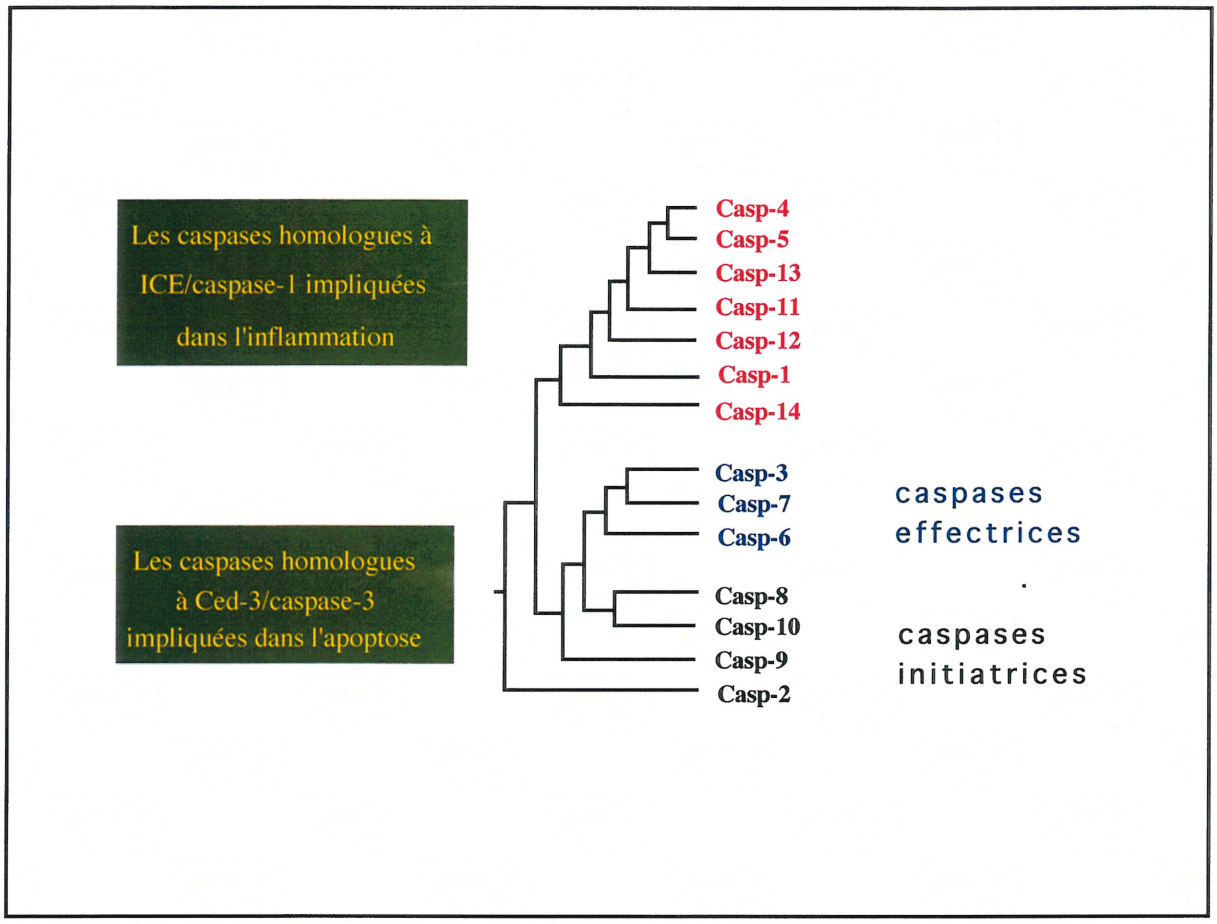

Figure 4: Les protéines de la famille des Ced-3/ICE/Caspases

actif (152, 153). Etant donné que les caspases clivent spécifiquement leurs substrats au niveau d'un résidu Asp, elles ont la capacité de s'autoactiver et/ou de s'activer mutuellement, ce qui aboutit à une activation protéolytique en cascade (154).

En plus de leurs sous-unités actives, les procaspases possèdent un prodomaine de longueur variable à leur extrémité NH2-terminale. Ces prodomaines permettent de diviser les caspases en deux sous-groupes: Les caspases initiatrices de l'apoptose (caspases-2, -8, -9, et -10) qui ont des prodomaines longs et les caspases effectrices (caspase-3, -6 et -7), qui ont des prodomaines courts. Les prodomaines des caspases initiatrices contiennent des séquences spécifiques d'interaction protéine-protéine telles que le domaine CARD *(caspase recrutment domain)* ou le domaine DED *(death effector domain)*. Ces séquences permettent aux procaspases d'être recrutées par des protéines adaptatrices au niveau des complexes d'initiation de l'apoptose. Le recrutement des procaspases initiatrices au niveau de sites spécifiques entraîne l'oligomérisation des proenzymes et leur activation par autocatalyse (155). Ainsi, l'activation du récepteur de mort cellulaire CD95/Apo-1/Fas entraîne le recrutement de la procaspase-8 par les protéines adaptatrices Fadd/Mort-1 ou Flash pour former le DISC *(death inducing signaling complex)*. L'interaction Fadd/Mort-1/procaspase-8, qui implique les domaines DED présents sur les deux protéines, permet l'activation de la procaspase-8 et l'initiation de la cascade protéolytique qui mène à l'apoptose (156-159). De façon similaire, la protéase Ced-3 de *C. elegans* et la procaspase-9, se lient respectivement aux protéines adaptatrices Ced-4 et Apaf-1 ou Card-4/Nod-1 par des interactions CARD-CARD. Ces interactions entraînent l'oligomérisation des protéases et leur activation en présence de cofacteurs (26, 36, 160, 161). La caspase-12 semble initier, par un mécanisme inconnu, la cascade protéolytique émergeant d'un stress au niveau du réticulum endoplasmique (162).

Une fois les caspases initiatrices activées au niveau des complexes d'initiation de l'apoptose, celles-ci clivent et activent les caspases effectrices dans une réaction en cascade qui amplifie le signal. Les caspases effectrices clivent à leur tour des substrats spécifiques dans la cellule, ce qui aboutit aux changements morphologiques caractéristiques de l'apoptose. Les substrats des caspases peuvent être inactivés par la protéolyse, c'est le cas, par exemple, de certaines protéines impliquées dans le maintien de l'intégrité du génome (PARP, ATM, DNA-PK), des protéines du cytosquelette (fodrin, actin, lamine), de l'inhibiteur de l'endonucléase DFF40/CAD (DFF45/ICAD), ou des protéines anti-apoptotiques Bcl-2 et Bcl-xL. D'autres protéines cellulaires telles que Bid ou la kinase MEKK1 peuvent être activées par les caspases et leur protéolyse contribue à amplifier les signaux de mort cellulaires (20). L'activation

des caspases à un niveau critique apparaît comme le point de non retour du processus apoptotique et le clivage protéolytique, par les caspases, de substrats cellulaires, est nécessaire à l'accomplissement de la phase ultime de l'apoptose.

1.5 Les mécanismes de régulation de l'apoptose

1.5.1 La mitochondrie

Les mitochondries jouent un rôle central dans l'activation de l'apoptose. L'ouverture des mégacanaux mitochondriaux dits *"mitochondrial permeability transition pores"*, la perte du potentiel transmembranaire mitochondrial ($\Delta\Psi m$) ainsi que la translocation de protéines apoptogéniques de l'espace intermembranaire mitochondrial vers le cytoplasme a été mis en évidence dans la plupart des cellules en apoptose. Ces changements au niveau des mitochondries sont des événements précoces qui induisent l'activation des caspases suite à un stimulus de mort cellulaire (19, 121). Parmi les facteurs apoptogéniques qui sont relâchés par les mitochondries on peut citer le cytochrome c, la flavoprotéine AIF *(apoptosis inducing factor)* ainsi que certaines procaspases telles que procaspase-9, -3 et -2 (163-166). Le relâchement de ces protéines ainsi que les autres altérations observées au niveau des mitochondries, sont régulés par les protéines de la famille des Ced-9/Egl-1/Bcl (167).

L'utilisation d'un système acellulaire a permis au groupe de X. Wang de déterminer trois facteurs nécessaires à l'activation de l'apoptose. Ces trois facteurs sont la protéine mitochondriale cytochrome c, Apaf-1, un homologue humain de Ced-4 et la caspase-9 (32, 36, 163). Dans un extrait cytosolique, en présence de dATP, Apaf-1, le cytochrome c et la procaspase-9 forment un complexe trimoléculaire, appelé apoptosome, qui induit l'activation de la procaspase-9. La liaison du cytochrome c à Apaf-1 permet l'oligomérisation de Apaf-1 et le recrutement de la procaspase-9 par des interactions entre les domaines CARD présents chez les deux protéines. L'agrégation de plusieurs molécules de procaspases-9 au niveau de l'apoptosome entraîne l'activation de la procaspase-9 par autocatalyse (161, 168-170). Une fois activée, la caspase-9 amplifie le signal de mort cellulaire en activant les caspases-3 et -7 qui à leur tour activent d'autres caspases en aval dans la cascade apoptotique. L'activation en cascade des caspases induite par le cytochrome c aboutit au clivage de substrats spécifiques et à la fragmentation de noyaux exogènes incubés dans l'extrait (36, 171). L'activation de l'apoptose par le complexe Apaf-1/caspase-9 a également été mise en évidence *in vivo* ainsi que dans les cellules en culture. Les souris déficientes du gène

apaf-1 ou *caspase-9* présentent des anomalies développementales majeures au niveau du système nerveux central, dues à une diminution de la mort cellulaire par apoptose au cours de la différentiation neuronale. Les thymocytes dérivés des souris *apaf-1*$^{-/-}$ ou *caspase-9*$^{-/-}$ démontrent une résistance à l'apoptose induite par l'étoposide et les radiations ionisantes (172-175). D'autre part, la surexpression de Apaf-1 dans les cellules promyélocytiques humaines HL-60, sensibilise les cellules à l'apoptose induite par le taxol et l'étoposide en favorisant l'activation des caspases-9 et -3 (176). Le cytochrome c est localisé dans l'espace intermembranaire de la mitochondrie et sa translocation dans le cytoplasme est en général nécessaire à l'activation des caspases et l'initiation de l'apoptose. La libération du cytochrome c, suite à un stimulus de mort cellulaire, est contrôlée par les protéines de la famille des Ced-9/Egl-1/Bcl. Les protéines qui exercent une activité anti-apoptotique, telles que Bcl-2 et Bcl-xL, inhibent la libération du cytochrome c et l'activation subséquente des caspases, alors que celles qui exercent une activité pro-apoptotique, telles que Bax-α, Bak ou Bid, favorisent sa libération (39, 40, 90, 111, 123, 177-180). La protéine anti-apoptotique Bcl-xL inhibe également l'activation de la procaspase-9 en interagissant avec le complexe cytochrome c/Apaf-1/procaspase-9 et cette interaction est antagonisée par les protéines pro-apoptotiques telles que Bax-α ou Bak (37, 38).

Le mécanisme par lequel le cytochrome c quitte l'espace intermembranaire de la mitochondrie après un stimulus de mort cellulaire est encore obscur. L'ouverture des mégacanaux mitochondriaux et la chute du potentiel transmembranaire ($\Delta\Psi m$) de la mitochondrie sont des événements souvent associés à la libération du cytochrome c et peuvent être régulées positivement ou négativement par les protéines de la famille des Ced-9/Egl-1/Bcl (90, 111, 123, 177, 178, 180, 181). La chronologie des altérations mitochondriales induites par un stimulus de mort cellulaire reste cependant controversée et la libération du cytochrome c peut précéder ou succéder la chute du potentiel transmembranaire de la mitochondrie, selon les systèmes utilisés (111, 177, 178, 181, 182).

Des études récentes suggèrent que certaines protéines de la famille des Ced-9/Bcl, telles que Bax-α, Bak, Bcl-2 ou Bcl-xL, régulent la libération du cytochrome c en modulant l'activité des mégacanaux mitochondriaux (110-114, 183). Les mégacanaux mitochondriaux sont localisés au niveau des sites d'interaction de la membrane interne et externe de la mitochondrie. Ce sont des complexes multiprotéiques dont les composants centraux sont la translocase de nucléotides adényliques (ANT) et le canal anionique voltage-dépendant (VDAC) qui sont

impliqués dans l'échange ADP/ATP entre la matrice mitochondriale et le cytoplasme (184). Suite à un stimulus de mort cellulaire, la transition de perméabilité, caractérisée par l'ouverture des mégacanaux mitochondriaux, induit la dissipation du potentiel transmembranaire mitochondrial ($\Delta\Psi m$) et la rupture de l'homéostasie ionique et osmotique de la mitochondrie. Ces événements entraînent le gonflement de la matrice mitochondriale, la rupture de la membrane externe et la libération du cytochrome c et d'autres protéines localisées dans l'espace intermembranaire mitochondrial (185-187). Des études récentes suggèrent que les protéines Bax-α et Bcl-2, favorisent ou inhibent respectivement la perte du potentiel transmembranaire mitochondrial et la libération du cytochrome c en interagissant avec la protéine ANT des mégacanaux mitochondriaux et en modulant son activité de canal (110, 183, 188). D'autres travaux publiés récemment suggèrent que certaines protéines de la famille des Ced-9/Bcl régulent la perméabilité des membranes mitochondriales et la libération du cytochrome c en interagissant avec la protéine VDAC des mégacanaux mitochondriaux. En effet, la reconstitution de l'activité canal de VDAC dans des liposomes a permis de montrer que les protéines pro-apoptotiques Bax-α et Bak favorisent l'ouverture de VDAC et permettent le passage du cytochrome c à travers le canal alors que les protéines Bcl-xL et Bcl-2 maintiennent le canal dans une conformation fermée, imperméable au cytochrome c (111-114). Une autre étude réalisée par le même groupe, montre que les protéines pro-apoptotiques Bid et Bik qui possèdent un domaine BH-3 unique, induisent la libération du cytochrome c indépendamment de la perméabilité de transition. Ces résultats permettent de mieux comprendre les données contradictoires concernant le rôle de $\Delta\Psi m$ dans l'initiation de l'apoptose. Selon le stimulus apoptotique que perçoit la cellule, différents membres pro-apoptotiques de la famille des Ced-9/Egl-1/Bcl transmettent le signal de mort cellulaire à la mitochondrie. Le relâchement du cytochrome c induit par l'interaction de Bax-α ou Bak avec la protéine VDAC est accompagné d'une perte du potentiel transmembranaire de la mitochondrie. En revanche, les protéines Bid et Bik induisent la libération du cytochrome c par un mécanisme alternatif indépendant de l'ouverture des mégacanaux mitochondriaux (113). La protéine pro-apoptotique Bax-α possède la capacité de former des canaux ioniques dans des membranes artificielles (117). De plus, certains travaux montrent que l'addition de la protéine recombinante Bax-α sur des mitochondries purifiées entraîne la libération du cytochrome c indépendamment de l'activité des mégacanaux mitochondriaux et sans induire une augmentation du volume mitochondrial (90, 189). Ces résultats suggèrent l'hypothèse supplémentaire que la protéine pro-apoptotique Bax-α forme des canaux dans la membrane mitochondriale, directement perméables au

cytochrome c (190). La formation de ces canaux pourrait également causer une instabilité de la membrane externe de la mitochondrie en diminuant la tension linéaire de la bi-couche phospholipidique, ce qui favoriserait le relâchement du cytochrome c (191). La capacité de Bax-α de former des canaux ioniques peut être inhibée par Bcl-2 (192).

1.5.2 Les récepteurs de mort cellulaire

La liaison de ligands spécifiques sur certains récepteurs exprimés à la surface cellulaire peut induire l'activation des caspases et la mort cellulaire par apoptose. Ces récepteurs, appelés récepteurs de mort cellulaire, constituent une famille de protéines qui comprend CD95/Apo-1/Fas, TNF-R1, DR3/Tramp/Wsl-1/Apo-3/Lard, DR4/Trail-R1/Apo-2, DR5/Trick2/Trail-R2 et DR6 (193). Les récepteurs de mort cellulaire sont caractérisés par la présence d'un domaine DD *(death domain)* dans leur région cytoplasmique qui permet le recrutement de protéines qui possèdent des motifs homologues. Les mécanismes de transduction des signaux de mort cellulaire par les récepteurs stimulés font l'objet de recherches intensives. A l'heure actuelle, la cascade d'activation de l'apoptose induite par le récepteur CD95/Apo-1/Fas est la mieux caractérisée. L'activation du récepteur CD95/Apo-1/Fas induite par le ligand FasL, entraîne l'agrégation du récepteur à la surface de la membrane cellulaire et la formation d'un complexe multiprotéique intracellulaire appelé DISC, qui transmet le signal à l'intérieur de la cellule. Le DISC comprend la protéine adaptatrice Fadd/Mort-1 ainsi que la procaspase-8. La protéine adaptatrice Fadd/Mort-1 est recrutée au niveau du récepteur par des interactions impliquant les domaines DD homologues présents sur les deux protéines. La protéine Fadd/Mort-1 possède un domaine DED *(death effector domain)* à son extrémité N-terminale et recrute à son tour la procaspases-8 par des interactions homologues DED-DED. Des études récentes ont montré que l'oligomérisation de plusieurs molécules de procaspases-8 au niveau de la protéine adaptatrice induit l'activation de la procaspase-8 par autocatalyse (159). L'activation de la caspase-8 au niveau du DISC initie la cascade d'activation des caspases responsables de l'exécution du programme de mort cellulaire (194). Récemment, deux types cellulaires ont été définis, concernant l'activation de l'apoptose induite par le récepteur CD95/Apo-1/Fas. Dans les cellules de type I, la quantité de caspase-8 activée au niveau du récepteur est suffisante pour promouvoir directement l'activation des caspases effectrices en aval (caspase-3, -7 et -6) et générer les changements morphologiques caractéristiques de l'apoptose. Par contre, dans les cellules de type II,

la caspase-8 activée au niveau du DISC engage la voie mitochondriale d'activation de l'apoptose qui amplifie le signal de mort cellulaire initié au niveau du récepteur (Figure 5). Dans les cellules de type II, l'apoptose induite par l'engagement du récepteur CD95/Apo-1/Fas est inhibée par la surexpression des protéines Bcl-xL ou Bcl-2 et l'activité anti-apoptotique que celles-ci exercent au niveau de la mitochondrie (195-198). Plusieurs études suggèrent que le signal de mort cellulaire initié au niveau du récepteur CD95/Apo-1/Fas est transmis aux mitochondries par les protéines de la famille des Ced-9/Egl-1/Bcl. Deux groupes indépendants ont montré que la protéine Bid, qui possède un domaine BH-3 unique, est un substrat de la caspase-8 dans l'apoptose induite par le récepteur CD95/Apo-1/Fas. Le clivage de Bid par la caspase-8 au niveau du DISC entraîne la translocation du fragment C-terminal de la protéine (tBid) vers la mitochondrie où tBid induit la libération du cytochrome c (39, 40). Une autre étude suggère un modèle d'amplification de la cascade apoptotique initiée par le récepteur CD95/Apo-1/Fas qui implique la protéine Bax-α. Dans ce modèle, l'activation du récepteur entraîne la redistribution de la protéine Bax-α du cytosol vers les mitochondries où Bax-α induit la libération du cytochrome c (199). Une relocalisation des protéines pro-apoptotiques Bax-α, Bad et Bcl-xS au niveau des mitochondries a également été observée dans les cellules leucémiques CEM traitées avec TNF-α (200).

1.6 Apoptose, cancer et chimiothérapie

1.6.1 L'apoptose et le cancer

La néoplasie résulte d'un déséquilibre entre la prolifération cellulaire et la mort cellulaire et des dérégulations des mécanismes d'activation de l'apoptose contribuent à la fois à la carcinogénèse et à la progression des cellules tumorales (7, 201-203). Plusieurs altérations de gènes impliqués dans la régulation de l'apoptose ont été décrites dans les cancers humains. Des mutations dans le gène suppresseur de tumeurs *p53* sont impliquées dans 50% des cancers humains. Le gène *p53* code pour un facteur impliqué dans le contrôle du cycle cellulaire, la réparation de l'ADN et l'apoptose (204, 205). Des altérations de certains gènes de la famille des *ced-9/bcl* ont également été identifiées dans les cancers humains, suggérant que cette famille de gènes joue un rôle dans la carcinogénèse et/ou la progression tumorale. La surexpression du gène *bcl-2*, suite à la translocation chromosomique t(14;18), est impliquée dans la plupart

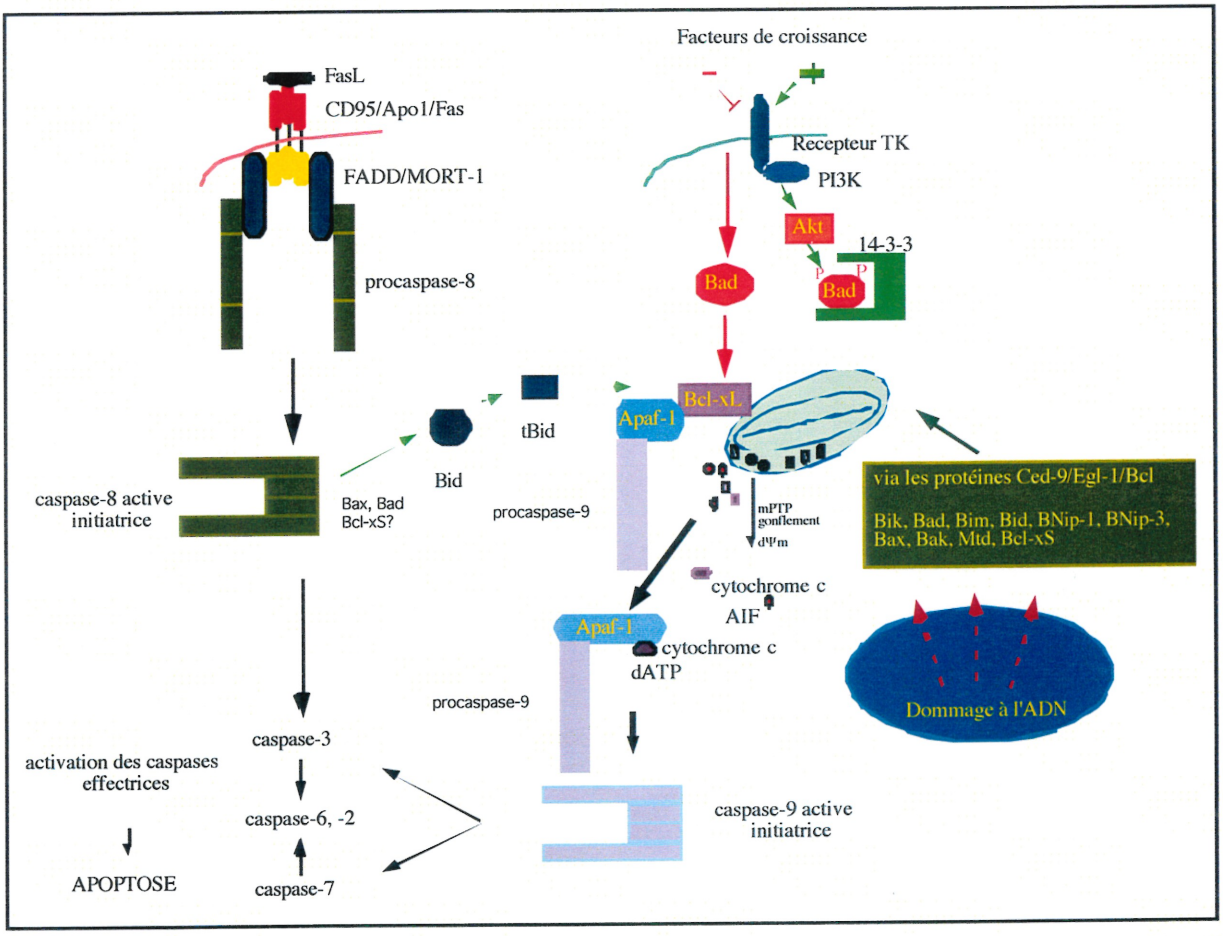

Figure 5: Les voies d'activation de l'apoptose

des lymphomes folliculaires humains (41). Une inactivation du gène pro-apoptotique *bax* par des mutations ponctuelles ou un changement du cadre de lecture est également observée dans plusieurs types de cancers (206-208). La surexpression de la protéine Bcl-2, Bcl-xL, Mcl-1 ou Bfl-1 a été détectée dans une panoplie de tumeurs humaines d'origine variée (203).

La contribution des gènes *ced-9/bcl* à la carcinogénèse et la progression des cellules tumorales a également été mise en évidence dans les cellules en culture ainsi que dans différents modèles animaux. Les gènes *bcl-2, bcl-xL* ou *bfl-1* immortalisent et transforment les cellules en coopération avec d'autres oncogènes tels que *c-myc* ou *ras*. (42, 209-214). Les souris transgéniques qui surexpriment les protéines anti-apoptotiques Bcl-2 ou Bcl-xL dans les cellules T, B ou dans l'ovaire, présentent des hyperplasies des cellules cibles et développent généralement des tumeurs à un stade plus tardif (44, 215-219). Des hyperplasies des thymocytes et des cellules B sont également observées chez les souris déficientes du gène pro-apoptotique *bax* et certains modèles de souris transgéniques démontrent qu'une perte de l'activité du gène *bax* contribue au développement et à la croissance tumorale (220-222).

1.6.2 L'apoptose et la chimiothérapie

Les radiations ionisantes ainsi que la plupart des drogues utilisées dans le traitement des cancers, telles que les inhibiteurs de topoisomérases I et II (camptothécine (CPT), étoposide (VP-16)), les inhibiteurs de la polymérisation ou la dépolymérisation des microtubules (vinblastine, vincristine et taxol) ou les agents formant des ponts covalents dans l'ADN (cisplatine), induisent l'apoptose dans les cellules malignes. Des altérations au niveau des gènes impliqués dans le contrôle de l'apoptose peuvent rendre les cellules résistantes aux traitements (223-225). L'efficacité d'une drogue dépend non seulement de sa capacité d'induire des lésions toxiques dans la cellule mais également de la capacité de la cellule de détecter et de répondre aux dommages. Des drogues ayant des mécanismes d'action différents peuvent induire la même réponse cellulaire qui aboutit à l'activation de l'apoptose. Ainsi, des défaillances au niveau des mécanismes d'activation de l'apoptose peuvent aboutir à une résistance multi-drogue (108, 205). Les réponses des cellules aux altérations générées par les drogues anticancéreuses sont complexes et les cellules peuvent mourir rapidement par apoptose ou nécrose ou activer des points-contrôle du cycle cellulaire (226, 227). L'activation de ces points-contrôle entraîne généralement

des arrêts de la croissance cellulaire au niveau de phases spécifiques du cycle cellulaire. Ces arrêts sont interprétés comme un moyen, pour les cellules traitées, de réparer les dommages perçus avant de poursuivre leur progression dans le cycle cellulaire. La persistence des dommages à l'ADN peut également entraîner l'activation de l'apoptose après ces points-contrôle du cycle cellulaire. Les mécanismes moléculaires impliqués dans la décision d'une cellule, de déclencher rapidement le programme de mort cellulaire ou d'activer des points-contrôle du cycle cellulaire en réponse aux agents anticancéreux, restent encore énigmatiques. Néanmoins, il semble que cette décision dépende de plusieurs paramètres tels que la nature et l'intensité des dommages qu'a subit la cellule, la position de la cellule dans le cycle cellulaire et l'expression des gènes impliqués dans les mécanismes de reconnaissance et de réparation des dommages, le contrôle du cycle cellulaire et le contrôle de l'apoptose.

L'expression des gènes de la famille des *ced-9/egl-1/bcl* joue un rôle déterminant dans la réponse et la sensibilité des cellules tumorales aux traitements anticancéreux. La chimiosensibilité des cellules tumorales en culture est augmentée par la surexpression de protéines pro-apoptotiques telles que Bax-α, Bik, Bak, alors que les cellules qui surexpriment les protéines anti-apoptotiques Bcl-2, Bcl-xL, Mcl-1 ou Bfl-1 présentent une résistance accrue à l'apoptose induite par différentes drogues anticancéreuses (228, 229). La modulation de la réponse des cellules tumorales aux traitements anticancéreux, par certaines protéines de la famille des Ced-9/Bcl, a également été mise en évidence dans des modèles animaux (230, 231). Des études d'expression de la protéine Bax dans des adénocarcinomes métastasiques du sein ont révélé une corrélation entre le niveau d'expression de la protéine et la réponse des cellules tumorales aux traitements chimiothérapeutiques (232). Une étude d'expression de la protéine anti-apoptotique Bcl-xL établit également une corrélation entre la surexpression de Bcl-xL et le grade de malignité des adénocarcinomes du sein (233). D'autres travaux suggèrent que la suppression de l'apoptose induite par les agents anticancéreux, par les protéines Bcl-2 ou Bcl-xL, augmente l'instabilité génique des cellules tumorales et favorise l'accumulation de mutations secondaires pouvant conduire à une augmentation du grade de malignité des cellules tumorales (234, 235).

1.6.3 Les mécanismes d'activation de l'apoptose induite par la chimiothérapie

Bien que les mécanismes moléculaires par lesquels les agents anticancéreux transmettent les signaux de mort cellulaire soient peu connus, plusieurs études suggèrent que ces signaux convergent vers les mitochondries qui jouent un rôle central dans l'initiation de l'apoptose induite par les drogues anticancéreuses.

1) Les agents anticancéreux tels que les inhibiteurs de topoisomérase I et II (CPT et VP-16), la doxorubucine, ou les antimétabolites 1-β-D-arabino-furanosylcytosine (cytarabine) et 2-chloroadenosine induisent une perte du gradient électrochimique de la mitochondrie ($\Delta\Psi m$) qui peut être inhibée par les protéines anti-apoptotiques Bcl-2 ou Bcl-xL (122, 236).

2) La translocation du cytochrome c de la mitochondrie vers le cytoplasme est observée dans plusieurs lignées cellulaires traitées avec des agents anticancéreux et l'inhibition de la libération du cytochrome c par les protéines Bcl-2 ou Bcl-xL bloque l'apoptose induite par les drogues (177, 178, 236, 237).

3) La surexpression des protéines Apaf-1 ou caspase-9 sensibilisent les cellules à l'apoptose induite par le taxol et l'étoposide alors que les cellules déficientes du gène *apaf-1* ou *caspase-9* sont résistantes à l'apoptose induite par des dommages à l'ADN (173-175).

Certains travaux suggèrent que l'activation du récepteur de mort cellulaire CD95/Apo-1/Fas contribue à l'apoptose induite par les agents anticancéreux. En effet, dans certains modèles cellulaires, les drogues anticancéreuses telles que l'étoposide, le cisplatine, la doxorubicine ou la bléomycine, induisent une augmentation de l'expression du récepteur CD95/Apo-1/Fas et/ou de son ligand. Dans ces modèles, l'inhibition de la voie de signalisation du récepteur par des anticorps antagonistes, inhibe partiellement l'apoptose induite par les drogues (238-240). D'autre part, dans certains systèmes, les drogues semblent induire l'agrégation et l'activation du récepteur CD95/Apo-1/Fas, indépendamment de la liaison ligand-récepteur (241). La contribution du récepteur CD95/Apo-1/Fas dans l'apoptose induite par les drogues est cependant controversée par de nombreuses études qui démontrent également que l'inhibition de la voie de signalisation du récepteur ne modifie pas la chimiosensibilité des cellules (242-244). Plus récemment, certaines études ont démontré une augmentation de l'expression du récepteur DR5/Trail-R2 suite à des dommages à l'ADN et dans notre laboratoire nous avons observé une augmentation de l'expression des récepteurs DR4/Trail-R1/Apo-2 et DR3/Tramp/Wsl-1/Apo-3/Lard dans les cellules

promyélocytiques HL-60 traitées avec les inhibiteurs de topoisomérases I et II, suggérant la participation de récepteurs autres que CD95/Apo-1/Fas dans l'apoptose induite par les drogues (245, 246). La contribution des récepteurs de mort cellulaire à l'apoptose induite par les agents anticancéreux reste à être définie. Cependant, l'augmentation de l'expression des récepteurs et/ou des ligands à la surface des cellules ainsi que l'augmentation du taux de ligand sécrété par les cellules, suite à un dommage à l'ADN, pourrait contribuer à amplifier le signal de mort cellulaire induit par les agents anticancéreux en suscitant l'activation des récepteurs de façon autocrine ou paracrine dans la population de cellules traitées.

1.6.4 Les points-contrôle du cycle cellulaire induits par les agents anticancéreux

Le cycle cellulaire est composé d'une phase de synthèse de l'ADN (phase S) suivie de la ségrégation des chromosomes répliqués en deux ensembles égaux durant la mitose (phase M). La fin de la phase M et le début de la phase de synthèse de l'ADN sont séparées par un intervalle de temps appelé phase G1 *(gap 1)*. Un deuxième intervalle, appelé phase G2 *(gap 2)*, sépare la fin de la phase S du début de la mitose. Le cycle cellulaire est soumis à des mécanismes de surveillance ou des points-contrôle qui bloquent sa progression lorsque le passage à la phase subséquente est inapproprié. Les points-contrôle peuvent être activés par des signaux extracellulaires, tels que l'insuffisance de facteurs de croissance, ou des signaux intracellulaires tels que des altérations au niveau de l'ADN ou des anomalies au cours de la ségrégation chromosomique. L'activation des points-contrôle peut entraîner des arrêts du cycle cellulaire durant la phase de réplication, la mitose et au niveau des transitions G1/S et G2/M. Ces délais dans la progression du cycle cellulaire permettent aux cellules, dans la mesure du possible, de réparer les dommages perçus avant d'initier la phase suivante du cycle et assurent la fidélité de la division cellulaire (247, 248).

La progression du cycle cellulaire est régulée par l'activation et l'inactivation séquentielle des protéines kinase cycline-dépendantes (cdk) qui régulent spécifiquement les transitions d'une phase à l'autre du cycle cellulaire. Pour être actives les cdk doivent s'associer à des sous-unités de régulation positive appelées cyclines, qui sont synthétisées et dégradées périodiquement au cours du cycle cellulaire. Une fois associées à leur cycline partenaire, l'activité des complexes cdk-cycline est régulée par des phosphorylations positives ou négatives de résidus spécifiques localisés sur les kinases. Un niveau supplémentaire de régulation des

kinases cycline-dépendantes implique la compartimentalisation des cdk-cyclines ou de leur protéines régulatrices dans la cellule (249, 250 , 251). Plusieurs inhibiteurs des complexes cdk-cycline ont été identifiés. Ces inhibiteurs appartiennent à deux familles. La famille des INK4 qui comprend p15, p16, p18, et p19 et la famille des CIP/KIP qui comprend p21, p27 et p57. Les inhibiteurs de la famille des INK interagissent avec les kinases cdk4 et cdk6 impliquées dans la progression de la phase G1, alors que les inhibiteurs de la famille des CIP/KIP inhibent plusieurs complexes cdk-cycline tout en démontrant une plus grande affinité pour le complexe cdk2-cyclineE (252).

Les agents anticancéreux ont des mécanismes d'action variés et agissent à différentes phases du cycle cellulaire (253). Les inhibiteurs de topoisomérases, tels que la camptothécine ou l'étoposide, sont généralement actifs dans les cellules en phase S et les dommages à l'ADN qu'ils génèrent peuvent induire des arrêts en phase G1, G2 ou en phase S du cycle cellulaire (254). Les inhibiteurs des complexes cdk-cycline jouent un rôle important dans la régulation des points-contrôle du cycle cellulaire et plus particulièrement dans la régulation de la transition G1/S (252, 253). Chez les cellules p53 positives, l'arrêt en phase G1 induit par des dommages à l'ADN, est généralement le résultat d'une stabilisation du facteur de transcription p53. L'accumulation de p53 conduit à la transactivation du gène *p21* dont la protéine inhibe les complexes cdk4-cycline D, cdk6-cycline D et cdk2-cycline E impliqués dans la régulation de la progression de la phase G1 et la transition G1/S (255, 256). Bien que les cellules p53 négatives aient tendance à omettre le point-contrôle à la transition G1/S après un dommage à l'ADN, les arrêts en phase G1 peuvent avoir lieu en l'absence de p53, suggérant l'existence de mécanismes de régulation de la transition G1/S, p53-indépendants (257).

La progression du cycle cellulaire de la phase G2 à la mitose est régulée par la sérine-thréonine kinase cdc2/cdk1. Cdc2/cdk1 est activée par son association avec la cycline B1 et la phosphorylation du résidu Thr-161 par la kinase CAK/cdk7 *(CDK-activating kinase)*. Pendant la phase G2, le complexe cdc2/cdk1-cycline B1 est maintenu inactif par la phosphorylation des résidus Thr-14 et Tyr-15 localisés dans le site actif de la kinase. Ces phosphorylations négatives sont catalysées par des protéines kinase inhibitrices telles que Wee-1, Myt-1 et Mik-1. A la transition G2/M, la déphosphorylation des résidus Thr-14 et Tyr-15, catalysée par la phosphatase cdc25c, entraîne l'activation du complexe cdc2/cdk1-cycline B1 et le passage en mitose (251, 258). La stabilisation du complexe cdc2/cdk1-cycline B1 sous sa forme hyperphosphorylée inactive est impliquée dans l'arrêt du cycle cellulaire en phase G2 induit par des dommages à l'ADN (259-261). La régulation de la localisation

intracellulaire des protéines régulatrices de la kinase cdc2/cdk1 semble également jouer un rôle important dans la transition G2/M. La phosphorylation de la phosphatase cdc25c par la protéine kinase Chk-1, entraîne la séquestration de cdc25c dans le cytoplasme par la protéine 14-3-3 et inhibe l'activation du complexe cdc2/cdk1-cycline B1. L'activation de la kinase Chk-1 semble impliquée dans les arrêts en phase G2 induit par des dommages à l'ADN (262-267). Les inhibiteurs des complexes cdk-cycline, qui sont essentiellent impliqués dans les arrêts en phase G1, en particulier dans les cellules p53 positives, ne semblent pas jouer un rôle déterminant dans la régulation de la transition G2/M après un dommage à l'ADN (268). L'importance de p53 dans l'arrêt en phase G2 est également controversée, étant donné que la plupart des cellules p53 négatives maintiennent leur capacité d'effectuer des arrêts en phase G2 suite à des lésions génotoxiques (269, 270). Des travaux récents suggèrent cependant que p53 favorise l'arrêt du cycle cellulaire à la transition G2/M en régulant négativement la transcription de la cycline B1 et en augmentant la transcription de la protéine régulatrice 14-3-3. Suite à un dommage à l'ADN, p53 transactive également la protéine Gadd45 qui est impliquée dans les mécanismes de réparation de l'ADN mais qui, dans certains cas, inhibe également l'activation du complexe cdc2/cdk1-cycine B1 en interagissant avec la kinase (271-274).

1.6.5 Couplage fonctionel entre l'apoptose et le cycle cellulaire

L'homéostasie cellulaire résulte d'un équilibre entre la prolifération cellulaire et la mort cellulaire et implique une connection entre le contrôle du cycle cellulaire et les mécanismes de régulation de l'apoptose. Le couplage entre l'apoptose et le cycle cellulaire est très bien illustré dans le cas de la protéine p53. En effet, suite à des dommages à l'ADN, la protéine p53 peut transmettre des signaux de mort cellulaire qui aboutissent à l'activation de l'apoptose mais elle peut également activer les points-contrôle du cycle cellulaire et promouvoir des arrêts dans la croissance cellulaire (275-277). D'autres protéines impliquées dans la régulation de la prolifération cellulaire, telles que c-Myc, E2F-1 ou Rb, modifient également la sensibilité des cellules à l'apoptose, et certaines d'entre elles sont des substrats de caspases (202, 278, 279). Récemment, un domaine BH-3 a été identifié chez la protéine humaine Rad-9, impliquée dans le point-contrôle du cycle cellulaire à la transition G2/M. Le domaine BH-3 confère une nouvelle fonction pro-apoptotique à la protéine Rad-9. De plus, Rad-9 co-immunoprécipite avec Bcl-2 et Bcl-xL qui inhibent son activité pro-apoptotique (280).

La similarité des changements morphologiques observés durant l'apoptose et la catastrophe mitotique qui résulte d'une activation non contrôlée de la kinase cdc2/cdk1, a suggéré une implication potentielle de cdc2/cdk1 et des autres cdk dans l'apoptose (281). A l'heure actuelle, cette hypothèse est appuyée par plusieurs travaux qui mettent en évidence une corrélation entre l'activation de certaines cdk et l'apoptose induite par différents stimuli incluant les dommages à l'ADN. L'activation de cdc2/cdk1 est associée à l'apoptose induite par la fragmentine-2, la staurosporine, le taxol, et l'activation du récepteur CD95/Apo-1/Fas. Dans ces modèles, l'inhibition de l'activité de cdc2/cdk1 module l'apoptose induite par les différents stimuli (282-284). La surexpression de p16 ou p27, des inhibiteurs de kinases cycline-dépendantes, ainsi que l'expression d'une forme dominante négative de cdk4 ou cdk6, protègent les neurones de l'apoptose induite par la camptothécine, les radiations UV et l'agent antimétabolique 1-β-D-arabinofuranosylcytosine (285). La protéine p27 inhibe également l'apoptose induite par FasL, l'étoposide et la vinblastine dans les cellules leucémiques humaines U937 (286). Une étude réalisée par R. Ross et ses collaborateurs suggère que le clivage de p21 et p27 par la caspase-3, qui conduit à l'activation du complexe cdk2-cycline A, contribue à amplifier le signal apoptotique dans les cellules endothéliales humaines HUVEC privées de sérum (279). Alternativement, d'autres travaux montrent que la protéine p21 inhibe l'apoptose induite par FasL en séquestrant la procaspase-3 au niveau de la mitochondrie (287-289). Une étude récente suggère que les caspases participent au contrôle du cycle cellulaire dans un contexte indépendant de l'apoptose. Dans cette étude il est montré que p27 est clivé par les caspases-3 dans les cellules myéloides humaines U266 en phase G1 du cycle cellulaire. Le clivage de p27 par les caspases augmente son activité et semble contribuer à l'arrêt en phase G1 (290). Finalement, d'autres travaux suggèrent que l'activation préférentielle de certaines caspases semblent être impliquée dans la prolifération de cellules T stimulées *in vitro* (291, 292).

Certains travaux suggèrent que les protéines régulatrices de l'apoptose de la famille des Ced-9/Bcl participent également au contrôle du cycle cellulaire. Chez les souris transgéniques qui surexpriment Bax-α ou Bcl-2 dans les cellules T, la prolifération des thymocytes est perturbée. La protéine pro-apoptotique Bax-α augmente le taux de prolifération des thymocytes alors que la protéine anti-apoptotique Bcl-2 exerce l'effet opposé. La surexpression de Bcl-2 retarde l'entrée en phase S des cellules T suite à un stimulus mitogénique alors que Bax-α favorise l'entrée en phase S. Dans ce modèle, Bcl-2 et Bax-α influencent le cycle cellulaire en modulant, par un mécanisme inconnu l'expression de p27. Une autre étude montre que Bcl-2 et Bax-α

modulent la sensibilté des thymocytes à l'apoptose induite par des dommages à l'ADN en régulant l'activation de la kinase cdk2 (293-295).

Récemment E. F. Lind et ses collaborateurs ont montré que la surexpression de Bcl-2 dans les cellules FDC-P1 induit un arrêt du cycle cellulaire en phase G1 suite à la suppression de l'IL-3. Dans ce système, l'expression de Bcl-2 entraîne la déphosphorylation de la protéine Rb et l'inhibition de l'activité des facteurs de transcription E2Fs. Ces changement sont associés à une augmentation de l'expression de la protéine p130 et la séquestration des isoformes actives de E2F par la protéine p130 (296).

1.7 Objectif du projet

Des dérégulations de l'apoptose sont impliquées dans de nombreuses pathologies humaines incluant les maladies autoimmunes, les maladies neurodégénératives et le cancer (6-8). L'expression des gènes régulateurs de l'apoptose semble être impliquée dans le processus de transformation cellulaire et la progression des cellules tumorales mais détermine également la réponse et la sensibilité des cellules tumorales face aux thérapies anticancéreuses (7, 201-203, 223-225, 228).

Ces dernières années ont connu une véritable explosion des connaissances sur les mécanismes d'activation et de régulation de la mort cellulaire programmée dans les cellules de mammifères. Lorsque nous avons débuté nos travaux, la famille des *ced-9/bcl* était essentiellement constituée de cinq membres *(bcl-2, a-1, mcl-l, bcl-x et bax)* et seules caspases-1 et -2 avaient été identifiées (24, 82, 297). Quelques études avaient mis en évidence le rôle des Ced-9/Bcl dans la mort cellulaire programmée et suggéraient que le niveau d'expression de Bcl-2 module la sensibilité des cellules tumorales face à l'apoptose induite par les agents anticancéreux (44, 45, 298-301). Cependant, le rôle des autres membres de cette famille, ainsi que la fonction et le mode d'action de ces protéines dans la cascade apoptotique induite par les agents anticancéreux restaient à être élucidés.

L'objectif de ce travail consistait à étudier le rôle des protéines Bcl-xL et Bax-α dans la réponse des cellules tumorales aux lésions cytotoxiques induites par les agents anticancéreux et d'élucider le mécanisme d'action de ces protéines dans la cascade apoptotique induite par les drogues (Chapitre 2 et 3). Nous avons également identifié

et cloné une nouvelle isoforme de *bax*, appelée *bax-σ* ainsi qu'une nouvelle isoforme de *bcl-x* appelé *bcl-xES*. L'expression, le rôle, la fonction et le mécanisme d'action de ces protéines, dans l'apoptose induite par les agents anticancéreux ont été étudiés (Chapitre 4 et 5). Les mécanismes moléculaires impliqués dans la décision d'une cellule de déclencher l'apoptose ou d'activer un point-contrôle du cycle cellulaire furent également étudiés. Plus particulièrement, la fonction de Bcl-xL couplée à un point-contrôle à la transition G2/M du cycle cellulaire fût analysée (Chapitre 6).

CHAPITRE 2.

Bcl-xL modulates apoptosis induced by anticancer drugs and delays DEVDase and DNA fragmentation promoting activities

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Avant propos:

Ce travail consiste à étudier le rôle de la protéine Bcl-xL dans l'apoptose induite par les agents anticancéreux et de situer son activité dans la cascade apoptotique induite par un dommage à l'ADN. Nous montrons que l'expression de Bcl-xL diminue la sensibilité des cellules leucémiques humaines U937 à l'apoptose induite par plusieurs drogues ayant des mécanismes d'action différents. La résistance des cellules à l'effet des drogues est d'autant plus grande que le niveau d'expression de la protéine est élevé. Dans les cellules traitées avec la camptothécine, un inhibiteur de topoisomérase I, Bcl-xL n'interfère pas avec le mécanisme d'action primaire de la drogue. En revanche, Bcl-xL exerce une modulation négative de l'apoptose en retardant l'activation des caspases et des facteurs impliqués dans la fragmentation de l'ADN.

• G. Cimoli a contribué à la production de la protéine recombinante Bcl-xL(ΔTM) et A. Steyaert au sous-clonage de l'ADN complémentaire de Bcl-xL dans le vecteur d'expression pCEP4.

SUMMARY

Using an episomal eucaryotic expression vector, we derived three stable transfected human leukemic U-937 variant lines showing differential expression of the Bcl-xL protein. Preventive effect of Bcl-xL on cell death induced by various concentrations of camptothecin (DNA topoisomerase I inhibitor; CPT) was observed in the three lines with most pronounced effect in cells containing the highest level of Bcl-xL expression. These results show that increased cell death protection by Bcl-xL is correlated with its level of expression. The extent of DNA strand break formation and DNA synthesis inhibition following CPT treatments was similar in control and transfected U-937 cells suggesting that Bcl-xL acts downstream of CPT-DNA topoisomerase I-mediated DNA strand breaks. Modulation of cell death by Bcl-xL was also observed in cells treated with etoposide, vinblastine, paclitaxel and cis-platinum(II) diammine dichloride. To define whether Bcl-xL functions downstream or upstream of apoptogenic proteolytic cascade activation, we compared kinetics of DNA fragmentation in treated cells with kinetics of caspase 1-like, caspase 3-like and N-tosyl-L-phenylalanylchloromethyl ketone (TPCK)-sensitive activities. In CPT-treated U-937 cells, caspase 3-like and TPCK-sensitive activities promoting DNA fragmentation in a cell-free system were detected much more rapidly in extracts obtained from CPT-treated U-937 cells compared to those obtained from CPT-treated U-937-Bcl-xL variant cells. These results suggest that Bcl-xL delays their activation that correlates with the occurrence of DNA fragmentation. Addition of recombinant Bcl-xL in extracts containing DEVDase and TPCK-sensitive activities did not inhibit these activities suggesting that Bcl-xL acts primarily upstream of their activation in the apoptotic process. Taken together, these results suggest that Bcl-xL is a primary checkpoint that can block or delay transmission of cell death signals emerging from DNA damage and prevents activation of an apoptogenic proteolytic cascade.

Keywords: *apoptosis / Bcl-xL / caspases / N-tosyl-L-phenylalanylchloromethyl ketone / cancer chemotherapy*

INTRODUCTION

Programmed cell death (apoptosis) is a fundamental process essential for normal regulation of cell homeostasis [1, 2 , 3, 4]. Apoptosis is well defined by characteristic cellular morphological changes associated with internucleosomal DNA fragmentation which results from endonuclease activation [5-7]. Genetic studies in the nematode *Caenorhabditis elegans* have provided major evidence that specific genes can act as inhibitor or activator in the apoptotic process. One gene, Ced-9, has been found to repress apoptosis while two others, Ced-3 and Ced-4, are essential for apoptosis in *C. elegans* [8-12]. Recent studies in human cell lines have uncovered many Ced-9/Bcl-like and Ced-3/Ice-like gene products that regulate either negatively or positively the apoptotic threshold of a human cell. The human Ced-9/Bcl-like family includes a series of related gene products such as Bcl-2 [13-16], Bax [17], Bcl-x [18], Mcl-1 [19], A1 [20], Bak [21-23], Bad [24], Bfl-1 [25], Bik [26], Bcl-w [27], Brag-1 [28] Bid [29] and Hrk [30]. One of them, Bcl-2, has been found translocated [t(14:18)] in human B cell lymphoma and is thought to contribute to the development of B-cell neoplasia [31, 32]. Extensive works have been done to understand the function and role of Bcl-2. Overexpression of Bcl-2 is often associated with cell death protection induced by several stimuli including anticancer compounds [33-38]. Although the mechanisms and functions of all the others have been less studied, recent observations suggested that Bcl-xL [39-42] had protective effects upon anticancer drugs, while Bax-α [43-47] and Bcl-xS [48 , 49] accelerate cell death induced by anticancer drugs.

An emerging family of Ced-3/Ice-like cysteine proteases (caspases) has also been identified and cloned based on a conserved peptapeptide sequence (QACXG) that contains the active cysteine residue. The caspases are separated in three subfamilies based on their degrees of homology and by phylogenetic analysis; The ICE-related family includes Ice/caspase-1 itself with its spliced isoforms [9, 50], Tx/Ich-2/Ice rel-II/caspase-4 [51-53], Ty/Ice rel-III/caspase-5 [53, 54] and Ich-3/caspase-11 [55]; the CPP32-related family includes CPP32/Yama/Apopain/caspase-3 [56-58], Mch-2/caspase-6 [59], Mch-3/Ice-Lap3/Cmh-1/caspase-7 [60-62], Mch-4/caspase-10 [63], Mch-5/Flice/Mach/caspase-8 [63-65] and the third group includes Ich-1[l and s]/caspase-2 [66] and Ice-Lap6/Mch6/caspase-9 [67]. Recent studies have also revealed that serine-like protease activities play important role in the apoptotic process. Granzyme B, a granule serine protease injected in target cells by cytotoxic lymphocytes, triggers apoptosis in association with perforin proteins [68, 69]. AP24

an elastase-like serine protease induced by TNF- and UV ligh has been reported to activate apoptosis in U937 cells [70] and two other serine proteases have been partially isolated from cells undergoing apoptosis [71, 72]. Other studies using either cultured cells or cell-free systems have also shown the importance of yet unknown serine-like protease activities in the apoptotic process [73-80]. More recently, it has been also reported that a dichloro-isocoumarin-sensitive pathway may lead to DNA fragmentation in cells following VP16 [81] while trypsin-like protease activity appeared to activate apoptosis following CPT treatment in hepatoma, monocytic leukemia and B lymphoma cell lines [80, 82].

Chemotherapeutic drugs such as DNA topoisomerase I (top 1) and II (top 2) inhibitors (camptothecin, CPT; etoposide, VP16, respectively) trigger apoptosis in various cell lines [75, 83-89]. These drugs are known to stabilize a transient intermediate of topoisomerase reactions where enzymes are linked to the 3' (top 1) or 5' (top 2) terminus of a DNA duplex producing DNA single- or double-strand breaks [90-94]. These protein-linked DNA single- or double-strand breaks are pre-lethal DNA lesions that inhibit DNA metabolism such as DNA synthesis [95-98]. Similarly, all of the anticancer chemotherapeutic drugs used in clinic can induce apoptosis in various cell lines [99, 100]. However, the mechanisms by which these drugs activate programmed cell death are just beginning to be examined. The decision for a given cell population to undergo cell growth or cell death is influenced by a series of extrinsic and intrinsic signals. The Ced-9/Bcl-like and Ced-3/Ice-like gene products are intrinsic proteins regulating the decision of a cell to survive or die and executing the process of death itself, respectively. Thus, expression and activation of these genes might determine drug sensitivity and resistance [4, 16, 35-37, 101-103].

To further analyze the mechanisms of anticancer drug-induced apoptosis and to characterize the relation between Bcl-xL, caspases, N-tosyl-L-phenylalanylchloromethyl ketone (TPCK)-sensitive activities and DNA fragmentation activation, we developed three U-937 monocyte-like lines that expressed differential levels of the Bcl-xL protein. In this paper we show that the protective effect conferred by Bcl-xL upon CPT-induced apoptosis correlates with its level of expression and that Bcl-xL acts downstream of CPT-mediated DNA strand break formation and DNA damage-mediated DNA synthesis inhibition. Bcl-xL modulates apoptosis induced by a series of anticancer drugs and prevents the activation of caspase 3-related and TPCK-sensitive activities. Addition of recombinant Bcl-xL(ΔTM) protein to extracts containing active proteases is unable to inhibit

DEVDase activity and to prevent DNA fragmentation in a cell-free system. Taken together, these results suggest that Bcl-xL acts primarily downstream of the primary effect of chemotherapeutic drugs like CPT and functions upstream of apoptogenic proteolytic cascade by preventing their activation.

MATERIALS AND METHODS

Chemicals. [2-^{14}C]-thymidine (59 mCi/mmol), [methyl-^{3}H]-thymidine (78 Ci/mmol) and [^{35}-S]-methionine [^{35}-S]-cysteine (Tran^{35}S-label) were purchased from ICN BioMedicals (Costa Mesa, CA). 20-S-camptothecin lactone, etoposide, vinblastine, paclitaxel, cis-platinum(II) diammine dichloride and N-tosyl-L-phenylalanylchloromethyl ketone were purchased from Sigma Chemicals Co. (St-Louis, MO). The tetrapeptide derivatives Ac-Asp-Glu-Val-aspartic acid aldehyde (Ac-DEVD-CHO) and Ac-Tyr-Val-Ala-aspartic acid aldehyde (Ac-YVAD-CHO) and the fluorogenic peptide derivatives Ac-Asp-Glu-Val-Asp-amino-4-methylcoumarin (Ac-DEVD-AMC) and 4-(4-dimethyl-aminophenylazo)benzoyl-Tyr-Val-Ala-Asp-Ala-Pro-Val-5-[(2-amino-ethyl) amino]-naphthalene-1-sulfonic acid (DABCYL-YVADAPV-EDANS) were purchased from Bachem Bioscience Inc (King of Prussia, PA). All other chemicals were of reagent grade and purchased either from Sigma, ICN and Boehringer-Mannheim or from other local sources.

Cell culture, DNA labeling and drug treatment. The human U937 cell line was obtained from the American Type Culture Collection (Rockville, Md). Cells were grown in suspension culture at 37°C in the presence of 5% CO_2 in RPMI-1640 medium supplemented with 10% heat-inactivated fetal bovine serum, 2 mM glutamine, 100 U/ml penicillin and 100 μg/ml streptomycin. All cell culture products were purchased from Gibco-BRL Life Technologies (Grand Island, NY). For DNA labeling, U-937 cells were grown with [^{14}-C]-thymidine (0.02 μCi/ml) for one doubling-time and then chased in isotope-free medium overnight before drug treatment.

cDNA cloning, transfection and immunocytofluorometry. The human bcl-xL cDNA was cloned by RT-PCR from polyA(+) RNA using specific adapter primers containing *NotI* sequences at the ATG start codon and TAA stop codon. The amplified fragment was first cloned in pCRII vector (TA cloning system; Invitrogen, San Diego, CA) and then subcloned at the *NotI* restriction site in the eucaryotic expression vector pCEP4 (InVitrogen) modified to include hemagglutinin

epitote Tag sequences (HA-tag) and Kosak consensus sequences. Vectors were sequenced by ALF DNA sequencer (LKB-Pharmacia Biotech, Upsalla, Sweden) using both vector and internal primers. Purified pCEP4-HA-Bcl-xL construct was transfected in U-937 cells by electroporation at 0.27 kVolts (Gene Pulser, BioRad, Hercules, CA). Transfection efficencies were controlled by cotransfection with a reported plasmid expressing ß-galactosidase. Transfected cells were grown under hygromycin selection at 250 μg/ml, 500 μg/ml and 1.5 mg/ml for 2 to 3 months to obtain stable lines before performing the experiments. All restriction and modification enzymes were purchased either from Pharmacia Biotech or Gibco-BRL. Relative level of expression of HA-Bcl-xL proteins in the transfected variant lines was determined by cytofluorometry. Permeable control and HA-Bcl-xL-U937 cells were analyzed in the absence and presence of anti-HA rhodamine-conjugated antibodies (Boehringer-Mannheim) and the fluorescence intensity distribution detected using a Becton Dickinson FACSTAR Plus flow cytometer.

Protein extracts, Western blot, immunoprecipitation and densitometry analysis. To prepare cytosolic extracts, control and transfected U937 cells were first collected by centrifugation, washed twice with ice-cold phosphate-buffered saline (PBS) and then homogenized in lysis buffer (50 mM Tris pH 7,4; NaCl 150 mM; EGTA 1 mM; PMSF 1 mM; DTT 1 mM; NP40 0,1%; 0.15 U/ml aprotinin) at 4°C for 30 min. The extracts were then centrifuged and supernatants were collected as protein extracts. Protein concentrations were determined using the Bradford assay with bovine serum albumin as standard. For immunoprecipitation control and transfected U937 cells were washed in serum-free and methionine-free medium before metabolic labeling. Cells were then grown in [^{35}-S]-methionine -[^{35}-S]-cysteine (Tran^{35}S-label; ICN) (1.0 mCi/ml) for 3 h in a methionine-free medium supplemented with 10% dialyzed fetal calf serum. Cells were washed twice in ice-cold PBS and whole-cell extracts were prepared as above. Specific anti-HA peptide monoclonal antibodies were added for 60 min and immunoprecipitates were captured with protein A-Sepharose (Pharmacia Biotech) for 60 min. Immunoprecipitates were washed in lysis buffer without NP-40 and solubilized with SDS-polyacrylamide gel electrophoresis sample buffer. SDS-PAGE (10-18%) and Western blotting were done by standard methodology. Gels containing [^{35}-S]-labeled proteins were fixed with 10% glacial acetic acid and 30% methanol for 30 min, enhanced by soaking with a fluorography enhancing solution (EnHance, DuPont) for 60 min and then dried before film exposure and autoradiography. Affinity

purified antibodies to human Bcl-xL were purchased from Transduction Laboratories and antibodies to the peptide epitope derived from the hemagglutinin protein of human influenza virus (HA epitope tag) were obtained from Boehringer-Mannheim. Enhanced cheminulescence detection of peroxidase-labeled secondary antibodies was performed using Amersham Life Science reagents. Molecular weight markers were obtained from Bio-Rad. Relative densitometry analysis of autoradiograph was based on integrated density value (IDV) using AlphaImager 2000 scanner (Alpha Innovatech, San Jose CA).

DNA filter elution assay. We routinely use DNA filter elution assays to monitor and quantitate DNA fragmentation associated with apoptosis [86, 88, 104, 105]. Each sample (approximatively 0.5 X 10^6 [^{14}C]-thymidine-labeled cells) is loaded onto a protein-adsorbing filter (vinyl/acrylic copolymers filters, Metricel membrane, 0.8 um pore size, 25 mm diameter, Gelman Sciences Inc., Ann Arbor, MI) mounted in a Millipore filter holder connected to a 50 ml syringe (see figure in ref. [104, 105]). Cells are then washed with 5 ml of phosphate buffered saline (PBS). When the washing solution has dripped through by gravity, lysis are done with 5 ml of LS-10 solution (0.2% sodium sarkosyl, 2 M NaCl, 0.04 M EDTA, pH 10.0). After the lysis have dripped through by gravity, it is washed from the filters with 5 ml of 0.02 M EDTA (pH 10.0). A single wash is sufficient to collect more than 90% of the remaining DNA fragments. The filter is then processed as described [86, 88, 104, 105]. Briefly, it was placed in a scintillation vial to which 1.0 ml of 1 N HCl is added. The vial is sealed and heated for 1 h at 60°C to depurinate the DNA. After removing the vials from the oven, 2.5 ml of 0.4 N NaOH is added and allowed to stand 1 h at room temperature to release the labeled DNA from the filters. Radioactivity was counted by liquid scintillation spectrometry in each fraction (loading wash, lysis, EDTA wash, filter). DNA fragmentation was determined as the fraction of DNA in the loading wash fraction + lysis fraction + EDTA wash fraction relative to total DNA (loading wash + lysis + EDTA wash + filter). Results are expressed as percent DNA fragmentation in treated cells compared to DNA fragmented in control untreated cells (background) using the formula:

$$(F\text{-}F0/1\text{-}F0) \times 100$$

Where F and F0 represent DNA fragmentation in treated and control cells, respectively.

KCl-SDS precipitation assay for protein-DNA complexes. The *in vivo* formation of covalent topoisomerase I-DNA complexes was quantitated using the

KCl-SDS precipitation assay previously described by Rowe et al. [90]. [^{14}C]-thymidine-labeled cells were treated with various concentrations of CPT for 30 min at 37^{0}C. Typically 0.5-1.0 x 10^6 cells (corresponding to 10,000 c.p.m.) were pelleted by centrifugation (1,000g, 3 min, 4°C), rapidly lysed by adding 1 ml of a prewarmed (65^{0}C) lysis solution containing 1.25% SDS, 5 mM EDTA (pH 8.0) and 0.4 mg / ml salmon sperm DNA and then transferred to a microfuge tube containing 250 µl of 0.325 M KCl. After vortexing for 10 s, sample was incubated on ice for 10 min and centrifuged for 10 min (10,000g, 4^{0}C). The pellet was resuspended in a prewarmed (65^{0}C) washing buffer (10 mM Tris-HCl pH 8.0, 1 mM EDTA pH 8.0, 100 mM KCl, 0.1 mg / ml salmon sperm DNA), incubated at 65^{0}C for 10 min, cooled on ice and centrifuged as above. After a second identical washing procedure, the pellet was dissolved in 250 µl water at 65^{0}C and radioactivity was counted by liquid scintillation spectrometry.

Measurement of thymidine incorporation. Control and transfected U937 cells were prelabeled with [^{14}-C]-thymidine (0.02 µCi/ml) for 24 h and then chased in isotope-free medium overnight before drug treatment. Cells were incubated in CPT containing medium for 30 min. After drug treatment, cells were washed twice with complete medium and rates of nucleotide incorporation were measured by 10 min pulse experiments with [^{3}-H]-thymidine (10 µCi/ml) as described [98]. Nucleotide incorporation was stopped by adding 10 ml of ice-cold PBS and cells were quickly pelleted by centrifugation (1,000g, 3 min, 4°C). Acid-insoluble nucleotides were precipitated on ice with 10% trichloroacetic acid. The precipitates were dissolved in 0.4 N NaOH and radioactivity was monitored by scintillation spectrometry. Results were expressed as the ratio of [^{3}-H]/[^{14}-C] for treated cells over the ratio of [^{3}-H]/[^{14}-C] of untreated cells [98].

Caspase activity determination. Cytosolic extracts are prepared by washing control and transfected U937 cells twice by centrifugation/resuspension in 10 ml ice-cold PBS (without Ca^{++} and Mg^{++}). Cells are then homogenized at 4°C for 30 min in a lysis buffer containing 100 mM Hepes (pH 7.5), 5 mM EDTA, 5 mM DTT, 20% glycerol and 0.5% NP-40. Samples are then centrifuged (13,000 g for 10 min at 4°C) and supernatants collected as cytosolic extracts. Caspase activities were measured as described previously [106] by monitoring fluorescence continuously in a dual luminescence fluoremeter (LS 50B Perkin Elmer) using excitation wavelength of 360 nm and emission wavelenght of 490 nm for the substrate

DABCYL-YVADAPV-EDANS and excitation wavelength of 380 nm and emission wavelenght of 460 nm for the substrate Ac-DEVD-AMC . Reactions were carried out in cuvettes and temperature maintained at 37° using a water-jacketed sample compartment. The assay mixture contained 100 mM Hepes (pH 7.5), 20% (v/v) glycerol, 5 mM DTT, 5 mM EDTA and 200 μM fluorogenic peptides. Enzyme activities were determined as initial velocities and expressed as relative intensity / min / mg. Typically, 200 μg of extracted proteins per assay was used to monitor caspase activities.

DNA fragmentation promoting activities in cell free system. Cytosolic extracts and isolated nuclei are prepared by washing control and transfected U-937 cells twice by centrifugation/resuspension in 10 ml ice-cold PBS (without Ca^{++} and Mg^{++}) and incubated on ice for 10 min at a density of 1.0×10^7 cells / ml in a lysis buffer containing 10 mM Hepes (pH 7.4), 20 mM NaCl, 80 mM KCl, 5 mM $MgCl_2$, 5 mM EGTA, 1 mM DTT, 0,1 mM PMSF, 0.15 U/ml aprotinin, 10% glycerol and 0.1% NP-40. Samples are then centrifuged (2,000g for 10 min at 4°C) and supernatants collected as cytoplasmic fractions. Pellets (nuclei) are then washed twice by centrifugation/resuspension in the lysis buffer without detergent. Cytoplasm from untreated or treated cells is then incubated with isolated nuclei from labeled untreated cells at 37°C for 30 min in the absence or presence of various putative inhibitors at the indicated concentrations. DNA fragmentation is then measured by DNA filter elution assays as described above [86, 88, 104, 105]. Typically, 200 μg of cytoplasmic proteins per assay was used to monitor DNA fragmentation promoting activities.

Recombinant Bcl-x(ΔTM) protein. The human Bcl-xL cDNA without the sequences coding for it carboxyl hydrophobic domain (TM), was first amplified by PCR using specific adapter primers containing a *NdeI* sequence at the ATG start codon and *BamHI* sequence at the TAA added stop codon. The PCR product was inserted in the pCRII vector (TA cloning system; InVitrogen, San Diego, CA) and then subcloned in the bacterial expression vector pET-14b(+)-His-TAG (Novagen; Madison WI) between the *NdeI* and *BamHI* sites. Vectors were sequenced by ALF DNA sequencer (LKB-Pharmacia Biotech) using both vector and internal primers. *E. Coli* BL21 (DE3) was transfected with purified plasmids and recombinant protein expression induced for up to 15 h by adding 100 μM IPTG to exponentially growing bacteria at room temperature. The bacteria were collected by centrifugation and resuspended in 5 mM imidazole, 0.5 M NaCl , 20 mM Tris-HCl (pH 7.9) and samples

were sonicated on ice. Following centrifugation at 12,000 g for 20 min, bacterial lysates were then applied to a charged and equilibrated Chelating Sepharose (Pharmacia Biotech) chromatography column. The column was then washed with 10 volumes of 5 mM imidazole, 0.5 M NaCl , 20 mM Tris-HCl (pH 7.9) followed by 6 volumes of 50 mM imidazole, 0.5 M NaCl , 20 mM Tris-HCl (pH 7.9). The bound protein was eluted with 6 volumes of 1 M imidazole, 0.5 M NaCl , 20 mM Tris-HCl (pH 7.9). Individual fractions were collected and purity of recombinant His_8-Bcl-xL(ΔTM) protein preparations were determined by SDS-polyacrylamide gel electrophoresis. The eluted recombinant protein was found predominently monomeric by centrifugal filtration on a 30,000 NMWL Millipore filter unit and immunoreacts with bcl-xL antibodies (data not show).

RESULTS

U-937/bcl-xL transfected lines. Consensus Kosak and hemagglutinin epitope tag sequences (HA) were first inserted in the eucaryotic expression vector pCEP4 at *HindIII* - *NotI* restriction sites. The human Bcl-xL cDNA was then amplified by RT-PCR and inserted in the modified pCEP4 vector (Fig. 1). After transfection, U-937 cells were selected as bulk culture under various concentrations of hygromycin (250 μg/ml, 500 μg/ml, 1.5 mg/ml) and selections were maintained for 2 to 3 months before performing the experiments. Western blot analysis using anti-HA epitope tag monoclonal antibodies, suggests that the HA-Bcl-xL protein is expressed at different levels with respect to hygromycin concentration used for the selection (Fig 2a; left panel). [^{35}S]-methionine[^{35}S]-cysteine labeled HA-Bcl-xL protein was also immunoprecipitated using anti-HA epitope tag monoclonal antibodies and visualized on SDS polyacrylamide gel electrophoresis following immunoprecipitation (Fig. 2a; middle panel). The endogenous level of Bcl-xL protein in U-937 cells is very low compared to HA-Bcl-xL protein and level of expression of HA-Bcl-xL increased with hygromycin selection (Fig 2a; right panel). The relative amount of HA-Bcl-xL protein was quantitated and normalized to the amount of endogenous Bcl-xL protein by densitometry of autoradiograph. Relative ratios of HA-Bcl-xL protein level normalized to endogenous Bcl-xL protein level are of 0 to 1, 3.3 to 1, 4.2 to 1 and 6.3 to 1 in control and transfected lines selected at 250 μg / ml, 500 μg / ml and 1.5 mg / ml hygromycin, respectively. Cytofluorometric assessment of HA-Bcl-xL protein level was also done using anti-HA rhodamine-conjugated monoclonal antibodies as shown in Fig. 2b. Mean fluorescence intensities are displaced from 8.35 to 24.58,

39.24 and 46.98 from control to transfected lines selected at 250 μg / ml, 500 μg / ml and 1.5 mg / ml hygromycin, respectively.

Protective effect of Bcl-xL upon apoptosis and DNA fragmentation. Several studies have shown that CPT, a DNA topoisomerase I inhibitor, activates the morphological changes and internucleosomal DNA fragmentation associated with apoptosis [84-89, 107]. We routinely used a filter DNA binding assay to monitor and quantitate kinetics of DNA fragmentation in cells undergoing apoptosis [86, 88, 104, 105]. Short treatments (30 min) with CPT at various concentrations induced apoptosis in U-937. Both kinetic and extent of DNA fragmentation were most pronounced in cells treated at the highest CPT concentration (Fig. 3). All stable transfected U-937 cells selected at 250 μg/ml (inversed triangles), 500 μg/ml (filled squares) and 1.5 mg/ml (filled triangles) were less susceptible to CPT-induced DNA fragmentation (Fig. 3). The protective effect of Bcl-xL was most pronounced in cells expressing the highest level of the Bcl-xL protein. At 0.1 μM and 0.5 μM CPT, the protective effect was nearly complete in the two lines showing the highest level of bcl-xL, even 24 h after drug treatment (Fig. 3). At 1.0 μM CPT, protection was almost complete for 8 h in the line expressing highest level of Bcl-xL protein. 24 h after drug treatment, the extent of DNA fragmentation in the Bcl-xL transfected line selected at 1.5 mg/ml hygromycin, was less than 40% compared to more than 85% in CPT-treated U-937 control cells (Fig. 3). These results show a direct relation between the amount of Bcl-xL protein and the degree of protection against CPT-induced apoptosis and DNA fragmentation.

CPT traps DNA topoisomerase I-cleavable complexes and induces DNA strand breaks. These enzyme-linked DNA adducts on active replication forks inhibit DNA synthesis. DNA replication inhibition is a most widely explored processes to be implicated in the cytotoxic mechanism of action of DNA topoisomerase I inhibitors [94-98]. To determine whether Bcl-xL protein acts primarily upstream or downstream of CPT-mediated DNA strand break formation and its translated effect on DNA synthesis, the formation of protein-DNA complexes and rates of DNA synthesis were measured immediately after CPT treatments by KCl-SDS precipitation assays and thymidine pulse incorporation, respectively. Table 1 shows that the amount of precipitable protein-DNA complexes mediated by various concentrations of CPT was similar in U-937 and transfected cells showing that Bcl-xL does not interfere with the formation of CPT-induced cleavable complex. Furthermore, Table 2 shows that immediately following CPT treatment at 0.1 μM , 0.5 μM and 1.0 μM, rates of DNA synthesis were reduced to approximately 80%, 69% and 55% compared to that of

control untreated cells, respectively. The extent of DNA synthesis reduction was similar in all transfected U-937 cells treated with 0.1μM, 0.5 μM and 1.0 μM CPT with rates to approximately 72%, 64% and 54% to that of transfected untreated cells, respectively. These resutls suggest that Bcl-xL does not interfere with the primary mechanism of action of CPT and does not prevent CPT-induced DNA strand break formation and DNA synthesis inhibition.

Modulation of cell death by Bcl-xL was also observed in cells treated continuously for 8 h with series of anticancer drugs that act primarily by different mechanisms of action (etoposide (VP-16), a DNA topoisomerase II inhibitor; vinblastine, a tubulin polymerization inhibitor; paclitaxel, a microtubule depolymerization inhibitor; cis-platinum(II) diammine dichloride, a DNA adducts inducing agent) (Fig. 4). The extent of protection conferred by Bcl-xL was nearly complete for all drugs at or below 10 μM. At 100 μM, protective effects were observed in transfected U-937 cells treated with paclitaxel and vinblastine and reduced protection was observed at high doses of cis-platinum diammine dichloride and VP-16 (Fig. 4). Taken together, these results suggest that Bcl-xL acts downstream of the primary mechanisms of action of these different drugs and seems to confer a multidrug resistance modulation.

Effect of Bcl-xL on the activation of apoptogenic protease cascade. A series of caspases is involved in the apoptotic process [102, 103] and recent studies have also revealed the importance of serine-like protease activities during DNA topoisomerase inhibitor-induced apoptosis [70-80, 82]. To determine whether Bcl-xL protein functions primarily upstream of a proteolytic cascade preventing its activation, first caspase activities were monitored using the specific fluorogenic peptide derivatives DABCYL-YVADAPV-EDANS and Ac-DEVD-AMC, substrates of caspase 1-like and caspase 3-like, respectively. Caspase 3-like activity was detected rapidly in cytosolic extracts obtained from U937 cells treated with 0.1 μM (Fig. 5a, left panel) and 0.5 μM CPT (Fig. 5a, right panel). In contrast a slower increase in caspase 3-like activity was detected in cysolic extracts obtained from HA-Bcl-xL U937 cells with most prononced difference in cells treated at 0.1μM CPT. No increase in DABCYL-YVADAPV-EDANS hydrolysis was detected following CPT treatment in both cell lines treated with 0.5 μM CPT suggesting that caspase 1-like activity is neither activated nor involved in the apoptotic process induced by CPT in U937 cells. To test whether Bcl-xL was preventing caspase 3-like activition or directly interfering with its activity, we have produced and purified to homogeneity recombinant Bcl-xL(ΔTM) protein. Bcl-xL(ΔTM) protein is used for these studies because

full-length recombinant Bcl-xL proteins are highly insoluble. Nevertheless, Bcl(ΔTM)-related proteins remain active *in vitro*. [108-110]. Fig. 5b (left panel) shows a SDS-polyacrylamide gel electrophoresis of various fractions in the purification of the recombinant Bcl-xL(ΔTM) protein. Addition of recombinant Bcl-xL(ΔTM) protein in extracts containing DEVDase activity has no effect whereas the tetrapeptide derivative Ac-Asp-Glu-Val-aspartic acid aldehyde (Ac-DEVD-CHO) completely inhibits DEVDase activity (Fig. 5b, right panel). TPCK at 10 μM has no significant inhibitory effect on DEVDase activity. Taken togheter, these results showed that Bcl-xL delays the activation of caspase 3-like activity but has no direct inhibitory effect on this cell-death protease.

To further characterize the effect of Bcl-xL, we used a cell-free system that we have developed previously. This cell-free system has been proven convenient to measure and detect DNA fragmentation promoting activities in cytosolic extracts prepared from cells undergoing apoptosis. These activated extracts contain factors that trigger DNA fragmentation typical of apoptosis when incubated with isolated nuclei obtained from control untreated cells [78, 80, 89, 104, 111]. However, these extracts do not digest plasmid DNA suggesting the requirement for nuclear factors to cause DNA fragmentation (results not shown). Recently, we have reported that distinct DNA fragmentation promoting activities present in these extracts lead to high molecular weight and oligonucleosome-sized DNA fragmentation pathway [80, 112]. Blocking caspase activities *in vivo* abrogates completely the appearance of DNA fragmentation induced by CPT; however, blocking caspase activities *in vitro*, in extracts obtained from CPT-treated cells using high concentration of the tetrapeptide derivative Ac-DEVD-CHO (200 μM), attenuates only slightly the extent of high molecular weight DNA fragmentation. These observations suggest that Ac-DEVD-CHO could prevent the accumulation of DNA fragmentation promoting factors *in vitro* but could not inhibit directly those that are already activated in extracts obtained from CPT-treated cells [80, 112]. Furthermore, addition of 10 μM TPCK *in vivo* abrogates completely the extent of oligonucleosome-sized DNA fragmentation without interfering with DEVDase activity and the occurrence of high molecular weight DNA fragmentation in CPT-treated cells; addition of 10 μM TPCK *in vitro*, in extracts obtained from CPT-treated cells, causes a similar effects and markely reduced the extent of oligonucleosome-sized DNA fragmentation but does not attenuate the extent of high molecular weight DNA fragments in the cell-free system [80, 112].

We therefore tested the effect of adding purified Bcl-xL on the activity of those DNA fragmentation promoting factors in cell-free system. Cytosolic extracts of CPT-treated U-937 cells prepared 2 h and 4 h after drug treatment can trigger DNA fragmentation when incubated with isolated control nuclei in the cell-free system (Fig. 6; upper panels). Cytosolic extracts prepared 1 h after drug treatment show no activity in the cell-free system consistent with lack of detectable DNA fragmentation induced by CPT in cultured cells. Activities in the 2 h extracts are inhibited almost completely by N-tosyl-L-phenylalanylchloromethyl ketone (TPCK), and slightly inhibited by the tetrapeptide derivative Ac-Asp-Glu-Val-aspartic acid aldehyde (Ac-DEVD-CHO) a caspase 3-like inhibitor (Fig. 6; upper panels). Activities in the 4 h extract are inhibited by Ac-DEVD-CHO and TPCK. The tetrapeptide Ac-Tyr-Val-Ala-aspartic acid aldehyde (Ac-YVAD-CHO), a caspase 1-like inhibitor, has no effect either in the 2 h or 4 h extracts (Fig. 6; upper panels). The TPCK-sensitive activities in those extracts remain uncharacterized as yet. Although TPCK could have a broad spectrum of activities, at the concentration used in this study (10 μM) it does not inhibit significantly DEVDase activity in cultured cells and in cell-free system (Fig. 5b, right panel) [80] and neither prevents PARP, Lamin A and B cleavage in cell-free system [113] but abrogates the oligonucleosome-sized DNA fragmentation both in *vivo* and *in vitro* [80, 112].

In cytosolic extracts of CPT-treated Bcl-xL-transfected U-937 cells, no DNA fragmentation promoting activities were detected 2 h and 4 h after drug treatments in the cell-free system (Fig. 6; lower panels) consistent with the protective effect confered by Bcl-xL in CPT-treated cultured cells. However, 8 h after drug treatment activities are found in the cell-free system consistent with the beginning of DNA fragmentation observed in cultured cells. Furthermore, activities in the 8 h extracts were prominently inhibited by TPCK and by Ac-DEVD-CHO (Fig. 6; lower panels). Taken together these results show that Bcl-xL protein acts by preventing the activation of these DNA fragmentation promoting activities. Addition of 20 μg recombinant Bcl-xL(ΔTM) protein in these extracts fails to inhibit these activities and DNA fragmentation in the cell-free system whereas Ac-DEVD-CHO and TPCK showed potent inhibition (Fig. 6; upper and lower panels). These results suggest that Bcl-xL does not affect those activities. Taken together, our results indicate that Bcl-xL acts upstream of proteolytic cascades involved in the apoptotic process and DNA fragmentation pathway preventing their activations. Thus, Bcl-xL seems to be an important control point that can block signals that will otherwise activate these proteases.

DISCUSSION

The human Bcl-x gene belongs to the Ced-9/Bcl-like family that regulates cell survival and cell death. Alternative splicing of the Bcl-x gene results in at least two distincts bcl-x mRNAs, bcl-xL and bcl-xS [18]. A third form of spliced mRNAs, nammed Bcl-xES, has been detected also in a series of haematopoietic cell lines including U-937 cells; its role and function are under investigation [114]. The first study with Bcl-x gene products indicated that Bcl-xL inhibits cell death upon growth factor withdrawal while Bcl-xS has an opposite effect, inhibiting the protective effect confered by Bcl-2 [18]. Immunohistochemical analysis of Bcl-x expression showed that Bcl-x proteins are expressed in a variety of cell types predominently in a variety of neuronal, haematopoietic, reproductive and epithelial cells [115, 116]. The central role of Bcl-x regulating the apoptotic threshold of haematopoietic cells and central nervous system tissues has been revealed by gene knock-out studies in mice [117]. Gene transfer studies in human cell lines have indicated also that high expression of Bcl-xL confers a protective effect from apoptosis in B lymphocytes exposed to gamma-irradiation and reactive oxygen species [118]. Multidrug resistance variant cell lines have been isolated recently based on Bcl-xL expression rather then P-glycoprotein expression [39], and other studies have reported that Bcl-xL provides resistance to ionizing radiation and various cytotoxic drugs in leukemia and neuroblastoma cells [40, 42]. Similarly, overexpression of the Bcl-xS protein was reported to sensitisize human breast carcinoma cells to chemotherapeutic drugs [49, 119]. Although all these studies brought major evidence that Bcl-xL has a protective effect upon induced-apoptosis, its relation with other components of the apoptotic machinery is unknown.

In this study, by selecting transfected U-937 variants that express differential level of the Bcl-xL protein, we show that its protective effect is directly related to its level of expression. Ordered balance within the ratio of effector and repressor proteins regulating the decision of a cell to survive or die has already been suggested to form an important checkpoint for cell death [120]. Our resuls are consistent with that model and say that the level of expression of the Bcl-xL protein might be an important mechanism regulating chemotherapeutic drug sensitivity and resistance; its observed high expression level in human tumours suggests that Bcl-xL might play an important role in treatment outcome.

Our results show also that Bcl-xL does not interfered with the primary mechanism of action of anticancer drugs such as CPT, the level of protein-DNA cleavable complexes and extent of DNA synthesis reduction have been found similar in

transfected and control cells following CPT treatment. Although signals emerging from unfinished replication and damaged DNA in these cells are yet unknown, this study indicates that Bcl-xL protein is a control point of these signals and influences the decision of a cell to survive or die. Similarily, Bcl-xL was shown to significantly inhibit taxol-induced apoptosis in human HL60 cells without affecting the tubulin polymerization and mitotic arrest due to taxol [42].

Activation of apoptotic proteases is a critical step in dying cells. Series of caspases and somes serine-like protease activities were reported to play a central role in executing apoptosis [70-72, 78-80, 82, 102, 103]. Few viral proteins could interact and inhibit directly the activated caspases. The *Baculovirus* protein p35 is a potent inhibitor of caspases [121-123], while the *Poxvirus* CrmA gene product encodes a protease inhibitor of caspase 1 and 8 and the serine protease granzyme B [124-127]. More recently, X-linked IAP was reported to inhibit active cell-death proteases [128]. In this study, we investigate the relation between Bcl-xL and the activation of caspases and TPCK-sensitive activities. We examined whether Bcl-xL prevents the activation of the proteases required in the execution phase of apoptosis or, whether Bcl-xL interacts with active proteases, thus inhibiting their activities.

Our experiments using a cell-free system in combination with purified recombinant Bcl-xL(ΔTM) protein, provide evidence that in cells protected from apoptosis by Bcl-xL, the activation of DEVDase, TPCK-sensitive and DNA fragmentation promoting activities was prevented and delayed. These activities were inhibited in the cell-free system by TPCK and Ac-DEVD-CHO while Ac-YVAD-CHO, a caspase -1-like protease inhibitor has no potent inhibitory effect. Addition of recombinant Bcl-xL(ΔTM) protein in activated extracts has no effect indicating that once activated, Bcl-xL cannot directly inhibit these proteases. Our cell-free system was developed to study the biochemical pathways involved in DNA fragmentation using cytosolic extracts activated by DNA damaging agents and protein kinase inhibitors [78, 89, 104, 105, 111]. Similar cell-free systems were developed independently by others, using either S/M phase cytoplasmic extracts [129] or activated cytosolic extracts from *Xenopus laevis* oocytes [130]. More recently, Lazebnik *et al.* have shown that neither PARP, Lamin A and B cleavage were inhibited in the presence of TPCK in their cell-free system extracts [113]. These results indicated that the TPCK-sensitive activities found in cell-free system are not related to the caspases involved in PARP, Lamin A and B cleavage. Furthermore, recent studies have revealed that TPCK does not inhibit caspase 3 activity in cultured

cells and in cell-free system but prevents the oligonucleosome-sized DNA fragmentation associated with apoptosis suggesting that TPCK-sensitive activities are involved in DNA fragmentation pathway *downstream* of caspase 3 [80]. Recently, a study of Cosulich et al. using the *Xenopus laevis* eggs indicated that Bcl-2 prevents apoptotic chromatin condensation and DNA cleavage only when added prior to the activation of the caspases [131]. In recent studies, others have reported that Bcl-2 also prevents the activation of cysteine proteases in cultured cells [132, 133]. Our results with Bcl-xL in human cultured cells and using a cell-free system are in agreement and suggest that Bcl-xL, as Bcl-2, functions by preventing the activation of these proteases. Such a conserved process must then be regulated tightly to avoid unwanted cellular initiation of the proteolytic cascade.

In summary, our results suggest that Bcl-xL is a primary checkpoint that can block or delay transmission of cell death signals emerging from DNA damage and prevents activation of an apoptotic protease cascade. Recent progress suggests that the Ced-9/Bcl-like proteins might function more specifically on the opening of mitochondrial megachannels and the release of apoptogenic protease activators from mitochondria such as the apoptosis-inducing factor (AIF) and cytochrome c [134]. The identification of the cell death signals emerging from DNA damage to mitochondria and understanding how Bcl-xL block these signals and prevents the activation of proteases will help to design new therapeutic protocols to control tumor growth.

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Table 1. Formation of protein-DNA complexes induced by camptothecin in control and transfected U937 Cells

CPT	U-937	U-937-xL$^{1.50}$
Control	183 ± 20	183 ± 16
0.1 μM	920 ± 101	980 ± 149
0.5 μM	1723 ± 317	1751 ± 176
1.0 μM	1818 ± 279	1904 ± 243

[^{14}C]-thymidine labeled cells were treated with various concentrations of CPT for 30 min. Formation of protein-DNA complexes was measured by the KCl-SDS precipitation assay described in "Materials and Methods". Values are expressed as precipitable counts (cpm) and represent the means ± S.E. of 5 determinations. The total cpm per assay were determined to be 1.0 x 10^4. U-937-xL$^{1.50}$ mean U-937 cells transfected with Bcl-xL and selected at 1.5 mg/ml hygromycin.

Table 2. Rates of DNA synthesis after campthotecin in control and transfected U937 Cells

CPT	U-937	U-937 -xL$^{0.25}$	U-937-xL$^{0.50}$	U-937-xL$^{1.50}$
0.1 μM	80.9 ± 9.2	73.7 ± 11.7	71.2 ± 7.8	73.8 ± 15.8
0.5 μM	69.8 ± 9.1	65.0 ± 15.1	64.5 ± 6.4	63.7 ± 10.4
1.0 μM	55.0 ± 5.0	58.7 ± 8.9	54.2 ± 6.2	51.7 ± 9.2

Cells were treated with various concentrations of CPT for 30 min. [^{3}H]-thymidine incorporation were determined by 10 min pulse experiments. Values are expressed as % of DNA synthesis relative to control untreated cells and represent the means ± S.E. of 2 independent experiments performed in triplicate (n=6). U-937 -xL$^{0.25}$, U-937 xL$^{0.50}$ and U-937-xL$^{1.50}$ mean U-937 cells transfected with Bcl-xL and selected at 0.25 mg/ml, 0.50 mg/ml and 1.5 mg/ml hygromycin, respectively.

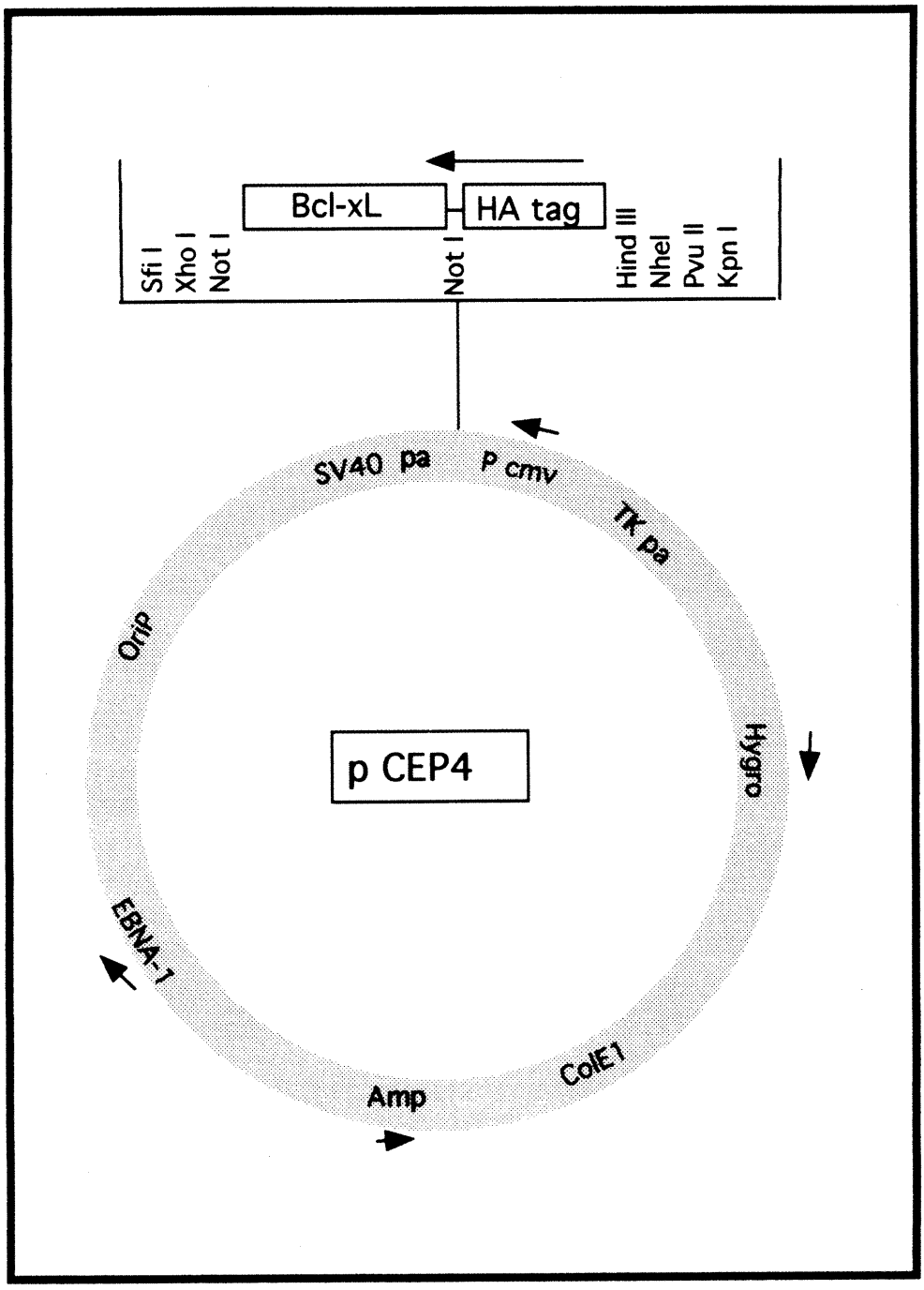
Bcl-xL
HA tag
Sfi I
Xho I
Not I
Not I
Hind III
Nhel
Pvu II
Kpn I
SV40 pa
P cmv
TK pa
Orip
p CEP4
Hygro
EBNA-1
Amp
ColE1

Figure 1

Fig. 1. Schematic representation of pCEP4-HA-BCL-xL expression vector.

The pCEP4 vector was first modified by adding Kosak and HA epitope tag sequences at the *HindIII - NotI* restriction sites; Bcl-xL ORF cDNA was then inserted in frame with the HA epitope tag sequences at the unique*NotI* restriction site. Vector was sequenced prior to transfection.

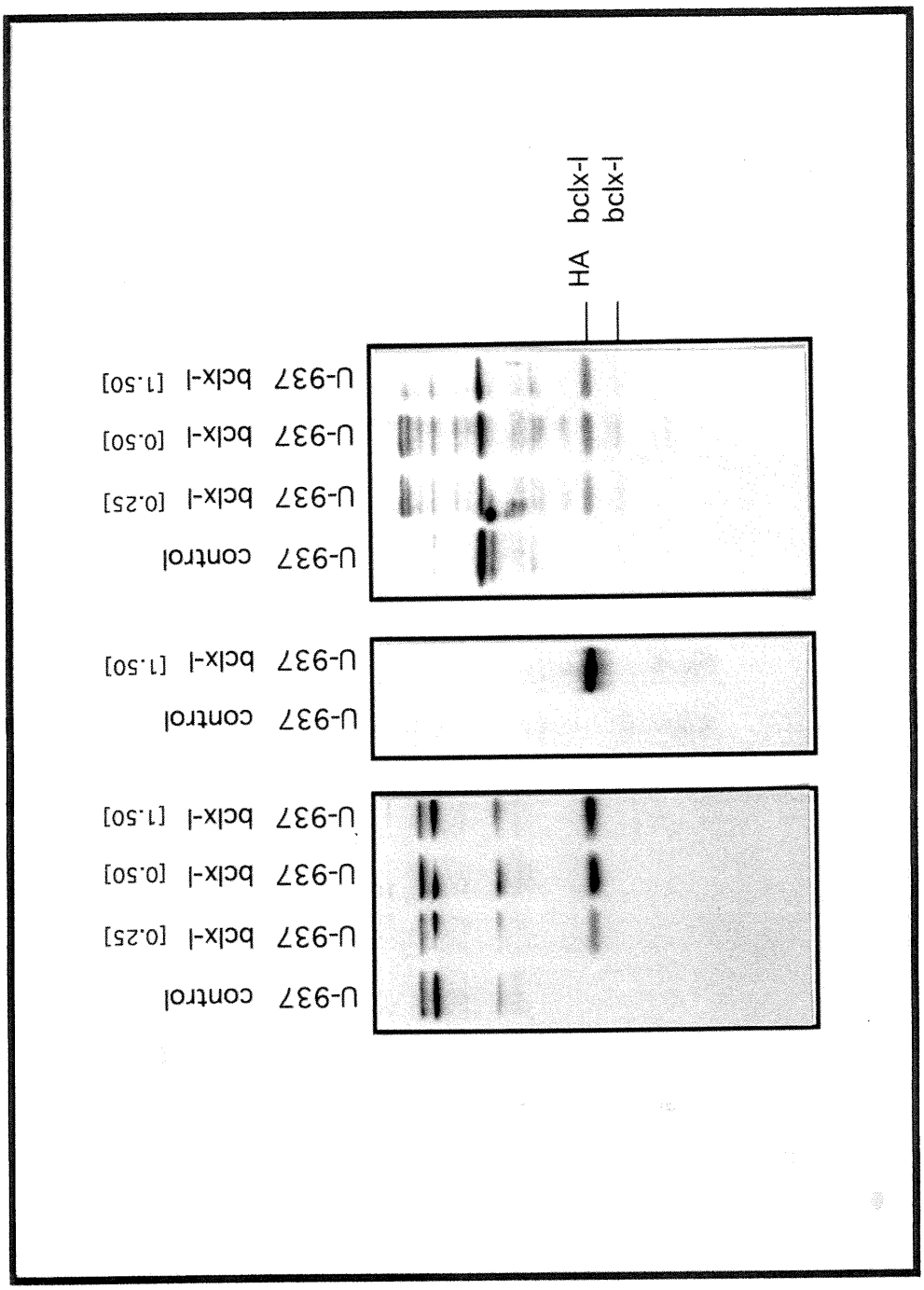
HA
bclx-l
bclx-l
U-937 bclx-l [1.50]
U-937 bclx-l [0.50]
U-937 bclx-l [0.25]
U-937 control
U-937 bclx-l [1.50]
U-937 control
U-937 bclx-l [1.50]
U-937 bclx-l [0.50]
U-937 bclx-l [0.25]
U-937 control

Figure 2a

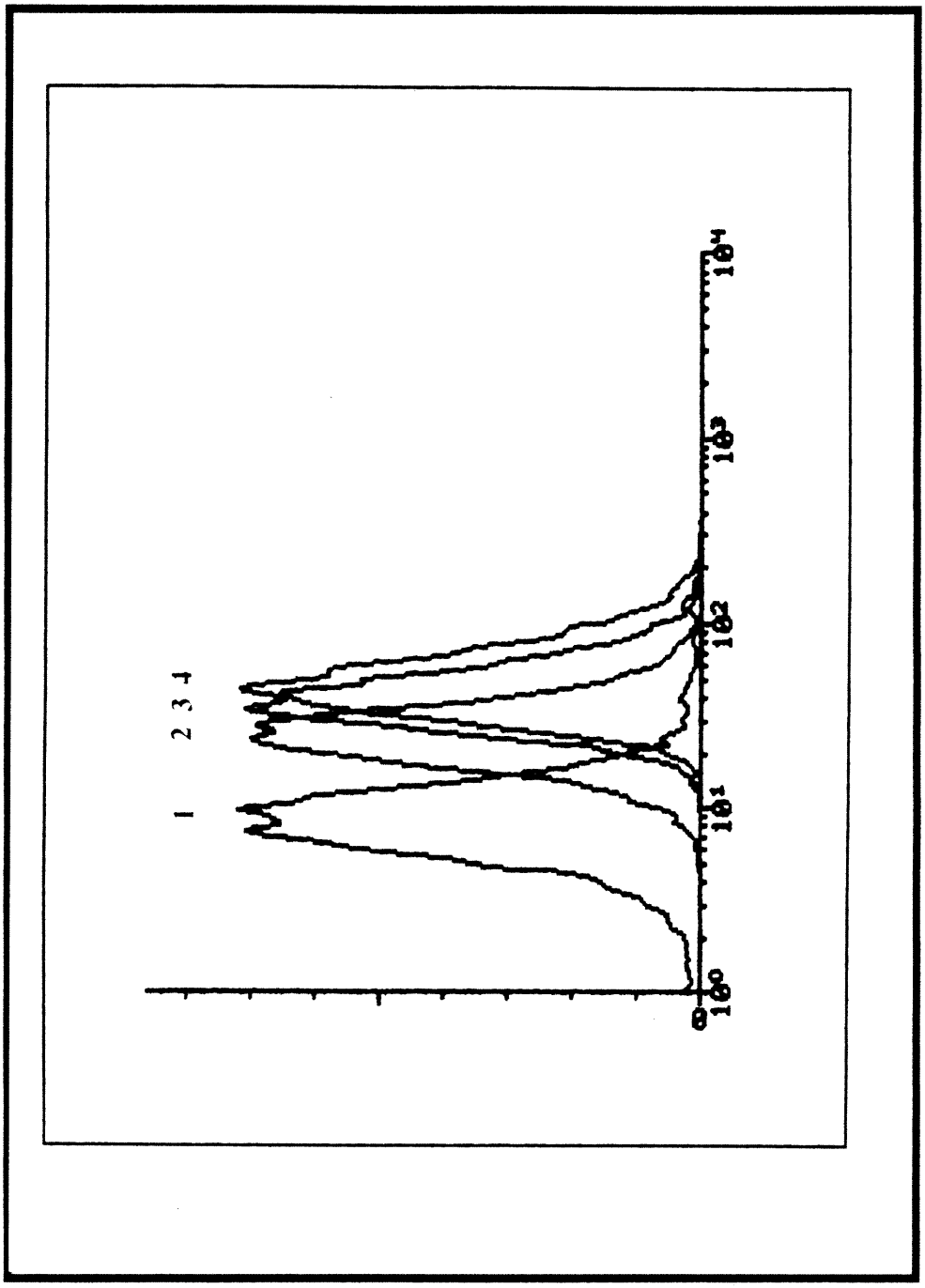

Figure 2b

Fig. 2. Expression of Bcl-xL in control and Bcl-xL-transfected U-937 cells.

A) Whole-cell extracts were prepared as described in Materials and Methods from U-937 and Bcl-xL-transfected U-937 cells selected at 0.25 mg/ml, 0.50 mg/ml and 1.5 mg/ml hygromycin. HA-Bcl-x proteins were detected by using anti-HA peptide monoclonal antibodies *(Left Panel)* and from [^{35}S]-methionine -[^{35}S]-cysteine-labeled cells by immunoprecipitation using anti-HA peptide monoclonal antibodies captured with protein A-Sepharose *(Middle panel)*. Both HA-bcl-xL and intrinsic Bcl-xL proteins were detected using anti-Bcl-xL polyclonal antibodies *(Right Panel)*.

B) Flurorescence intensity distribution of permeable cells was analyzed in the presence of 10 μg / ml anti-HA rhodamine-conjugated monoclonal antibodies (x axis: rhodamine fluorescence; y axis: U937 cells counts). Control U-937 cells (1, stained); HA-Bcl-xL U937 cells selected at 0.25 mg/ml hygromycin (2, stained); HA-Bcl-xL U937 cells selected at 0.50 mg/ml hygromycin (3, stained). HA-Bcl-xL U937 cells selected at 1.50 mg/ml hygromycin (4; stained).

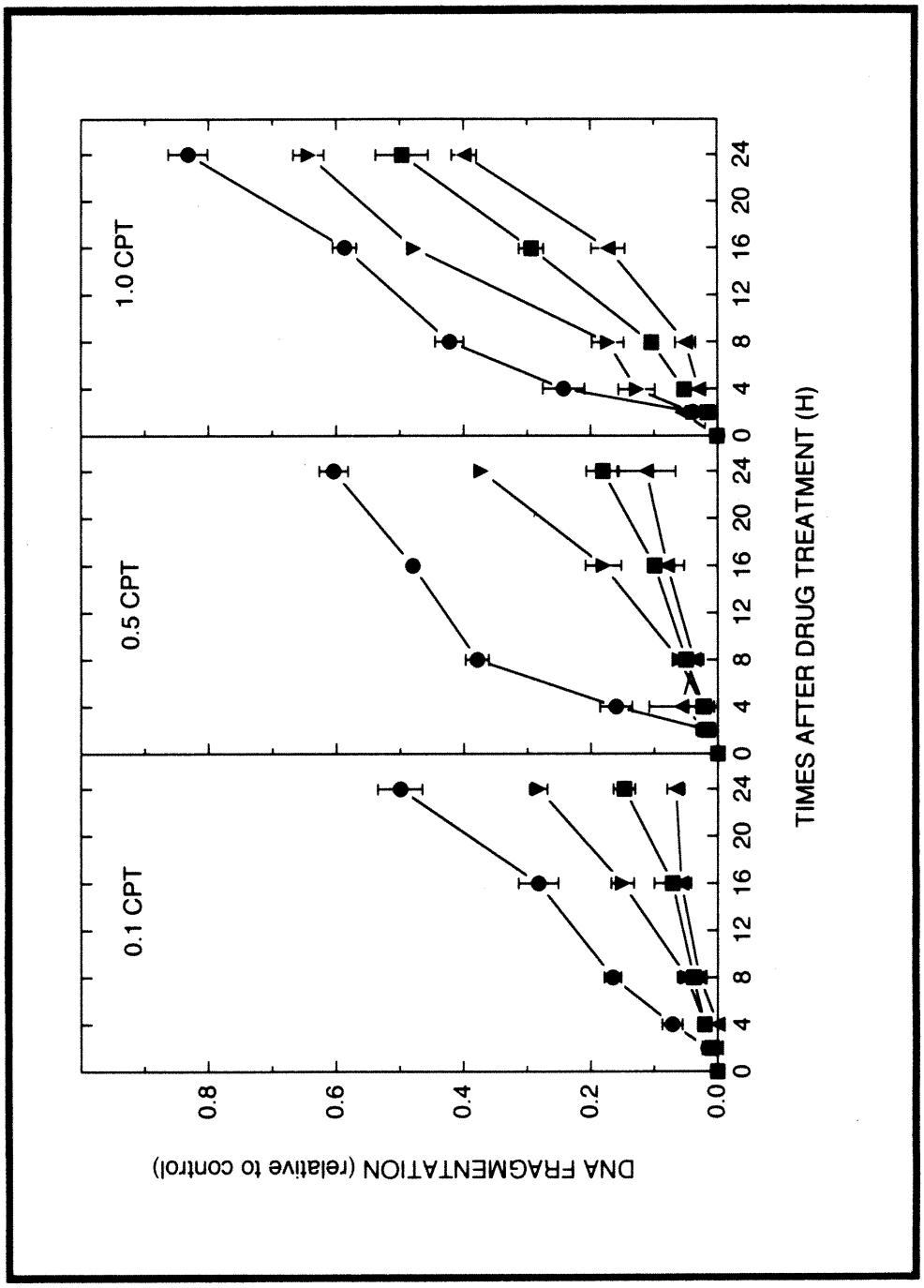
DNA FRAGMENTATION (relative to control)
0.0
0.2
0.4
0.6
0.8
0.1 CPT
0.5 CPT
1.0 CPT
0 4 8 12 16 20 24
TIMES AFTER DRUG TREATMENT (H)

Figure 3

Fig. 3. Inhibition of CPT-induced DNA fragmentation in Bcl-xL transfected U-937 cells.

[^{14}C]Thymidine-labeled cells were treated for 30 min with 0.1 (left panel), 0.5 (middle panel) or 1.0 μM CPT (right panel). After drug removal, cells were incubated in drug free-medium and at the indicated times (x axis; hour), DNA fragmentation was determined by filter elution assays. Results are expressed as percent DNA fragmentation. Points represent the means +/- SE of four independent experiments performed in duplicate. Symbols are U-937 cells (●); Bcl-xL-transfected U-937 cells selected at 0.25 mg/ml (▼), 0.50 mg/ml (■) and 1.5 mg/ml hygromicin (▲).

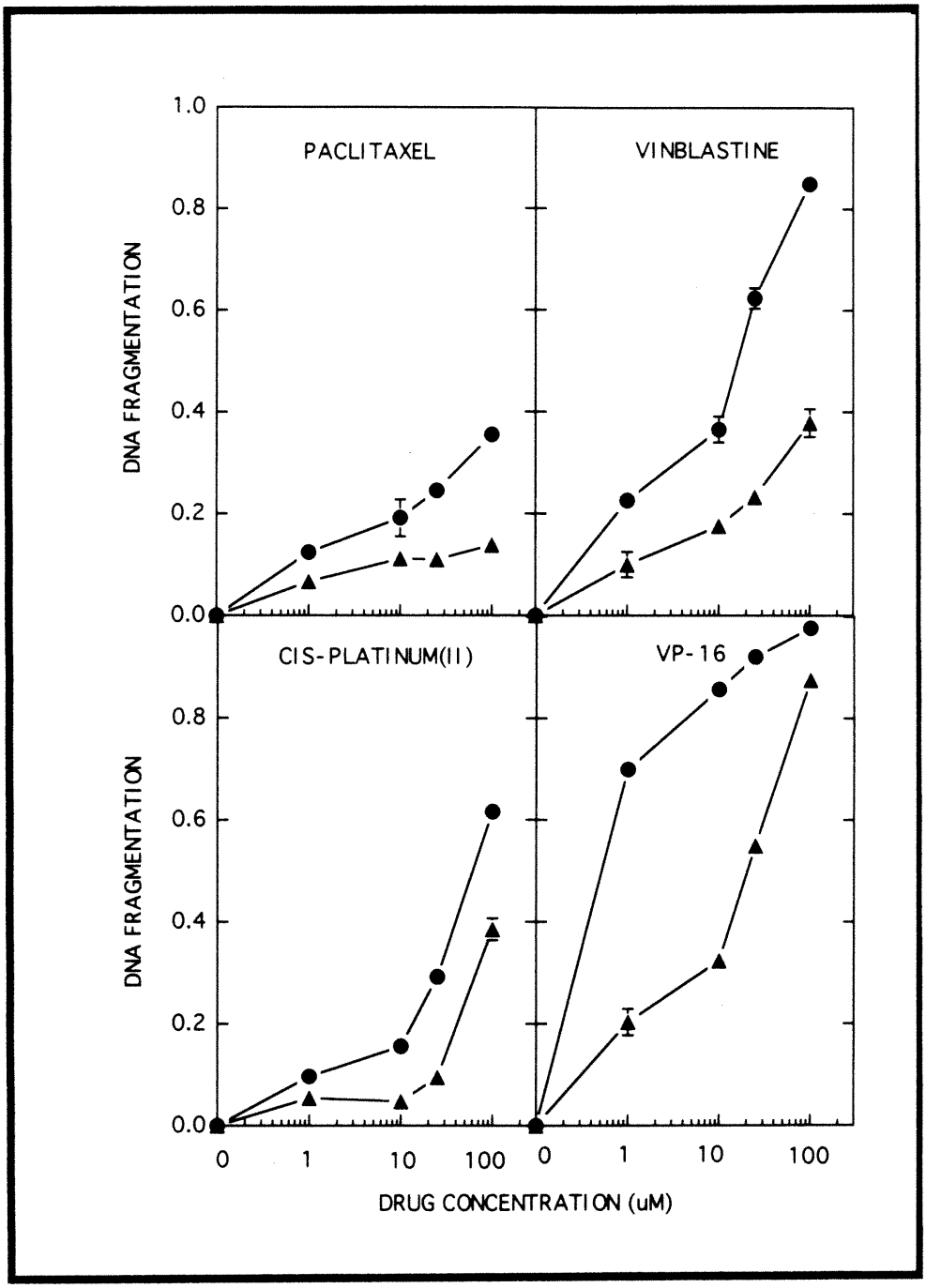
PACLITAXEL
VINBLASTINE
CIS-PLATINUM(II)
VP-16
DNA FRAGMENTATION
DNA FRAGMENTATION
1.0
0.8
0.6
0.4
0.2
0.0
0
1
10
100
DRUG CONCENTRATION (uM)

Figure 4

Fig. 4. Inhibition of DNA fragmentation in Bcl-xL transfected U-937 cells following paclitaxel, vinblastine, cis-platinum and VP-16 treatments.

[^{14}C]Thymidine-labeled cells were treated for 8 hours with various concentrations of paclitaxel (upper left panel), vinblastine (upper right panel), cis-platinum (lower left panel) and VP-16 (lower right panel). DNA fragmentation was determined by filter elution assays. Results are expressed as percent DNA fragmentation. Points represent the means +/- SE of two experiments performed in triplicate. Symbols are U-937 cells (●) and bcl-xL-transfected U-937 cells selected at 1.5 mg/ml hygromicin (▲).

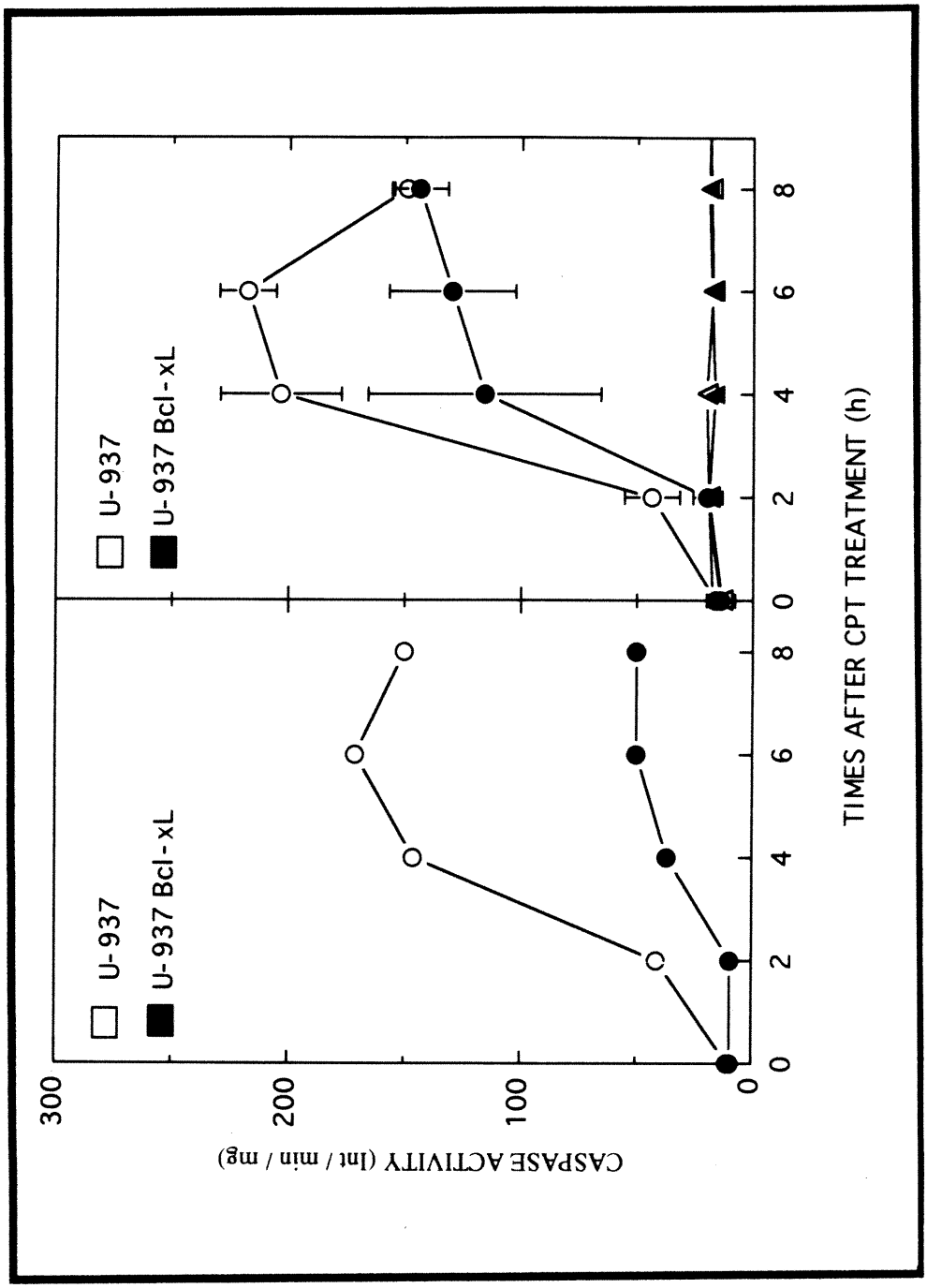
CASPASE ACTIVITY (Int / min / mg)
300
200
100
0
U-937
U-937 Bcl-xL
0
2
4
6
8
TIMES AFTER CPT TREATMENT (h)

Figure 5a

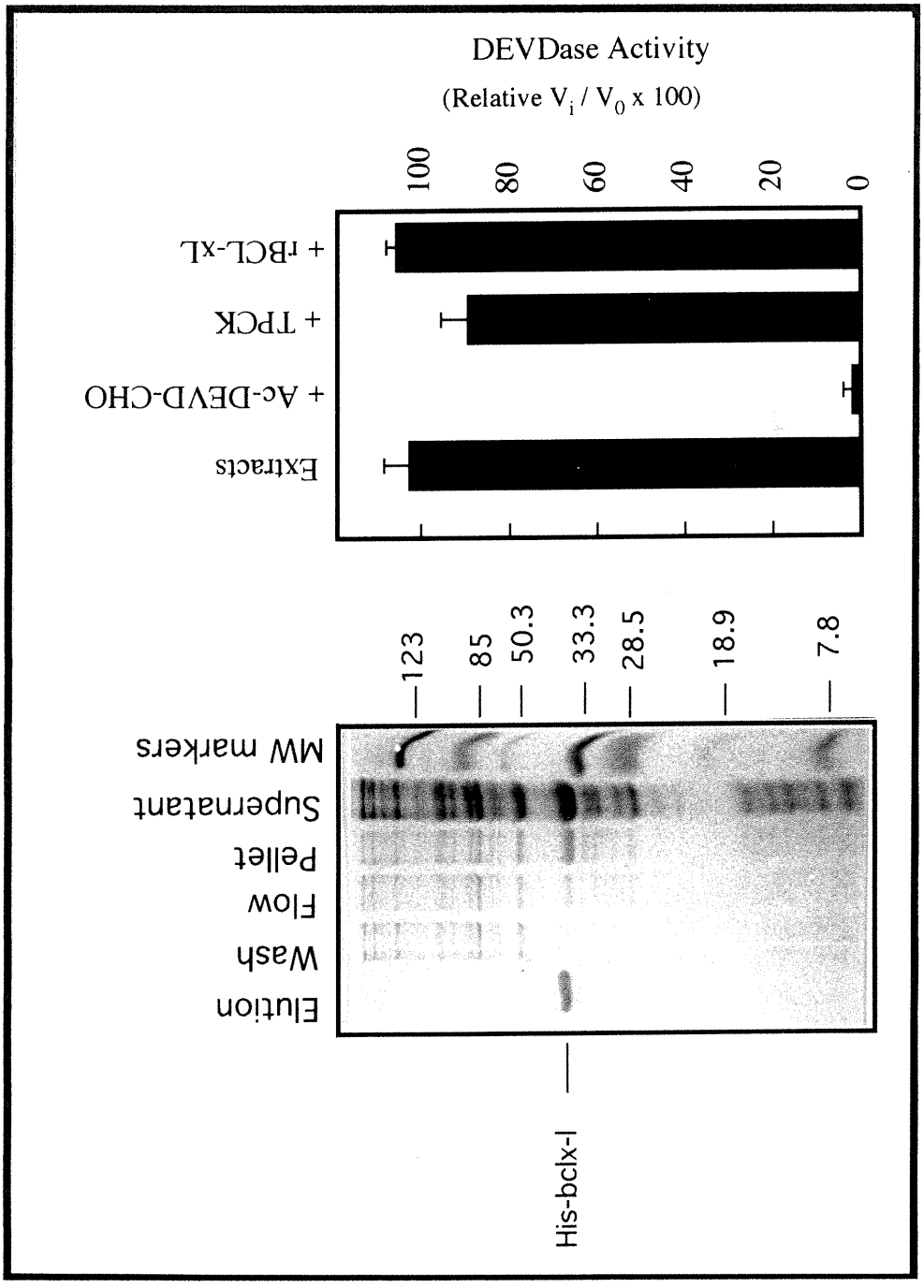
DEVDase Activity
(Relative V_i / V_0 x 100)
100
80
60
40
20
0
Extracts
+ Ac-DEVD-CHO
+ TPCK
+ rBCL-xL
123
85
50.3
33.3
28.5
18.9
7.8
MW markers
Supernatant
Pellet
Flow
Wash
Elution
His-bclx-l

Figure 5b

Fig. 5a. Caspase-1-like and caspase-3-like activities in control and Bcl-xL-transfected U-937 cells following CPT treatment.

Cytosolic extracts were prepared from control (open symbols) and transfected U-937 cells (close symbols) at the indicated times following 30 min treatment with 0.1 μM CPT (left panel) and 0.5 μM CPT (right panel). Caspase 3-like activity was monitored continuously at 37°C by detecting fluorescence emission in the presence of Ac-DEVD-AMC (○ , ●) at an excitation wavelength of 380 nm and emission wavelenght of 460 nm. Caspase 1-like activity was monitored in the presence of DABCYL-YVADAPV-EDAN (Δ , ▲) using excitation wavelength of 360 nm and emission wavelenght of 490 nm. Enzyme activities were measured as initial velocities and expressed as relative intensity / min / mg. Points represent the means +/- SD of three independent experiments (right panel) and represent values of a single experiment (left panel).

Fig. 5b. The effect of Bcl-xL(ΔTM) on DEVDase activity.

Left panel: SDS-polyacrylamide gel electrophoresis of various fractions in the purification of recombinant Bcl-xL(ΔTM) protein. From right to left, lanes are a) molecular weight markers; b) bacterial lysate supernatant (soluble fraction); c) bacterial pellet (insoluble fraction); d) column flow through; e) column wash and f) eluted bcl-xL protein. Gel was stained with Comassie Blue R-250.

Right panel: DEVDase activity was measured by monitoring the hydrolysis of Ac-DEVD-AMC in the absence and presence of 200 μM Ac-DEVD-CHO, 10 μM TPCK and 20 μg Bcl-xL(ΔTM). Data are expressed as percent relative activity (v_i / v_0) and represent the means +/- SE of three independent determinations. The amount of Bcl-xL(ΔTM) protein added per assay represent 10% of the total extracts (20 μg Bcl-xL(ΔTM) protein in 200 μg of extracted proteins).

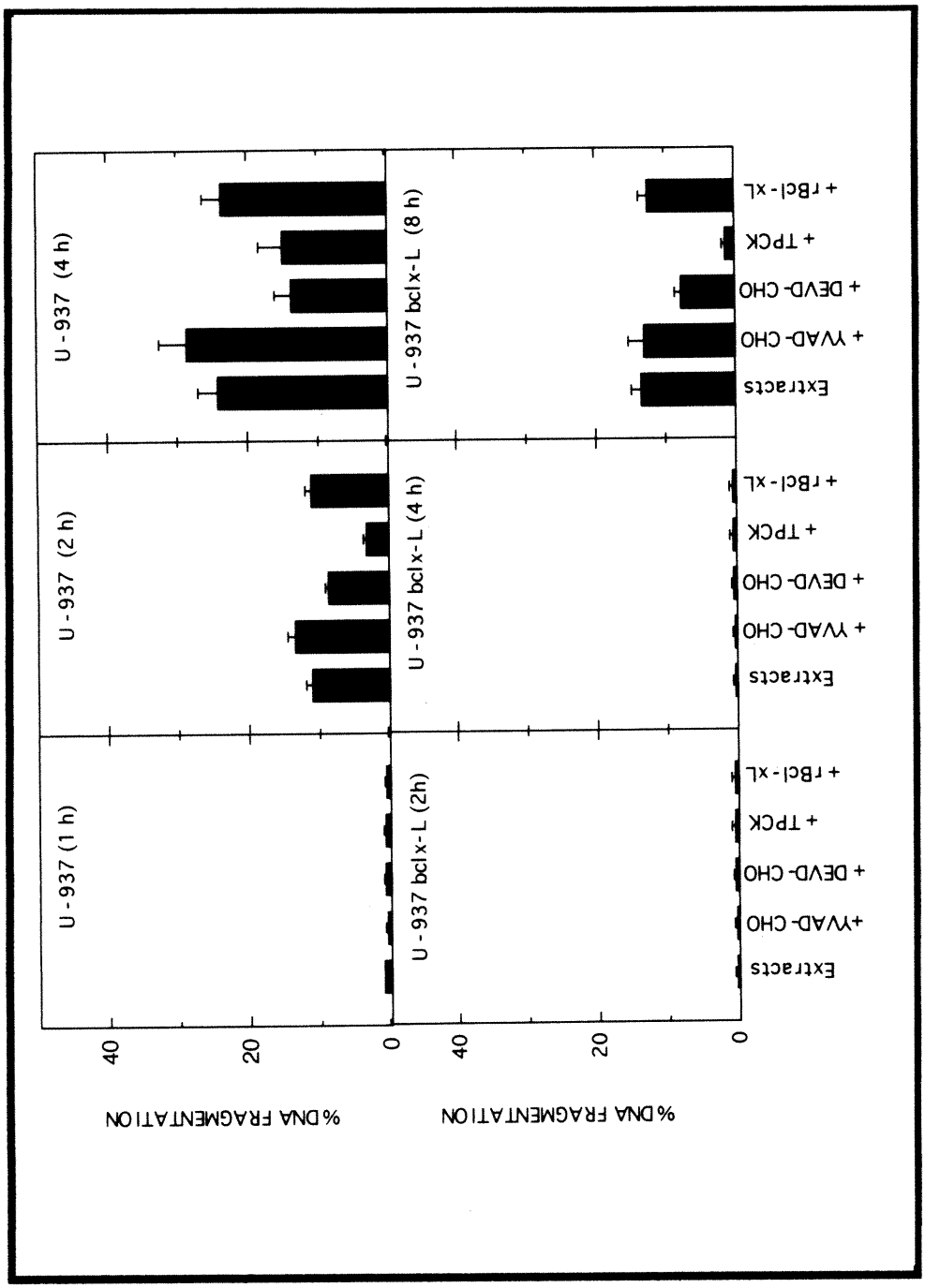
U - 937 (1 h)
U - 937 (2 h)
U - 937 (4 h)
U - 937 bclx-L (2h)
U - 937 bclx-L (4 h)
U - 937 bclx-L (8 h)
Extracts
+YVAD- CHO
+ DEVD- CHO
+ TPCK
+ rBcl - xL
0
20
40
% DNA FRAGMENTATION

Figure 6

Fig. 6.The effect of Bcl-xL(ΔTM) on DNA fragmentation promoting activities in a cell-free system.

Cytosolic extracts were prepared from U-937 cells (upper panels) and Bcl-xL-tranfected U-937 cells (lower panels) at the indicated times following CPT treatment (0.5 μM for 30 min). [^{14}C]Thymidine-labeled isolated nuclei from untreated cells were then incubated for 30 min with cytosolic extracts alone or in the presence of 10 μM TPCK, 200 μM Ac-YVAD-CHO, 200 μM Ac-DEVD-CHO or purified recombinant Bcl-xL(ΔTM) protein (20 μg Bcl-xL(ΔTM) protein in 200 μg of extracted proteins). DNA fragmentation was determined by filter elution assays. Results are expressed as percent DNA fragmentation. Bars represent the means +/- SE of four independent experiments performed in triplicate. The Bcl-xL transfected U-937 line used in these experiments was the one selected at 1.5 mg/ml hygromycin.

CHAPITRE 3.

Bax-α promotes apoptosis induced by cancer chemotherapy and accelerates the activation of caspase 3-like cysteine proteases in p53 double mutant B lymphoma Namalwa cells

Cell Death Diff. **5, 506-516, 1998**

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Avant propos:

Dans ce travail nous étudions le rôle de la protéine Bax-α dans l'apoptose induite par les agents anticancéreux. L'expression de Bax-α dans les cellules de lymphome B Namalwa sensibilise les cellules à l'apoptose induite par la camptothécine, l'étoposide et la vinblastine. Contrairement à Bcl-xL, Bax-α exerce une activité pro-apoptotique dans les cellules traitées en favorisant l'activation des caspases impliquées dans la phase d'exécution de l'apoptose.

• A. Steyaert a contribué au clonage de l'ADN complémentaire de Bax-α. G. Cimoli a amorçé des travaux pour produire une protéine recombinante. Ses efforts ont été soulignés par sa présence comme co-auteur sur le manuscrit

SUMMARY.

Stable transfected human p53 (mt/mt) B lymphoma Namalwa variant lines showing differential expression of the Bax-α protein were derived under hygromycin selection. Overexpression of Bax-α in these variant cells accelerates cell death induced by short or continuous treatments with various concentrations of camptothecin, etoposide, vinblastine and shows no accelerating cell death activity in cis-platinum and paclitaxel-treated cells. Activation of apoptosis and oligonucleosome-sized DNA fragmentation was observed in the variant lines with more pronounced effect in cells containing high level of Bax-α protein. These results suggest that increased cell death mediated by anticancer drugs correlates with Bax-α level of expression and that Bax-α sensitizes Namalwa cells treated at low drug concentrations. The extent of DNA synthesis inhibition following DNA topoisomerase inhibitor treatments was similar in control and all transfected Namalwa cells suggesting that Bax-α acts downstream of DNA topoisomerase-mediated DNA strand breaks. To define further the relation between Bax-α expression and apoptosis activation, kinetics of caspase activation was measured in drug-treated cells. Caspase activities were measured using specific fluorogenic peptide derivatives DABCYL-YVADAPV-EDANS and Ac-DEVD-AMC, substrates of the caspase 1-like and caspase 3-like families, respectively. In control and Bax-α transfected Namalwa cells no increase in caspase 1-like activity was detected following camptothecin and etoposide treatments. In contrast, a significant difference in Ac-DEVD-AMC hydrolysis activity was observed in Bax-α transfected Namalwa cells compared to that of control Namalwa cells after camptothecin and etoposide treatment. Increased caspase 3-like activity correlated also with poly(ADPribosyl) polymerase cleavage. Taken together, these results suggest that Bax-α sensitize B lymphoma cells to series of anticancer drugs and accelerates the activation of apoptotic protease cascade.

Key Words: Bax; cancer chemotherapy; caspase; lymphoma; apoptosis

INTRODUCTION

Programmed cell death (apoptosis) is a fundamental process essential for normal regulation of cell homeostasis and seems to play important role in tumor development and progression (Raff et al., 1994; Thompson, 1995; Wyllie, 1997). All of the anticancer drugs used in clinic can induce apoptosis in various cell lines but the mechanisms by which these drugs activate programmed cell death are just beginning to be examined (Hickman, 1996; Reed, 1995). Several studies have provided major evidence that specific genes can act either as inhibitor or activator in the apoptotic process. The human Ced-9/Bcl-like family includes a series of related gene products that either inhibits apoptosis such as Bcl-2 (Hockenbery et al., 1990; Korsmeyer, 1992; Reed, 1997; Vaux et al., 1988), Bcl-xL (Boise et al., 1993), Mcl-1 (Kozopas et al., 1993), A1 (Lin et al., 1993), Bag (Takayama et al., 1995; Takayama et al., 1996), Bfl-1 (Choi et al., 1995), Bcl-w (Gibson et al., 1996) and Brag-1 (Das et al., 1996), while others such as Bax (Oltvai et al., 1993), Bcl-xS (Boise et al., 1993), Bak (Chittenden et al., 1995; Farrow et al., 1995; Kiefer et al., 1995), Bad (Yang et al., 1995), Bik (Boyd et al., 1995), and Bid (Wang et al., 1996) are associated with the activation of apoptosis. Bcl-2 has been found translocated [t(14:18)] in human B cell lymphoma and is thought to contribute to the development of B-cell neoplasia (Clarke et al., 1986; Tsujiimoto et al., 1984) while bcl-xL is often highly expressed in B lymphoma tumor cells (Xerri et al., 1996). Bcl-2 and Bcl-xL are also associated with cell death protection after treatment with anticancer compounds (Datta et al., 1995; Dole et al., 1995; Fang et al., 1995; Hockenbery, 1995; Ibrado et al., 1996; Korsmeyer, 1995; Reed, 1995) and recent observations suggested that Bax-α (Chresta et al., 1996; Krajewski et al., 1995; Sakakura et al., 1996; Thomas et al., 1996; Wagener et al., 1996) and Bcl-xS (Ealovega et al., 1996; Sumantran et al., 1995) may inhibit protective effects conferred by Bcl-2 and Bcl-xL and accelerate cell death induced by some anticancer drugs.

An emerging family of Ced-3/Ice-like cysteine proteases (caspases) has been also identified and several studies have revealed their importance in executing the process of cell death itself. The caspases are separated into three subfamilies based on their degrees of homology and by phylogenetic analysis; the ICE-related family includes Ice/caspase-1 itself with its spliced isoforms (Alnemri et al., 1995; Yuan, 1993), Tx/Ich-2/Ice rel-II/caspase-4 (Faucheu et al., 1995; Kamens et al., 1995; Munday et al., 1995), Ty/Ice rel-III/caspase-5 (Faucheu et al.,1996; Munday et al., 1995) and Ich-3/caspase-11 (Wang et al., 1996); the CPP32-related family includes CPP32/Yama/Apopain/caspase-3 (Fernandes-Alnemri et al., 1994; Nicholson et al.,

1995; Tewari et al., 1995), Mch-2/caspase-6 (Fernandes-Alnemri et al., 1995), Mch-3/Ice-Lap3/Cmh-1/caspase-7 (Duan et al., 1996a; Fernandes-Alnemri et al., 1995; Lippke et al., 1996), Mch-4/caspase-10 (Fernandes-Alnemri et al., 1996), Mch-5/Flice/Mach/caspase-8 (Boldin et al., 1996; Fernandes-Alnemri et al., 1996; Muzio et al., 1996) and the third group includes Ich-1[l and s]/caspase-2 (Wang et al., 1994) and Ice-Lap6/Mch6/caspase-9 (Duan et al., 1996b).

Chemotherapeutic drugs such as DNA topoisomerase I (top 1) and II (top 2) inhibitors (camptothecin, CPT; etoposide, VP16, respectively) trigger apoptosis in various cell lines (Barry et al., 1990; Bertrand et al., 1991; Bertrand et al., 1993; Del Bino et al., 1992; Jaxel et al., 1988; Kaufmann, 1989; Solary et al., 1993; Walker et al., 1991). These drugs are known to stabilize a transient intermediate of topoisomerase reactions where enzymes are linked to the 3' (top 1) or 5' (top 2) terminus of a DNA duplex producing DNA single- or double-strand breaks (Gupta et al., 1995; Pommier, 1997). The unknown signals emerging from DNA damage induced by chemotherapy drugs like DNA topoisomerase inhibitors are influenced by a series of checkpoints and are related to specific gene expression. Although it has been proposed that Bax-α is a wild-type p53 response gene, DNA topoisomerase inhibitors are able to trigger apoptosis in p53 mutated and null cells. Thus, to analyze further the relation between Bax-α expression, drug sensitivity and caspase activation, we have developed human B lymphoma Namalwa variant lines that express differential levels of Bax-α protein.

In this paper we show that Bax-α accelerates apoptosis induced by CPT (DNA topoisomerase I inhibitor), VP16 (DNA topoisomerase II inhibitor) and vinblastine (a tubulin polymerization inhibitor) even at very low drug concentration. The cell death promoting activity of Bax-α correlates also with its level of expression. Bax-α does not interfere with the primary mechanism of action of DNA topoisomerase inhibitor CPT and acts downstream of DNA damage-mediated DNA synthesis inhibition. However, Bax-α acts upstream of caspase and DNA fragmentation induction and accelerates the activation of caspase 3-like at low drug concentration. Taken together, these results suggest that Bax-α acts primarily downstream of the primary effect of chemotherapy drugs such as CPT and sensitizes B lymphoma cells to a series of anticancer drugs. Bax-α functions also upstream of caspase 3-like in the apoptotic process and accelerates their activation.

RESULTS

Expression of Bax-α and Bcl-xL in Namalwa cell lines. The human B lymphoma Namalwa cells are p53 double mutant cells (O'Connor et al., 1993) that contain two copies of the Epstein-Barr virus (EBV) genome integrated in opposite orientations on chromosome 1 (Lawrence et al., 1988). The Namalwa cells are characterized by their absence of EBV episomes and the lack of virus replication in response to inducers (Klein & Dombos, 1973; Matsuo et al., 1984). These cells express nuclear antigen proteins belonging to the EBNA-3 family, whose expression is proposed to play a role to maintain the lymphoblastoid cells in an immortalized state (Luka et al., 1978; Sawada et al., 1989) although a causal role for EBV in tumor development remains obscure (Horner et al., 1995). However, these cells do not express the latent membrane proteins of the LMP family (Sample et al., 1989) and the Bcl-2 homolog protein BHFR1, whose expression is primarily linked with virus replication and lytic induction (Pearson et al., 1987) and more often associated with nasopharyngeal carcinoma (Horner et al., 1995). Moreover, these cells show high molecular weight and oligonucleosome-sized DNA fragmentation patterns typical of apoptosis after short DNA damaging agent treatments (Sane et al., 1997) and express members of the Ced-9/Bcl-like family, such as Bcl-2, Bax-α and Bcl-xL (Fig. 1, lower panels). To analyze the effect of Bax-α expression on drug response and caspase activation in human B lymphoma cells, we have developed Namalwa variant lines that express differential levels of Bax-α protein. A Namalwa variant line that overexpress Bcl-xL was also derived to compare their effects. The human Bax-α and Bcl-xL cDNAs were first amplified by RT-PCR and inserted in a modified pCEP4 vector containing consensus Kosak and hemagglutinin epitope tag sequences (HA-tag). After transfection, Namalwa cells were selected as bulk culture in hygromycin (500 μg / ml, 1.5 mg / ml) and selection was maintained for about 2 mounths to obtain stable lines prior performing the experiments. Control Namalwa cells did not survive under hygromycin selection. Western blot analysis indicates that the HA-Bax-α protein is expressed at different levels with respect to hygromycin concentration (Fig. 1, upper left panel). The highest expression of HA-Bax-α has been found in transfected cells selected at 1.5 mg/ml hygromycin. High level of expression of HA-Bcl-xL was also achieved in cells selected at 1.5 mg/ml hygromycin (Fig. 1, upper righ panel). Protein expression was also visualized by immunohistochemical staining and micrographs show that all cells express the HA-epitope tagged Bax-α and Bcl-xL proteins (Fig. 2).

Effect of Bax-α upon apoptosis and DNA fragmentation. To test Bax-α activity in the selected transfected cell lines, we first determined HA-Bax-α potency to promote cell death in cells grown in low serum concentration (0.1%) or triggered with an anti-CD95/Fas antibodies for 24 h (Fig. 3). We routinely used a filter DNA binding assay to monitor and quantitate kinetics of DNA fragmentation in cells undergoing apoptosis (Bertrand et al., 1995; Bertrand & Pommier, 1995; Bertrand et al., 1991). Higher expression of Bax-α in Namalwa cells increases rates of DNA fragmentation both in cells grown in low serum concentration (0.1%) and in the presence of anti-CD95/Fas antibodies for 24 h (Fig. 3). The amount of DNA fragmentation is highest in cells expressing the highest level of Bax-α protein.

To analyze the effect of Bax-α upon anticancer drug-induced apoptosis we first evaluated the effect of CPT, a DNA topoisomerase I inbibitor, in control and Bax-α transfected Namalwa variant lines. Several studies have shown that CPT activates the morphological changes and internucleosomal DNA fragmentation associated with apoptosis. Short treatments (30 min) with CPT at moderate concentrations (0.1 μM - 0.5 μM) induced apoptosis in control Namalwa cells while low concentration of CPT such as 0.01 μM does not trigger apoptosis significantly (Fig. 4). However in the Bax-α transfected Namalwa cells, both the kinetic and extent of DNA fragmentation were more pronounced after CPT treatment at low concentration (Fig. 4). Moreover, the promoting cell death activity of Bax-α after such treatment was more pronounced in cells selected at 1.5 mg / ml hygromycin, expressing the highest level of Bax-α. At 0.01 μM and 0.05 μM CPT, the cell death promoting effect of Bax-α in the transfected lines was relatively more pronounced compared to the effect of CPT in control Namalwa cells. At higher CPT concentration (0.1 μM and 0.5 μM) the difference between the CPT-treated control and Bax-α transfected Namalwa cell line selected at 1.5 mg / ml hygromycin seems to be less pronounced (Fig. 4) and no significant differences were detected in the Bax-α variant line selected at 0.5 mg / ml hygromycin when compared to Namalwa cells (data not show). These results indicate a direct relation between the amount of Bax-α and the degree of apoptosis induced by low concentration CPT. Thus, higher level of Bax-α sensitizes these cells to low drug concentration. For comparison, Namalwa cells transfected with HA-Bcl-xL and selected at 1.5 mg / ml hygromycin, are completely resistant to CPT-induced apoptosis (Fig. 4; open circles).

CPT traps and stabilizes an intermediate of DNA topoisomerase I reaction when the enzyme is covalently linked to DNA. Stabilization of these cleavable complexes induces DNA strand breaks. These enzyme-linked DNA adducts on active

replication forks inhibit DNA synthesis. DNA replication inhibition is one of the most widely explored process to be implicated in the cytotoxic mechanism of action of DNA topoisomerase I inhibitors (Gupta et al., 1995). To determine whether Bax-α acts primarily upstream or downstream of CPT translated effect on DNA synthesis, rates of DNA synthesis were measured by thymidine pulse incorporation immediately after CPT treatments. Table 1 shows that in Namalwa control cells, immediately following CPT treatment at 0.01 μM, 0.05 μM, 0.1 μM and 0.5 μM, DNA synthesis rates were reduced to approximately 85, 75, 65 and 59% to that of control untreated cells, respectively. The extent of DNA synthesis reduction was similar in the two transfected Namalwa cells treated with the same concentrations of CPT with rates of DNA synthesis of approximately 80, 72, 64 and 57% to that of Bax-α transfected untreated cells, respectively. These results suggest that Bax-α does not interfere with the primary mechanism of action of CPT and does not amplify CPT-induced DNA synthesis inhibition.

Modulation of cell death by Bax-α was also observed in cells treated with VP-16 (DNA topoisomerase II inhibitor) and vinblastine (tubulin polymerization inhibitor). The death promoting activity of Bax-α was more pronounced in the variant line expressing the highest level of Bax-α (Fig. 5) at low VP-16 and vinblastine concentrations. While the control Namalwa cells do not show significant DNA fragmentation when treated at or below 20 μM VP-16 and vinblastine, in the high Bax-α expressing cells more than 70% DNA fragmentation was detected 8 hours after drug treatment (Fig. 5). As with CPT, these results show that higher level of Bax-α sensitizes these cells to low VP-16 and vinblastine concentrations. Conversely, overexpression of Bax-α did not significantly sensitize these cells to paclitaxel and cis-platinum(II) diammine dichloride treatment either 8 h or 24 h after drug treatment (Fig. 5). To confirm the specificity of the DNA fragmentation observed by the filter assay, we visualized the intensity of the oligonucleosome-sized DNA fragments caused by these drugs in Namalwa and Namalwa-Bax cells (Fig. 6). Overexpression of Bcl-xL in these cells completely abrogates the occurrence of DNA fragmentation (Fig. 6).

Effect of Bax-α on caspase activation. A series of caspases are involved in the apoptotic process (Kumar & Lavin, 1996; Patel et al., 1996). To determine whether Bax-α accelerates caspase activation, we monitored caspase 1-like and caspase 3-like activities using specific fluorogenic peptide substrates. In cytosolic extracts obtained from Namalwa cells, a slow increase in caspase 3-like activity was detected after 0.05 μM CPT or 10 μM VP16 treatment (Fig. 7). The kinetic of caspase

3-like activity correlates with the slow activation of DNA fragmentation following CPT and VP16 treatment in control Namalwa cells (Fig. 4 and 5). In contrast, in Bax-α transfected Namalwa cells a rapid increase in caspase 3-like activity was detected following CPT and VP16 treatment (Fig. 7). These results suggest that caspase 3-like activity correlates with occurrence of DNA fragmentation and that Bax-α accelerates caspase 3-like activation. No increase in DABCYL-YVADAPV-EDANS hydrolysis was detected following CPT and VP16 treatment in both cell lines suggesting that caspase 1-like activity is neither activated nor involved in the apoptotic process induced by CPT and VP16 in these cells. Moreover, caspase 3 activation was not detected in cells overexpressing the HA-Bcl-xL protein after CPT and VP-16 treatment (data not show). These observations are consistent with the protective effect conferred by Bcl-xL in these cells.

To validate these findings, kinetic of poly(ADPribosyl) polymerase (PARP) cleavage, a known substrate of caspase 3-like activity (Lazebnik et al., 1994), was monitored in control and Bax-α transfected Namalwa cells treated with VP-16. Figure 8 shows that PARP cleavage and the appearance of the small 24 kDa fragment occurs much more extensively in VP16-treated Bax-α transfected Namalwa cells compared to that of VP16-treated control Namalwa cells. Taken together, our results suggest that Bax-α accelerates the processing and activation of caspase 3-like at low drug concentrations.

DISCUSSION

The human Bax gene belongs to the Ced-9/Bcl-like family that regulates cell survival and cell death. Several mRNA isoforms have been isolated and derived likely from alternative splicing of a single Bax gene mapped to human chromosome 19q13.3-q13.4 (Apte et al., 1995; Oltvai et al., 1993). The first study with a Bax gene product indicated that Bax-α has a cell death promoting activity, inhibiting the protective effect confered by Bcl-2 in an interleukine (IL)-3 dependent cell line upon cytokine removal (Oltvai et al., 1993). Immunohistochemical analysis of Bax expression in mice showed that Bax proteins are expressed in a wide variety of cell types and predominently in a variety of epithelial cells, in the thymic medulla and in the germinal center lymphocytes of lymph nodes and in several population of reproductive and neuronal cells (Krajewski et al., 1994). Yeast two-hybrid assays indicated that Bax-α interacts and heterodimerizes with itself, Bcl-2, Bcl-xL, Mcl-1 and A-1 (Sedlak et al., 1995). Bax-deficient mice displayed thymocytes and B cells hyperplasia and disordered ovarian granulosa cells, atypical male germ cells and no mature haploid

sperm (Knudson et al., 1995). Thus, low Bax expression may result in hyperplasia and plays a role in tumor development.

In this study, by selecting transfected B-lymphoma Namalwa variant cells that express differential levels of Bax-α, we show that its effect on apoptosis is directly related to its level of expression. Reduced expression of Bax was associated recently with poor response rates to chemotherapy in women with metastatic breast adenocarcinoma (Bargou et al., 1995). Gene transfer studies in human breast cell lines showed that Bax-α sensitized these cells to radiation- and drug-induced apoptosis (Sakakura et al., 1997; Wagener et al., 1996). Other studies have indicated that the Bax:Bcl-2 ratio in human testicular tumours and B-cell chronic lymphocytic leukemia is related to cell susceptibility to undergo apoptosis (Chresta et al., 1996; McConkey et al., 1996; Thomas et al., 1996). Ordered balance within the ratio of effector and repressor proteins regulating the decision of a cell to survive or die has already been suggested to form an important checkpoint for cell death (Oltvai & Korsmeyer, 1994). Our results are consistent with that model and indicate that the level of expression of the Bax-α might be an important control point regulating anticancer drug sensitivity in tumor cells. Others have reported also that Bax-α can antagonize the protective effects of Bcl-xL during VP16-induced apoptosis in FL5.12 cells and interestingly in these cells Bax-α was also much less potent in promoting cell death induced by cisplatinum consistant with our observations in Namalwa cells (Simonian et al., 1996). Although our observations suggest that Bax-α does not promote paclitaxel-induced apoptosis in the B lymphoma Namalwa cells, others have reported that a mean of 10-fold increased level of murine Bax expression in human SW626 cancer cells promotes cell death induced by paclitaxel (Strobel et al., 1996). No rationale explanation could be provided to explain the different observations, but the difference in paclitaxel sensitivity between these cell lines may reflect the presence or absence of other unknown factors that are involved in paclitaxel-induced apoptosis.

Our results indicate that Bax-α does not interfere with the primary mechanism of action of anticancer drugs such as CPT, since the extent of DNA synthesis reduction is similar in all Bax-α transfected and control cells following CPT treatment. Although signals emerging from unfinished replication and damaged DNA are yet not fully understood, this study and others (Sakakura et al., 1997; Wagener et al., 1996), indicate that Bax-α is a control point and influences the decision of a cell to undergo apoptosis. It has been proposed that Bax-α is a wild-type p53 primary response gene in a p53-regulated pathway for apoptosis activation (Miyashita et al., 1994; Miyashita

& Reed, 1995). The Namalwa cells are p53 double mutant cells [codon 248 / exon 7; Arg to Gln / Arg to Trp] (O'Connor et al., 1993) and it is unlikely that mutated p53 plays a role in the induction of apoptosis in these lines. Nevertheless, gene transfer-mediated elevation of Bax-α sensitizes these cells to some but not all anticancer agents in a p53-independent manner. Moreover, the expression of EBNA-3 protein family in the Namalwa cells are unlikely to play a role in apoptosis regulation (Horner et al., 1995).

Activation of apoptotic proteases are known to be a critical step in dying cells. Series of caspases were reported to play a central role in executing apoptosis. In this study, we investigate the relation between Bax-α and the activation of caspases. We examined whether Bax-α accelerates the activation of the proteases required in the execution phase of apoptosis. Our experiments using fluorogenic substrates provide evidence that in cells undergoing apoptosis induced by CPT and VP-16, the activation of caspase-3 like was accelerated in Bax-α transfected cells. No evidence of caspase 1-like activation was detected in these cells treated with CPT and VP16. Caspase 3-like activation was also associated with PARP cleavage, a known substrate for these proteases (Lazebnik et al., 1994). Interestingly, the effect of Bax-α on caspase activation is opposite to that of Bcl-2 and Bcl-xL. Recent studies have reported that Bcl-2, like Bcl-xL, prevents the activation of caspases in cultured cells (Monney et al., 1996; Shimizu et al., 1996). Activation of caspase 3 in Bax-induced cell death was reported recently in several studies (Deveraux et al., 1997; Kitanaka et al., 1997; Xiang et al., 1996). Interestingly, blocking caspase 3-like activities by the protease inhibitor N-benzyloxycabonyl-Val-Ala-Asp-fluoromethylketone prevents the cleavage of nuclear and cytosolic substrates and DNA fragmentation but does not prevent cells from dying by a necrosis-like mode of cell death (Hirsch et al., 1997; Xiang et al., 1996).

In summary, our results indicate that Bax-α is a primary controlpoint that promotes transmission of cell death signals emerging from DNA damage in a p53-independent manner and accelerates activation of an apoptotic protease cascade. The identification of cell death signals triggered by DNA damage in p53 mutant cells and understanding how Bax-α promotes caspase activation and DNA fragmentation will certainly help to design new therapeutic strategies to circumvent drug resistance mechanisms in p53-independent pathway of apoptosis.

MATERIALS AND METHODS

Chemicals. Radioactive precursors [2-^{14}C]-thymidine (59 mCi/mmol), and [methyl-^{3}H]-thymidine (78 Ci/mmol) were obtained from ICN BioMedicals (Costa Mesa, CA). 20-S-camptothecin lactone, etoposide (VP-16), vinblastine, paclitaxel and cis-platinum(II) diammine dichloride were purchased from Sigma Chemicals Co. (St-Louis, MO) and dissolved in dimethyl-sulphoxide (at 10 mmol/l) prior to each experiment. The fluorogenic peptide derivatives 4-(4-dimethylaminophenylazo)benzoyl-Tyr-Val-Ala-Asp-Ala-Pro-Val-5-[(2-aminoethyl)amino]-naphthalene-1-sulfonic acid (DABCYL-YVADAPV-EDANS) and Ac-Asp-Glu-Val-Asp-amino-4-methylcoumarin (Ac-DEVD-AMC) were purchased from Bachem Bioscience Inc. (King of Prussia, PA). Antibodies to human CD95/Fas receptor were obtained from Upstate Biotechnology (Lake Placid, NY). All other chemicals were of reagent grade and purchased either from Sigma, ICN and Boehringer-Mannheim or from other local sources.

Cell culture and DNA labeling. The human Namalwa cell line, obtained from the American Type Culture Collection (Rockville, Md), were grown in suspension culture at 37°C in the presence of 5% CO_2 in RPMI-1640 medium supplemented with 10% heat-inactivated fetal bovine serum, 2 mM glutamine, 100 U/ml penicillin and 100 μg/ml streptomycin. Cell culture products were purchased from Gibco-BRL Life Technologies (Grand Island, NY). For DNA labeling, Namalwa cells were grown with [^{14}-C]-thymidine (0.02 μCi/ml) for 24 h and then chased in isotope-free medium overnight prior to drug treatment.

cDNA cloning and transfection. The human Bax-α and Bcl-xL cDNAs were cloned by RT-PCR from total RNA using specific adapter primers containing *NotI* sequences at the ATG start codon and TAA stop codon. The amplified fragments were first cloned in pCRII vector (TA cloning system; Invitrogen, San Diego, CA) and then subcloned at the *NotI* restriction site in the eucaryotic expression vector pCEP4 (InVitrogen) that has been modified to include consensus Kosak and hemagglutinin epitope Tag sequences (HA-tag) between the *BamHI* and *Not I* restriction sites. Vectors were sequenced by ALF DNA sequencer (LKB-Pharmacia Biotech, Upsalla, Sweden) using both vector and internal primers. Purified pCEP4-HA-Bax, pCEP4-HA-Bcl-xL constructs and pCEP4-mock vector were transfected in Namalwa cells by electroporation at 0.27 kVolts (Gene Pulser, BioRad, Hercules, CA). Transfected cells were grown under hygromycin selection at 500

μg/ml and 1.5 mg/ml for 2 to 3 months to obtain stable lines before performing the experiments. Control Namalwa cells do not survive under hygromycin selection. Drug sensitivity of control and pCEP4-mock transfected variant lines were identical. Restriction and modification enzymes were purchased either from Pharmacia or Gibco-BRL.

Western blot and immunohistochemistry analysis. For Western blots, control and transfected Namalwa cells were collected by centrifugation, washed twice with ice-cold phosphate-buffered saline (PBS) and then homogenized in a lysis buffer containing 5 mM HEPES pH 7.4, 160 mM KCl, 40 mM NaCl, 10 mM $MgCl_2$, 1 mM PMSF, 0.5% NP40 and a cocktail of protease inhibitors (Complete™, Boehringer-Mannheim) at 4°C for 30 min with agitation. After centrifugation, supernatants were collected as cytosolic protein extracts. Protein concentrations were determined using the Bradford assay with bovine serum albumin as standard. Antibodies to the peptide epitope derived from the hemagglutinin protein of human influenza virus (HA epitope tag) and to human PARP protein were obtained from Boehringer-Mannheim. Affinity purified antibodies to human Bcl-2 and Bax were purchased from Santa Cruz Biotechnology, Inc (Santa Cruz, CA) and antibodies to Bcl-xL were obtained from CalBiochem (San Diego, CA). Enhanced cheminulescence detection of peroxidase-labeled secondary antibodies was performed using Amersham Life Science reagents. Expression of HA -Bax and -Bcl-xL by immunohistochemistry was visualized using anti-HA mouse monoclonal antidodies (Boehringer-Mannheim) as a primary antibody (10 μg / ml), a biotinylated goat anti-mouse (Sigma) as secondary antibody (10 μg / ml), followed by a steptavidin-alkaline phosphatase complex (Sigma) and colorimetric reaction using FastRed TR/Naphthol AS-MX (Sigma). Briefly, cells were spread by centrifugation on microscope slides and fixed in a solution containing 50% acetone and 50% methanol. Permeable cells were then incubated for 30 min in PBS buffered solution containing 10 % (v/v) goat serum and 5 % (w/v) bovine serum albumin (Sol A). Cells were then incubated with the primary antibody diluted in Sol A for 30 min, followed by several washes with Sol A and then incubated with the secondary antibodies diluted in Sol A. After several washes in Sol A, cells were incubated with a streptavidin-alkaline complex, washed again and incubated with a solution of FastRed TR/Naphthol AS-MX according to the manufacturer instructions (Sigma). Cell were then analyzed under an Axioscop camera- equiped microscope (Carl Zeiss, West Germany).

DNA filter elution assay. DNA filter elution assays were designed to

monitor and quantitate DNA fragmentation associated with apoptosis (Bertrand & Pommier, 1995). Each sample (approximatively 0.5 X 10^6 [^{14}C]-thymidine-labeled cells) is loaded onto a protein-adsorbing filter (vinyl/acrylic copolymers filters, Metricel membrane, 0.8 um pore size, 25 mm diameter, Gelman Sciences Inc., Ann Arbor, MI) mounted in a Millipore filter holder connected to a 50 ml syringe. Cells are then washed with 5 ml of phosphate buffered saline (PBS). As soon as the washing solution has dripped through by gravity, lysis is performed with 5 ml of lysis solution (0.2% sodium sarkosyl, 2 M NaCl, 0.04 M EDTA, pH 10.0). After the lysis has dripped through by gravity, it is washed from the filters with 5 ml of 0.02M EDTA (pH 10.0). The filter is then processed as described (Bertrand & Pommier, 1995). Briefly, it was placed in a scintillation vial to which 1.0 ml of 1 N HCl is added. The vial is sealed and heated for 1 h at 60°C to depurinate the DNA. Then, 2.5 ml of 0.4 N NaOH is added and allowed to stand 1 h at room temperature to release the labeled DNA from the filters. Radioactivity was counted by liquid scintillation spectrometry in each fraction (loading wash, lysis, EDTA wash, filter). DNA fragmentation was determined as the fraction of DNA in the loading wash fraction + lysis fraction + EDTA wash fraction relative to total DNA (loading wash + lysis + EDTA wash + filter). Results are expressed as the percentage of DNA fragmented in treated cells compared to DNA fragmented in control untreated cells (background) using the formula:

$$(F\text{-}F0/1\text{-}F0) \times 100$$

where F and F0 represent DNA fragmentation in treated and control cells, respectively.

Analysis of DNA fragmentation by agarose gel electrophoresis. To visualize the oligonucleosome-sized DNA fragments, at specified times after drug treatment cellular DNA was extracted by a salting-out procedure as described previously (Miller et al., 1988). Electrophoresis was done in 1.6% agarose gel in Tris-acetate buffer (pH 8.0). Following electrophoresis DNA was visualized by ethidium bromide staining.

Measurement of thymidine incorporation. Control and transfected Namalwa cells were prelabeled with [^{14}-C]-thymidine (0.02 μCi/ml) for 24 h and then chased in isotope-free medium overnight prior to drug treatment. Cells were incubated in CPT containing medium for 30 min. After drug treatment, cells were washed twice with complete medium and rates of nucleotide incorporation were measured by 10 min pulse experiments with [^{3}-H]-thymidine (10 uCi/ml) as described (Bertrand et al., 1992). Nucleotide incorporation was stopped by adding 10 ml of ice-cold PBS and

cells were quickly pelleted by centrifugation (1 000g, 3 min, 4°C). Acid-insoluble nucleotides were precipitated on ice with 10% trichloroacetic acid. The precipitates were dissolved in 0.4 N NaOH and radioactivity was monitored by scintillation spectrometry. Results were expressed as the ratio of [^{3}-H]/[^{14}-C] for treated cells over the ratio of [^{3}-H]/[^{14}-C] of untreated cells (Bertrand et al., 1992).

Caspase activity determination. Cytosolic extracts are prepared by washing control and transfected Namalwa cells twice by centrifugation/resuspension in 10 ml ice-cold PBS (without Ca^{++} and Mg^{++}). Cells are then homogenized at 4°C for 30 min in a lysis buffer containing 100 mM Hepes (pH 7.5), 5 mM EDTA, 5 mM DTT, 20% glycerol and 0.5% NP-40. Samples are then centrifuged (13,000g for 10 min at 4°C) and supernatants collected as cytosolic extracts. Caspase activities were measured by monitoring fluorescence continuously in a dual luminescence fluoremeter (LS 50B Perkin Elmer) using excitation wavelength of 360 nm and emission wavelenght of 490 nm for the substrate DABCYL-YVADAPV-EDANS and excitation wavelength of 380 nm and emission wavelenght of 460 nm for the substrate Ac-DEVD-AMC. Reactions were carried out in cuvettes and temperature maintained at 37° using a water-jacketed sample compartment. The assay mixture contained 100 mM Hepes (pH 7.5), 20% (v/v) glycerol, 5 mM DTT, 5 mM EDTA and 200 μM fluorogenic peptides. Enzyme activities were determined as initial velocities and expressed as relative intensity / min / mg.

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Table 1. Rates of DNA synthesis after camptothecin in control and transfected Namalwa cells

CPT	Namalwa	Nam -bax $^{0.50}$	Nam -bax$^{1.50}$
0.01 μM	85.0 ± 9.9	80.5 ± 9.8	80.7 ± 3.8
0.05 μM	74.7 ± 5.8	73.7 ± 5.2	71.2 ±10.9
0.10 μM	65.5 ± 6.4	64.7 ± 2.9	63.5 ± 8.2
0.50 μM	59.2 ± 7.5	56.2 ± 4.8	57.5 ± 4.8

Cells were treated with various concentrations of CPT for 30 min. [3H]-thymidine incorporation were determined by 10 min pulse experiments. Values are expressed as % of DNA synthesis relative to control untreated cells and represent the means ± S.E. of 2 independent experiments performed in triplicate (n=6). Nam -bax$^{0.50}$ and Nam-bax$^{1.50}$ mean Namalwa cells transfected with Bax and selected at 0.50 mg/ml and 1.5 mg/ml hygromycin, respectively.

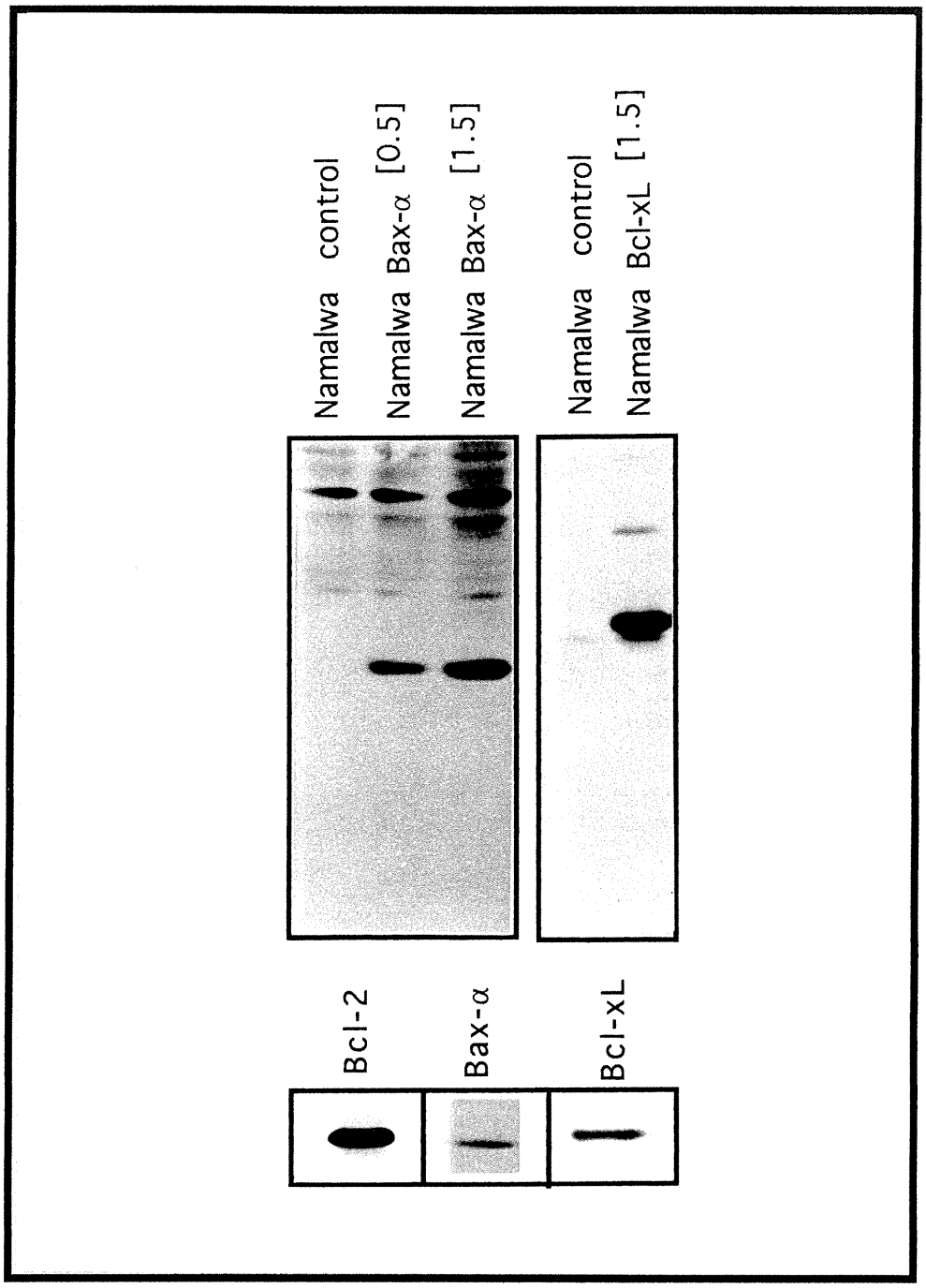
Namalwa control
Namalwa Bax-α [0.5]
Namalwa Bax-α [1.5]
Namalwa control
Namalwa Bcl-xL [1.5]
Bcl-2
Bax-α
Bcl-xL

Figure 1

Fig. 1. Expression of Bcl-related proteins in Nawalwa cells.

Upper panels. Cytosolic extracts were prepared as described in Materials and Methods from control, HA-Bax-α transfected Nawalwa cells selected at 0.50 mg/ml and 1.5 mg/ml hygromycin (left panel) and HA-Bcl-xL transfected cells selected at 1.5 mg/ml hygromycin (right panel). Following SDS-polyacrylamide gel electrophoresis and electrophoretic transfer, HA-Bax-α and HA-Bcl-xL proteins were detected using anti-HA peptide monoclonal antibodies. Visualization of signals was performed by ECL and autoradiography.

Lower panels. Cytosolic extracts were prepared from control Nawalwa cells. Following SDS-polyacrylamide gel electrophoresis and electrophoretic transfer, Bcl-2, Bax-α and Bcl-xL proteins were detected using specific antibodies. Visualization of signals was performed by ECL and autoradiography.

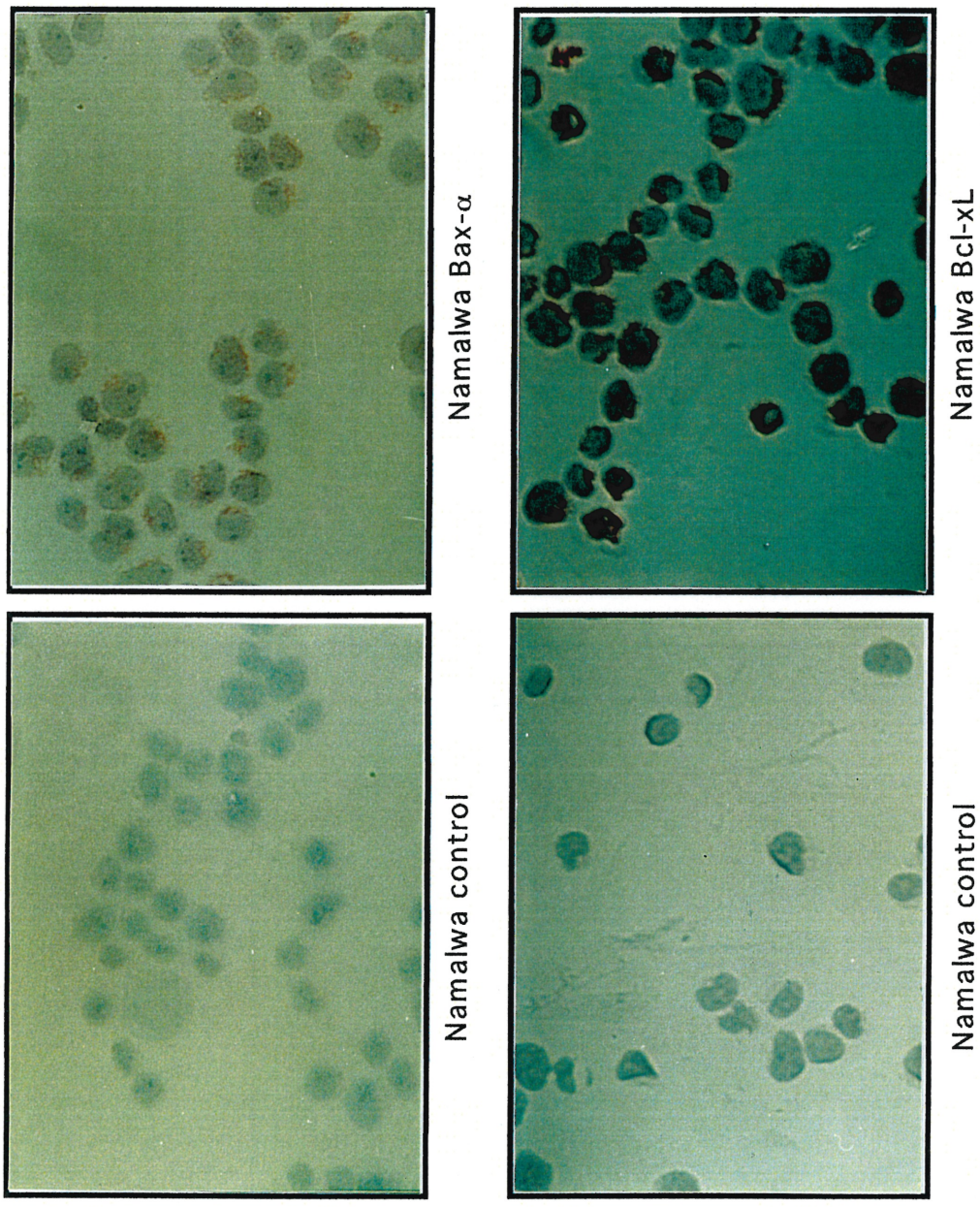
Namalwa Bax-α
Namalwa Bcl-xL
Namalwa control
Namalwa control

Figure 2

Fig. 2. Immunohistochemical revelation of the HA-Bax-α and HA-Bcl-xL proteins in transfected Namalwa variant cells.

Upper panels are control Namalwa (left) and HA-Bax-α transfected Namalwa cells (right). Lower panels are control Namalwa (left) and HA-Bcl-xL transfected Namalwa cells (right). HA-Bax-α and HA-Bcl-xL were detected using anti-HA mouse monoclonal antibodies as primary antibody and a biotinylated goat anti-mouse as secondary antibody, followed by a streptavidin-alkaline phosphatase complex and colorimetric reaction using FastRed TR/Naphtol AS-MX. Cell were analysed under Axioscop camera-equiped microscope.

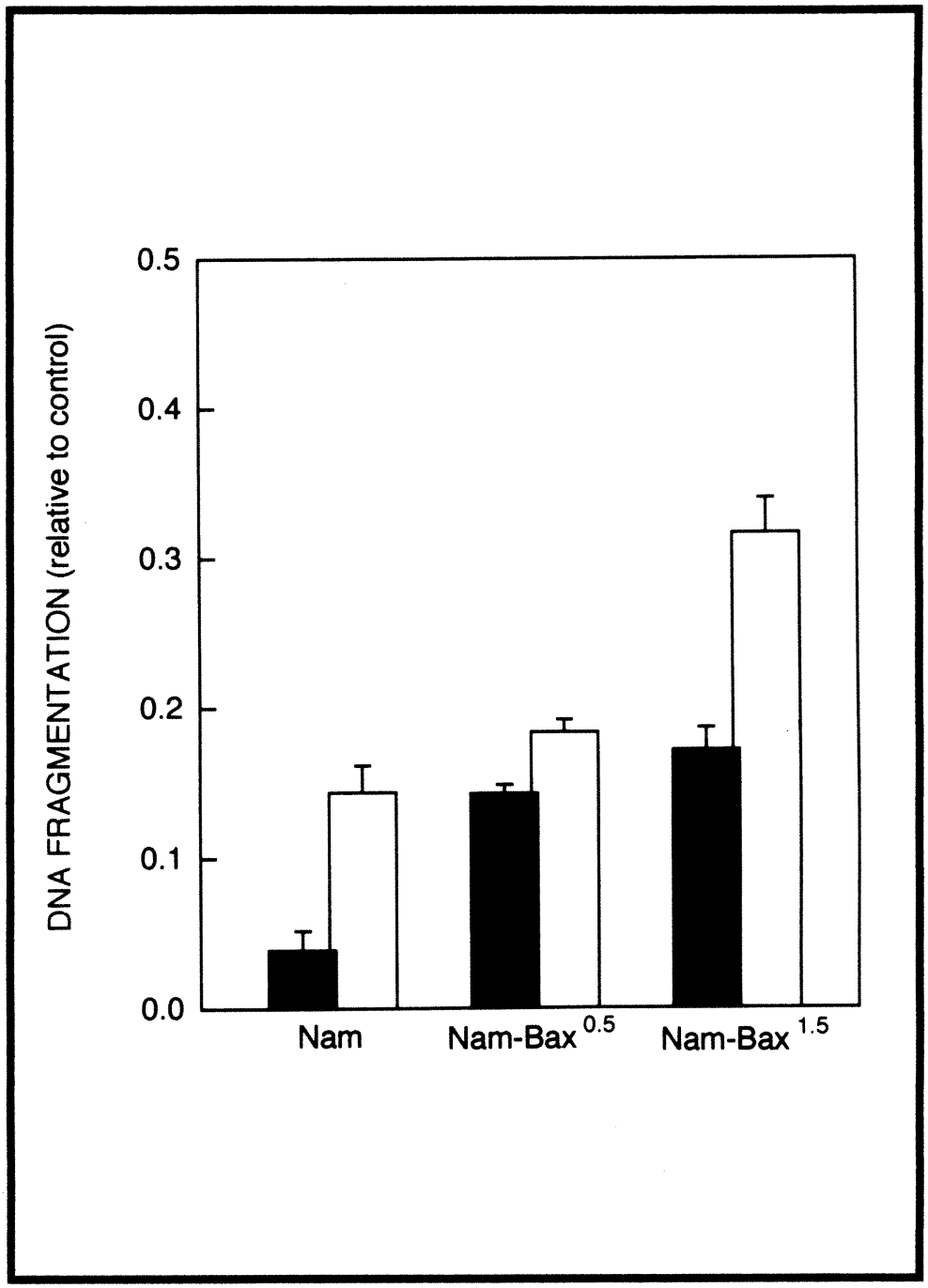
DNA FRAGMENTATION (relative to control)
0.5
0.4
0.3
0.2
0.1
0.0
Nam
Nam-Bax 0.5
Nam-Bax 1.5

Figure 3

Fig. 3. Bax-α promotes DNA fragmentation in cells grown in low serum and in presence of CD95\Fas antibodies.

[^{14}C]Thymidine-labeled cells were grown for 24 h in 0.1% heat inactivated serum (close bars) and in the presence of anti-CD95/Fas antibodies (open bars). DNA fragmentation was determined by filter elution assays. Results are expressed as percentage DNA fragmentation relative to untreated cells grown in 10% heat inactivated serum. Bars represent the means +/- SE of a triplicate experiment.

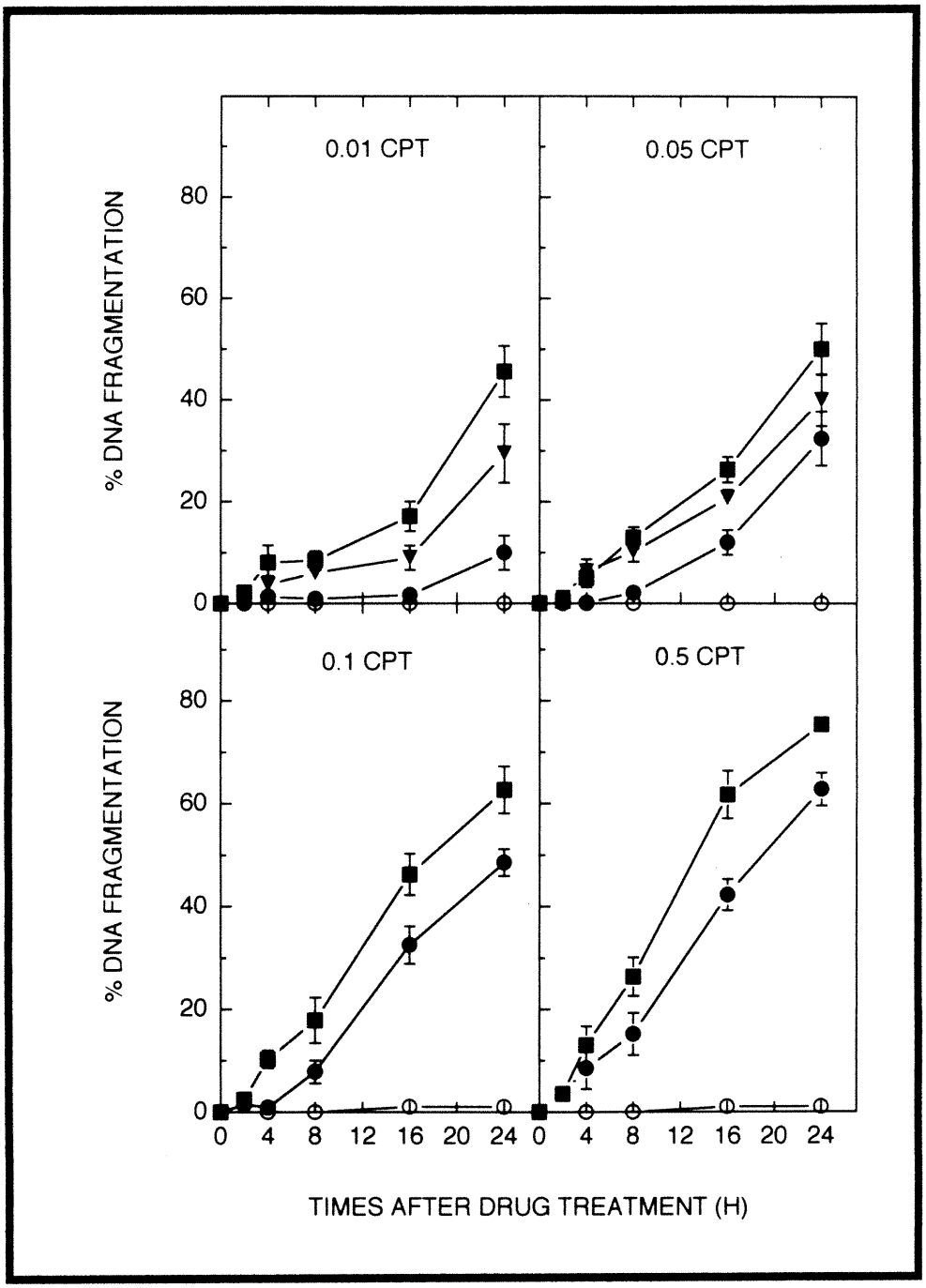
0.01 CPT
0.05 CPT
0.1 CPT
0.5 CPT
% DNA FRAGMENTATION
% DNA FRAGMENTATION
0
20
40
60
80
0 4 8 12 16 20 24 0 4 8 12 16 20 24
TIMES AFTER DRUG TREATMENT (H)

Figure 4

Fig. 4. The effects of overexpression of Bax-α in Namalwa cells treated at various CPT concentrations.

[^{14}C]Thymidine-labeled cells were treated for 30 min with 0.01 (upper left panel), 0.05 (upper right panel), 0.1 (lower left panel) and 0.5 μM CPT (lower right panel). After drug removal, cells were incubated in drug free-medium and at the indicated times (x axis; hour), DNA fragmentation was determined by filter elution assays. Results are expressed as a percentage of DNA fragmentation relative to that of untreated cells. Points represent the means +/- SE of four independent experiments performed in duplicate. Symbols are Namalwa cells (●); Bax-α transfected Namalwa cells selected at 0.50 mg/ml (▼) and 1.5 mg/ml hygromycin (■). For comparison, Bcl-xL transfected cells selected at 1.5 mg/ml hygromycin (○) were used. The difference in rate of DNA fragmentation between the variant lines and control cells was statistically significant at 4, 8, 16 and 24 h after drug treatment ($0.001 < p < 0.03$; student T test values; except for values at 24 h in HA-Bax selected at 0.5 mg/ml hygromycin).

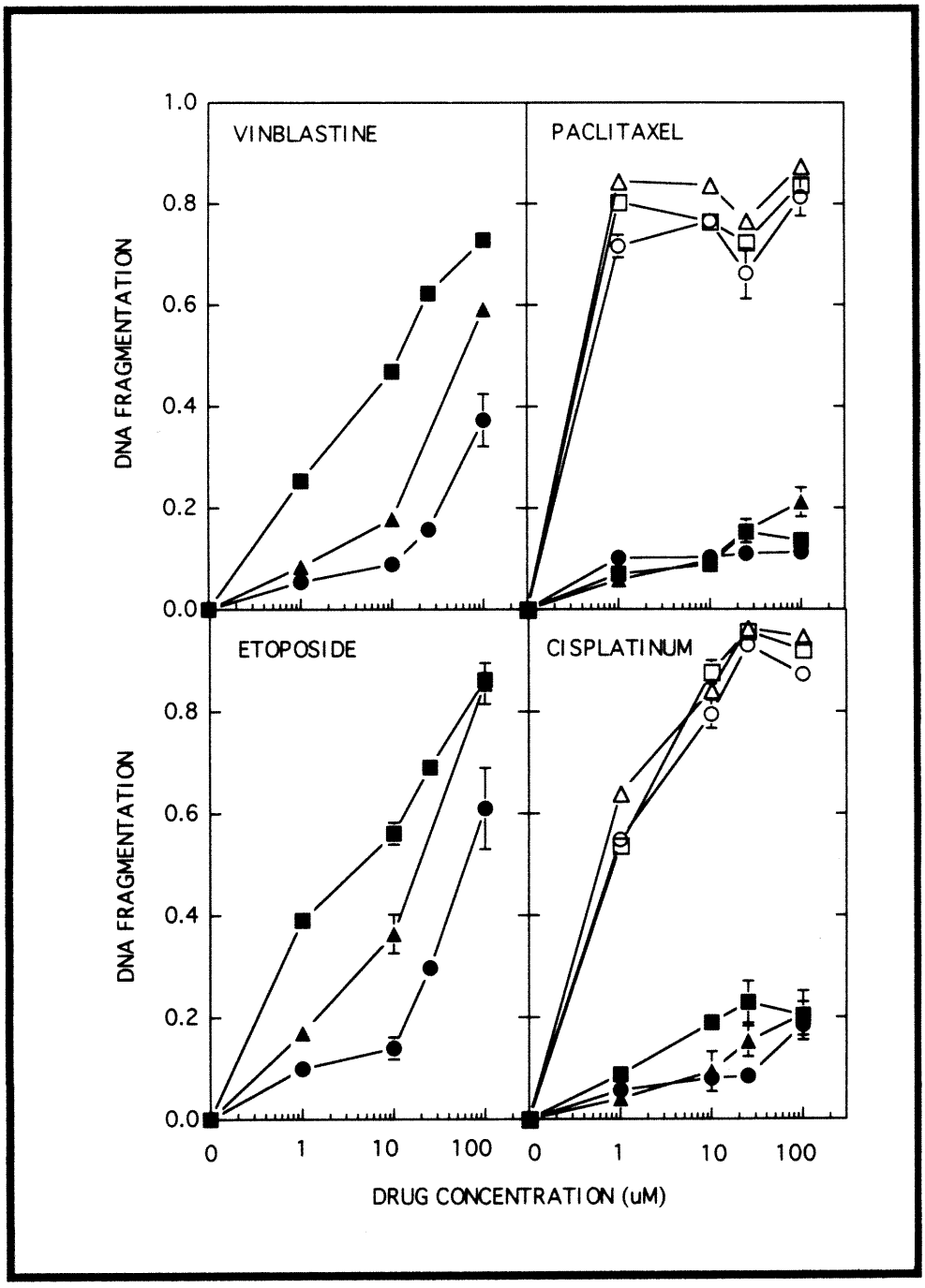
VINBLASTINE
PACLITAXEL
ETOPOSIDE
CISPLATINUM
DNA FRAGMENTATION
DNA FRAGMENTATION
1.0
0.8
0.6
0.4
0.2
0.0
0
1
10
100
DRUG CONCENTRATION (uM)

Figure 5

Fig. 5. Activation of DNA fragmentation in control and Bax-α transfected Namalwa cells following VP-16, vinblastine, paclitaxel and cis-platinum treatments.

[^{14}C]Thymidine-labeled cells were treated for 8 h (close symbols) or 24 h (open symbols) with various concentrations of vinblastine (upper left panel), VP-16 (lower left panel), paclitaxel (upper right panel) and cis-platinum (lower right panel). DNA fragmentation was determined by filter elution assays. Results are expressed as percentage DNA fragmentation relative to that of untreated cells. Points represent the means +/- SE of three experiments performed in triplicate. Symbols are Namalwa control cells (● , ○) and Bax-α transfected Namalwa cells selected at 0.5 mg/ml hygromicin (▲ , Δ) and 1.5 mg/ml hygromicin (■ , ❑). The difference in rate of DNA fragmentation between the variant lines and control cells was statistically significant for vinblastine and etoposide treated cells ($0.0001 < p < 0.03$; student T test values).

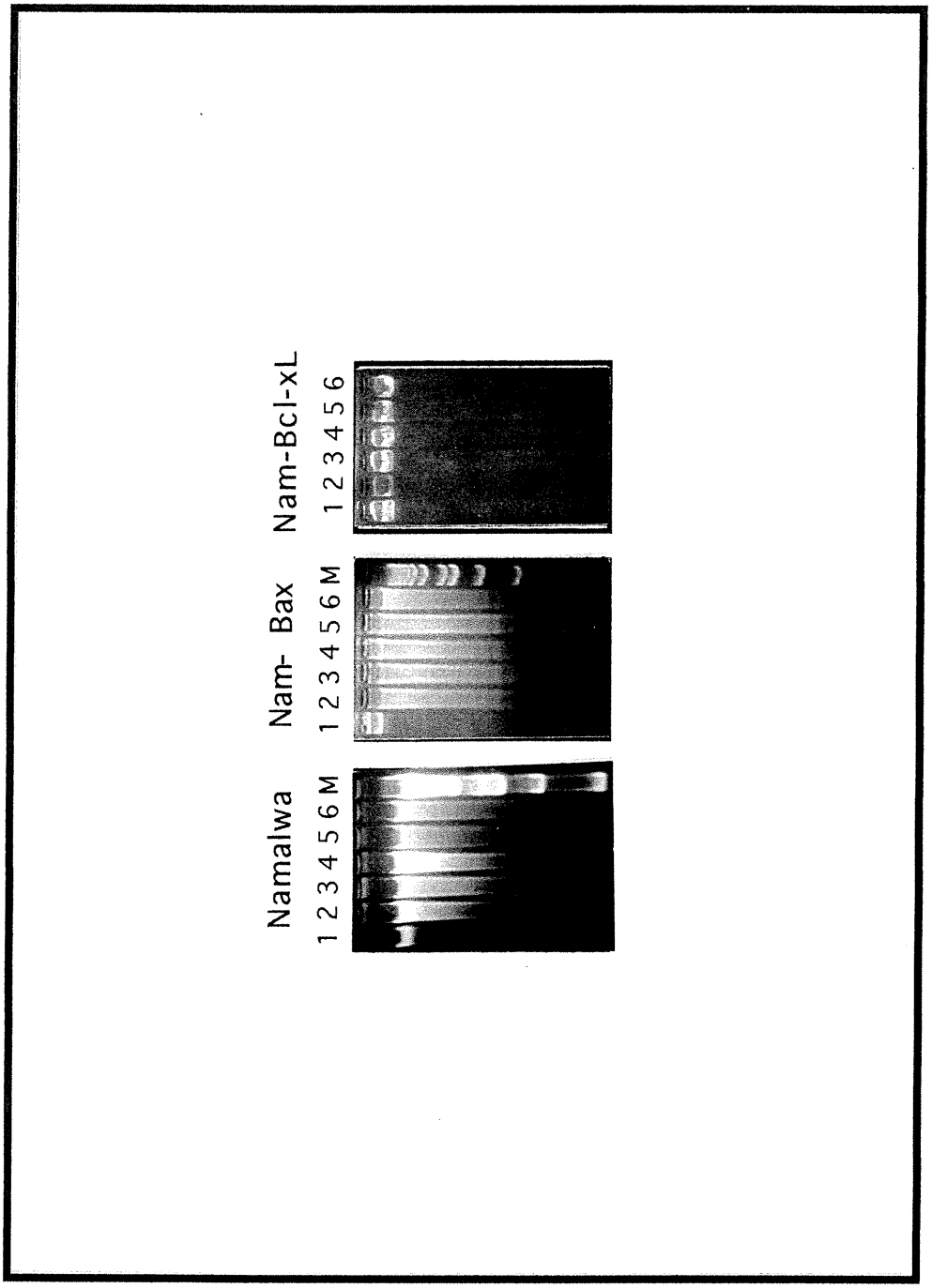

Figure 6

Fig. 6. Analysis of DNA fragmentation by agarose gel electrophoresis.

Oligonucleosome-sized DNA fragments were visualized following standard agarose gel electrophoresis followed by ethidium bromide staining. (1) untreated cells; (2) 0.05 μM CPT; (3) 1.0 μM VP-16; (4) 1.0 μM cisplatinum; (5) 1.0 μM vinblastine; (6) 1.0 μM paclitaxel. Treatments were of 16 h for Namalwa cells, 8 h for HA-Bax-α and 24 h for HA-Bcl-xL variant Namalwa cells.

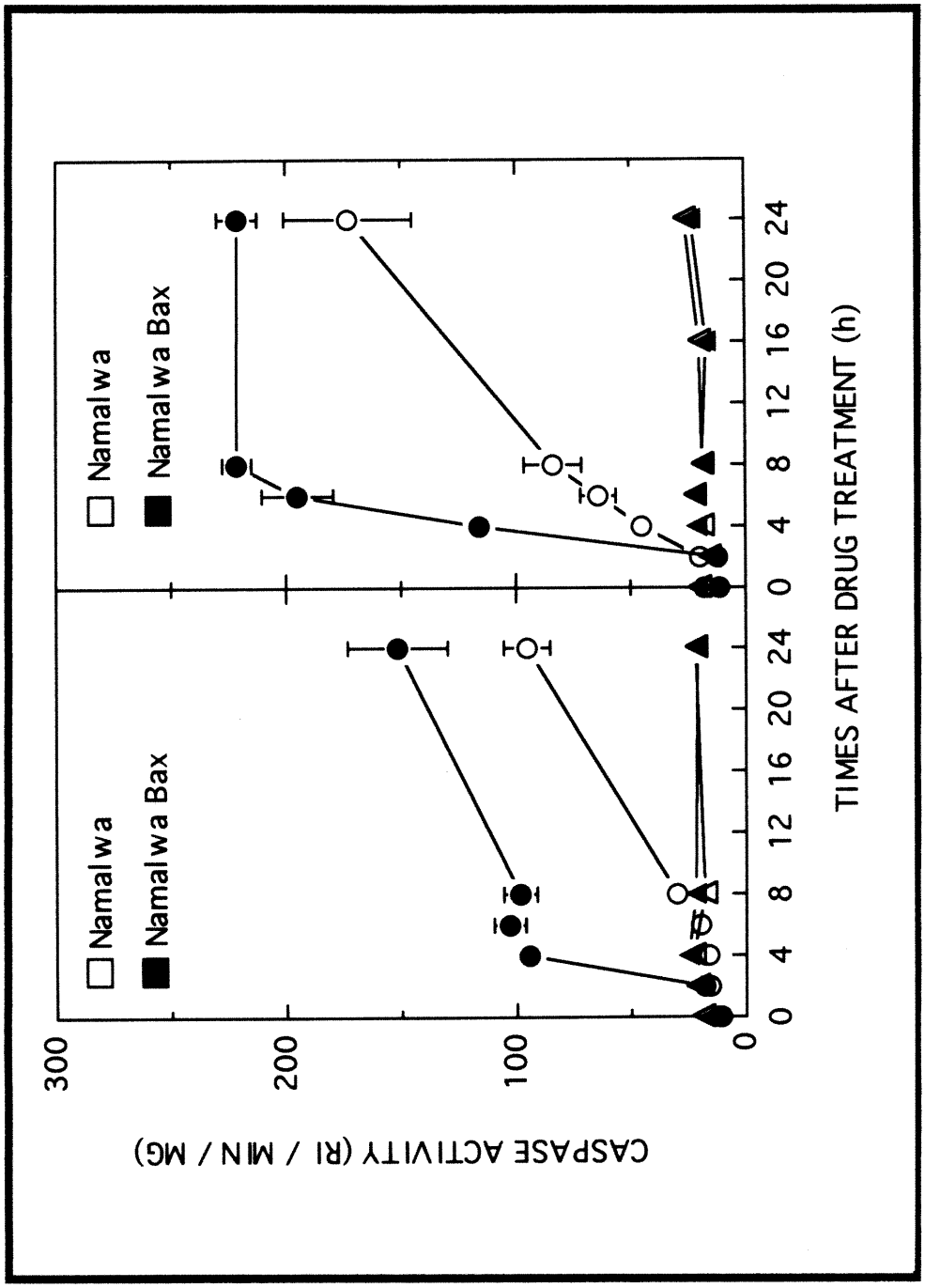
Namalwa
Namalwa Bax
Namalwa
Namalwa Bax
CASPASE ACTIVITY (RI / MIN / MG)
300
200
100
0
0 4 8 12 16 20 24
0 4 8 12 16 20 24
TIMES AFTER DRUG TREATMENT (h)

Figure 7

Fig. 7. Caspase 1-like and caspase 3-like activities in CPT and VP-16 treated control and Bax-α transfected Namalwa cells.

Cytosolic extracts were prepared from control (open symbols) and Bax-α tranfected Namalwa cells (close symbols) at the indicated times following CPT (0.05 μM; left panel) and VP16 (10 μM; right panel) treatment. Caspase activities were monitored continuously at 37°C by detecting fluorescence emission in the presence of DABCYL-YVADAPV-EDANS using excitation wavelength of 360 nm and emission wavelenght of 490 nm and Ac-DEVD-AMC at an excitation wavelength of 380 nm and emission wavelenght of 460 nm. Enzyme activities were measured as initial velocities and expressed as relative intensity / min / mg. Points represent the means +/- SE of three independent determinations. Symbols used are (▲ , Δ) caspase 1-like activity ; (● , ○) caspase 3-like activity.

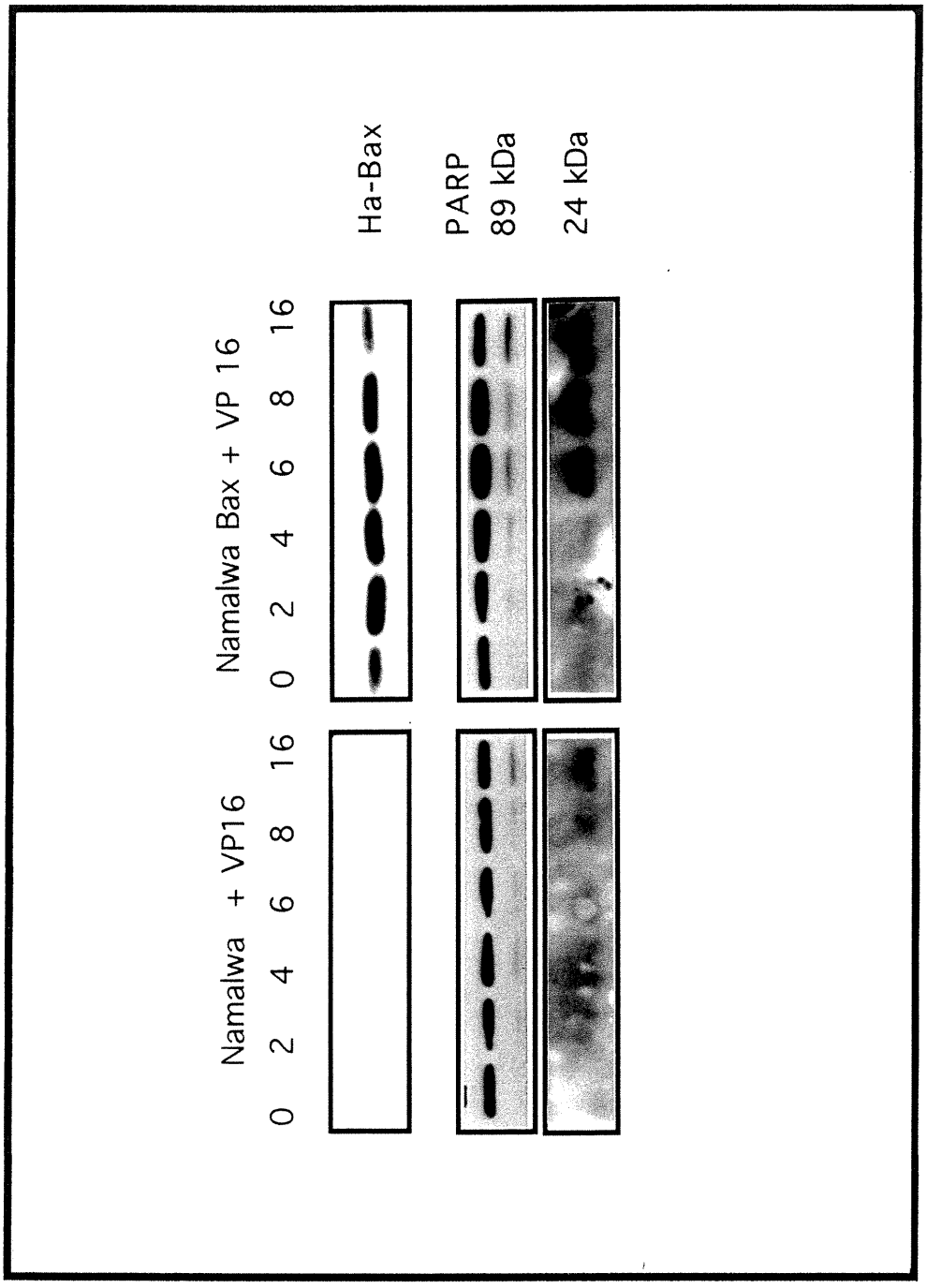
Namalwa + VP16
0 2 4 6 8 16
Namalwa Bax + VP 16
0 2 4 6 8 16
Ha-Bax
PARP
89 kDa
24 kDa

Figure 8

Fig. 8. PARP cleavage in control and Bax-α transfected Namalwa cells following VP-16 treatment.

Whole-cell extracts were prepared as described in Materials and Methods at the indicated times after VP-16 treatment (10 μM) from Namalwa (left panel) and Bax-α transfected Namalwa cells selected at 1.5 mg/ml hygromycin (right panel). Following SDS-polyacrylamide gel electrophoresis (12%) and electrophoretic transfert, HA-Bax-α expression and PARP cleavage were detected using specific antibodies. Visualization of signals was performed by ECL and autoradiography at different exposure times.

CHAPITRE 4.

Characterization of Bax-σ, a cell death-inducing isoform of Bax

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Avant propos:

Nous avous identifié et cloné une nouvelle isoforme de Bax appeléé Bax-σ. La protéine Bax-σ présente une structure semblable à celle de Bax-α mais présente une délétion de 13 acides aminés localisée entre le domaine BH-2 et le domaine C-terminal hydrophobe. Une analyse de l'expression des ARNm de Bax-σ montre que les transcrits sont exprimés dans des lignées cellulaires tumorales et des tissus sains d'origine variée.

Ce travail consiste à étudier la fonction de Bax-σ dans la mort cellulaire programmée. L'expression transitoire de Bax-σ dans les cellules de lymphome B Namalwa induit l'activation des caspases, la fragmentation de l'ADN et les changements morphologiques caractéristiques de l'apoptose. Nous montrons que Bax-σ ainsi que Bax-α activent les caspases en induisant la translocation du cytochrome c des mitochondries vers le cytoplasme. L'expression de Bax-σ dans les cellules Namalwa accroit également la sensibilité des cellules à l'apoptose induite par la camptothécine, la vinblastine et le taxol.

• C. Paquet a réalisé l'étude d'expression de Bax-σ, M. Beauchemin a mis au point le système de double-hybride dans la levure et J. Dever-Bertrand à contribué aux mesures des cinétiques de fragmentation de l'ADN.

ABSTRACT

The *Ced-9/Bcl*-like family of genes codes for proteins that have anti-apoptotic and pro-apoptotic activity. Several Bax isoproteins have been detected by 2-D gel electrophoresis, and a novel human member, designated as Bax-σ, has been identified and cloned from human cancer promyelocytic cells. Bax-σ contains BH-3, BH-1, BH-2 domains, putative α-5 and α-6 helices and the carboxy-terminal hydrophobic transmembrane domain but lacks amino acids 159 to 171 compared to Bax-α. mRNA expression analysis by reverse transcription-polymerase chain reaction and RNase protection assays have revealed that Bax-σ is expressed in a variety of human cancer cell lines and normal tissues. To investigate the potential role of Bax-σ in apoptosis, first its effects were compared to those of Bax-α by transient expression in human B lymphoma Namalwa cells. Both Bax-σ and Bax-α promoted apoptosis, as detected by DNA fragmentation and morphological analysis by electron microscopy. The apoptosis induced by Bax-σ and Bax-α was correlated with their expression, cytochrome c release and caspase activation. In a yeast 2-hybrid system, Bax-σ interacted with several Ced-9/Bcl family members but had no affinity for the human Egl-1 homologs Bik and Bad and the Ced-4 homolog Apaf-1. In human cells, Bax-σ function was counteracted by Bcl-xL overexpression, and co-immunoprecipitation experiments indicated that Bax-σ was associated with Bcl-xL. Furthermore, Bax-σ overexpression increased cell death induced by various concentrations of genotoxic agents with the most pronounced effect occurring at low camptothecin and vinblastine dose levels. Our results suggest that Bax-σ, a novel variant of Bax, encodes a protein with a pro-apoptotic effect and mode of action similar to those of Bax-α.

Keywords: Bax; mitochondria; caspase; DNA damage; apoptosis.

Abbreviations: MPT , mitochondrial permeability transition ; $\Delta\psi m$, mitochondrial inner transmembrane potential; ANT, adenine nucleotide translocator; VDAC, voltage-dependent anion channel ; RT-PCR, reverse transcriptase-polymerase chain reaction; HA-tag, hemagglutinin epitope tag sequences; Ac-DEVD-AMC, Ac-Asp-Glu-Val-Asp-amino-4-methylcoumarin; CPT, 20-S-camptothecin lactone; VP-16, etoposide; VINB, vinblastine; taxol, paclitaxel; cisPT, cis-platinum(II) diammine dichloride.

INTRODUCTION

Pro- and anti-apoptotic proteins of the Bcl-2 family play a critical role in controlling mitochondrial permeability transition (MPT), maintenance of mitochondrial inner transmembrane potential ($\Delta\psi m$), cellular redox equilibrium and caspase activation during apoptosis[1-3]. Although these proteins are found in the cytosol, most of them have a transmembrane domain that stabilizes them for intracellular membranes, primarily the outer mitochondrial membrane, endoplasmic reticulum and nuclear envelope[4-6].

Bax was the first described pro-apoptotic member of the family, and mRNA expression analysis showed its wide expression and splicing pattern that vary between lineages and cell types [7]. Bax-α mRNA contains exon-1, -2, -3, -4, -5, and -6 and encodes a protein of 192 amino acids that has BH-3, BH-2 and BH-1 domains, putative α-5 and α-6 helices and the carboxy-terminal hydrophobic transmembrane domain (TM)[7]. Bax-γ mRNA contains exon-1, -3, -4, -5 and -6 and differs by an alternative splicing of intron-5; the absence of exon-2 leads to a translational frameshift, resulting in a protein of 41 amino acids that lacks all the known functional domains of Bax-α[7]. Bax-β mRNA contains exon-1, -2, -3, -4, -5, intron-5 and exon-6; the resulting protein contains 218 amino acids and lacks the COOH terminus domain due to the presence of a stop codon within the coding sequence of intron-5[7]. Bax-δ lacks exon-3 but contains all the others and retains the same reading frame; the protein contains 143 amino acids and lacks the BH-3 domain[8]. Bax-ω mRNA contains exon-1, -2, -3, -4, -5, an unspliced intron-5 and exon-6; the resulting protein contains 221 amino acids and lacks the COOH- terminus domain due to a translational frameshift[9]. Finally, Bax-ε lacks the BH2 and the transmembrane domain[10]. The presence of 18-kDa and 16-kDa proteins, abundant in B-cell chronic lymphocytic leukemia and hemopoietic tissues, has been detected using anti-Bax antibodies[11, 12].

Extensive work has been done so far to understand the function and mode of action of Bax-α in mediating apoptosis. Bax-α itself can induce cell death when it is overexpressed. Moreover, overexpressed Bax-α accelerates apoptotic cell death following exposure to various death stimuli and can counteract the anti-apoptotic activity of Bcl-2[7, 13]. Currently-available data suggest that ordered balance between pro-apoptotic and anti-apoptotic activities of the Bcl-2 protein family regulates the decision of a cell to survive or die, forming a checkpoint upstream of mitochondrial

dysfunction and caspase activation[1, 7]. Redistribution of Bax-α from the cytosol to the mitochondria after exposure to cell death stimuli has been suggested as an important change promoting cell death[14-17].

Bax-α overexpression is associated with mitochondrial dysfunction that includes cytochrome c release and, in some instance, loss of $\Delta\psi m$[16, 18-20]. Three-dimensional structure analysis of Bcl-xL suggests that some Bcl-related proteins, such as Bax-α may possess pore- or channel-forming activity[21-25]. When added to artificial membrane bilayers, Bax-α can form pore-channels with several levels of conductance and ion selectivity that can be impeded by Bcl-2[23].

More recent observations have revealed that Bax-α binds to mitochondrial permeability transition pore complexes (MPTP) and cooperates with adenine nucleotide translocator protein (ANT) to increase mitochondrial membrane permeability and potently transduce cell death[26]. In reconstituted liposomes that contain the mitochondrial porin channel or voltage-dependent anion channel (VDAC), Bax-α accelerates VDAC opening in a manner that permits cytochrome c to pass directly through VDAC[20]. In contrast, other studies have indicated that Bax-α-induced cytochrome c release is independent of MPTP but highly dependent on Mg^{2+} ions[27]. Some have shown that the mitochondrial F_0F_1-ATPase proton pump is also required for Bax-α to transduce cell death[28]. Much less is known about the mechanism of action and functional role of the other Bax isoforms, including Bax-γ, Bax-β, Bax-δ and Bax-ε. Recently, Bax-ω was shown to increase basal cell death at a level similar to that of Bax-α but did not appear to potentiate death induced by other stimuli[9].

In this paper, we report the identification of several Bax isoproteins by 2-D gel electrophoresis and cloning of Bax-σ, a novel isoform of Bax expressed in a variety of human tissues and cancer cell lines. Bax-σ has a structure very similar to that of Bax-α with the exception of a fragment of 13 amino acids located between the BH-2 domain and the TM.

MATERIALS AND METHODS

Chemicals. Radioactive precursors [α-^{32}P]-dCTP (> 3,000 Ci/mmol), [α-^{32}P]-dUTP (800 Ci/mmol) and [2-^{14}C]-thymidine (59 mCi/mmol) were obtained from ICN BioMedicals (Costa Mesa, CA). 20-S-camptothecin lactone, etoposide, vinblastine, paclitaxel and cis-platinum(II) diammine dichloride were purchased from the Sigma Chemical Co. (St. Louis, MO) and dissolved in dimethyl-sulphoxide (at 10 mmol/l) prior to each experiment. The fluorogenic peptide derivative

Ac-Asp-Glu-Val-Asp-amino-4-methylcoumarin (Ac-DEVD-AMC) was bought from Bachem Bioscience Inc. (King of Prussia, PA). Hygromycin B was obtained from Boehringer-Mannheim (Laval, Que.). All other chemicals were of reagent grade and purchased either from Sigma and ICN or from local sources.

cDNA cloning of human Bax-α and Bax-σ. Human Bax-α and Bax-σ cDNAs were cloned by reverse transcription-polymerase chain reaction (RT-PCR) from mRNA obtained from human HL-60 cells using specific adapter primers containing *NotI* sequences at the ATG start codon (5'-primer GCGGCCGCATGGACGGGTCCGGGGAGCAG) and TGA stop codon (complementary 3'-primer GCGGCCGCTCAGCCCATCTTCTTCCAGAT). The amplified fragments were first cloned in pCRII vector (Invitrogen, Carlsbad, CA) and then subcloned at the *NotI* restriction site in the eucaryotic expression vector pCEP4 (Invitrogen) modified to include consensus Kosak and hemagglutinin epitope Tag sequences (HA-tag)[29, 30]. All vectors were sequenced by ALF DNA sequencer (LKB-Pharmacia Biotech, Upsalla, Sweden). Restriction and modification enzymes were purchased either from Pharmacia Biotech or Gibco-BRL Life Technologies (Grand Island, NY).

Cell culture, transfection and DNA labeling. All cell lines were obtained from the American Type Culture Collection (Manassas, VA) and grown in suspension culture at 37°C in the presence of 5% CO_2 in RPMI-1640 medium supplemented with 10% heat-inactivated fetal bovine serum, 2 mM glutamine, 100 U/ml penicillin and 100 μg/ml streptomycin. Cell culture products were purchased from Gibco-BRL Life Technologies. Cesium chloride-purified pCEP4-HA-Bax-α, pCEP4-HA-Bax-σ, pCDNA3-Bcl-xL-myc, pCDNA3-mock-myc and pCEP4-HA-mock vectors were transfected in Namalwa cells and/or Namalwa cells overexpressing Bcl-xL[30] by electroporation at 0.27 kVolts (Gene Pulser, BioRad, Hercules, CA). To obtain stable transfected lines, the cells were grown under hygromycin B selection at 1.5 mg/ml for 3 months before performing the experiments, as described elsewhere[29, 30]. For DNA labeling, Namalwa cells were grown with [^{14}C]-thymidine (0.02 μCi/ml) for 24 h, then chased overnight in isotope-free medium prior to electroporation or drug treatment[51].

Protein extracts, subcellular fractionation, co-immunoprecipitation and Western blot analysis. For sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), control and transfected cells were collected by centrifugation, washed once with ice-cold phosphate-buffered saline

(PBS), then homogenized in a lysis buffer containing 5 mM N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic aid (HEPES), pH 7.4, 160 mM KCl, 40 mM NaCl, 10 mM $MgCl_2$, 1 mM phenylmethylsulfonyl fluoride (PMSF), 1 mM dithiothreitol (DTT) and 0.5% NP-40 and a cocktail of protease inhibitors (Complete™, Boehringer-Mannheim) at 4°C for 30 min with gentle agitation. After centrifugation (10,000 g, 10 min), the supernatants were collected as protein extracts.

For 2-D gel electrophoresis, lysis was performed in buffer containing 8.8 M urea, 2% Triton, 0.25 M β-mercaptoethanol, 8.0 mM PMSF and a mixture of Pharmalyte 3-10 (Amersham Pharmacia Biotech, Piscataway, NJ). Isoelectric focusing (IEF) was run on Immobiline DryStrip gel (pH 3-10) with rehydration in appropriate IPG buffer using a Multiphor II apparatus according to the manufacturer's instructions (Amersham Pharmacia Biotech). Following IEF, the second dimension was run on ExcelGel SDS, gradient 8-18% (Amersham Pharmacia Biotech).

For subcellular fractionation, Namalwa cells were first swelled in deionized water for 4 min on ice. The samples were then adjusted to 220 mM mannitol, 70 mM sucrose, 5 mM HEPES, pH 7.4 and 1.0 mM EDTA and dounced briefly on ice. Nuclei (1,000 x g, 15 min) and mitochondria (10,000g, 15 min) were pelleted by sequential differential centrifugation. The supernatants were clarified further by centrifugation at 100,000g for 60 min (S-100 fraction)[31].

For immunoprecipitation, whole cell extracts were prepared in PBS containing 1 mM PMSF, 1 mM DTT and 1.0% NP-40. Specific anti-HA or anti-myc monoclonal antibodies were added for 3 h and immunocomplexes were captured with protein A/G-Sepharose (Santa Cruz Biotechnology Inc., Santa Cruz, CA). Antibodies against the peptide epitope derived from the hemagglutinin protein of human influenza virus (HA) (clone 12CA5) were obtained from Boehringer-Mannheim, and anti-myc tag antibodies (R950-25) from Invitrogen. Affinity-purified antibodies to human Bax (P-19 and P-20) were purchased from Santa Cruz Biotechnology Inc., and antibodies to cytochrome c (clone 7H8.2C12) were procured from PharMingen (San Diego, CA). Enhanced chemiluminescence (ECL) detection of peroxidase-labeled secondary antibodies was performed using Amersham Life Science reagents.

Expression studies by RT-PCR and ribonuclease protection assays. Total RNA was isolated from exponentially growing cells with TRIzol® reagent according to the manufacturer's procedure (Gibco-BRL), and first strand cDNA was synthesized from 2.0 μg of RNA employing Moloney murine leukemia virus reverse transcriptase (2.5 U) (Perkin-Elmer Biosystem, Branchburg, NJ) with

oligo dT(16) priming. cDNA from normal tissues was obtained from Invitrogen. Bax cDNAs were amplified with 50 ng of 5'-primer ATGGACG GGTCCGGGGAGCAG and 50 ng of complementary 3'-primer TCAGCCCATCTTC TTCCAGAT in a reaction mixture containing 200 μM dNTPs, 50 mM KCl , 10 mM Tris-HCl (pH 8.3) and 0.5 U Taq DNA polymerase (Perkin-Elmer Biosystem). The reaction mixtures were heated for 5 min at 95°C and amplified for 30 cycles with denaturation at 95°C for 1 min, annealing at 55°C for 45 sec and extension at 72°C for 45 sec. The amplified products were electrophoresed on a 1.6% agarose gel in Tris-acetate buffer (pH 8.0) and transferred by capillarity to GeneScreen nylon membranes (Dupont-NEM Research Products, Boston, MA). After DNA denaturation and fixation, blots were hybridized with purified human Bax-α cDNA probe labeled with [α -^{32}P]-dCTP by a random priming procedure. Hybridizations were undertaken overnight at 42°C in a solution containing 2x SSC, 2x Denhart, 2% (w/v) SDS, 50% (v/v) formamide and 100 μg/ml salmon sperm DNA. The blots were washed for 30 min at 42°C in 0.1x SSC and 0.1% (w/v) SDS, then for 45 min at 68°C in 0.05x SSC and 0.05% (w/v) SDS. DNA was visualized by autoradiography on Kodak X-AR film.

Expression of the isoforms was confirmed by RNase protection assays in some samples. Specific [α-^{32}P]-dUTP-labeled Bax-σ and Bax-α cRNA probes were prepared using the MAXIscript SP6 and T7 kit (Ambion, Inc., Austin, TX) and eluted from 5% polyacrylamide urea gel after electrophoresis. These probes were hybridized with total RNA (10 μg) for 16 h, then digested with RNase A and T1. The protected fragments were precipitated and then separated on 5% polyacrylamide urea gel. RNA double strands were visualized by autoradiography on Kodak X-AR film. ß-actin was used as control in the RT-PCR and RNase protection assays.

Yeast 2-hybrid system. The MatchMaker LexA 2-hybrid system (Clontech Laboratories Inc, Palo Alto, CA) was employed according to the manufacturer's instructions. Bax-σ deleted of its transmembrane domain, Bax-σ(ΔTM), was subcloned in the 2-hybrid plasmid pLexA vector. The Bcl family members Bcl-xL(ΔTM), Bcl-xS(ΔTM), Bcl-xES(ΔTM), Bfl-1(ΔTM), Bax-α(ΔTM), Bax-σ(ΔTM), Bax-κ(ΔTM), Bak(ΔTM), Bok(ΔTM), the human Egl-1 homolog Bik(ΔTM), Bad, and the human Ced-4 homolog Apaf-1 were subcloned in the 2-hybrid plasmid pB42AD. Sequential transformations were conducted by the lithium acetate method in yeast strain EGY48, and the cells were plated on histidine-deficient

(for pLexA marker), tryptophan-deficient (for pB42AD marker), and histidine/tryptophan/leucine-deficient (for LEU2 reporter gene assay) minimal SD induction/selection media. Apaf-1 cDNA was obtained from Dr. X. Wang (University of Texas Southwestern Center, Dallas TX)[36], Bok cDNA from Dr. A.J.W. Hsueh (Stanford University Medical School, Stanford CA)[52], Bad cDNA from Dr. S.J. Korsmeyer (Washington Medical School of Medicine, St. Louis MO)[53], Bfl-1 and Bik cDNAs from Dr. G. Chinnadurai (St. Louis University Medical Center, St. Louis, Mo)[54, 55], and Bak cDNA from Dr. G.I. Evan (Imperial Cancer Research Fund Laboratories, London, UK)[56]. All the others were cloned previously in our laboratory[30, 42, 57]. pLexA p53, pLex Lam, and pB42AD T from Clontech Laboratories served as positive and negative controls.

DNA filter elution assay. DNA filter elution assays were designed to monitor and quantitate DNA fragmentation associated with apoptosis[51]. 5 x 10^6 [^{14}C]-Thymidine-labeled cells were loaded onto protein-adsorbing filters (vinyl/acrylic copolymer filters, Metricel membrane, 0.8-μm pore size, 25-mm diameter, Gelman Sciences Inc., Ann Arbor, MI), and then washed with 5 ml of PBS. As soon as the washing solution dripped through by gravity, lysis was performed with 5 ml of lysis solution (0.2% sodium sarkosyl, 2 M NaCl, 0.04 M EDTA, pH 10.0). After the lysis solution has dripped through by gravity, the filters were washed with 5 ml of 0.02 M EDTA (pH 10.0). They were then processed as described[58]. Briefly, they were placed in scintillation vials to which 1.0 ml of 1 N HCl was added. The vials were sealed and heated for 1 h at 60°C to depurinate the DNA. Then, 2.5 ml of 0.4 N NaOH was added and allowed to stand for 1 h at room temperature to release labeled DNA from the filters. Radioactivity was counted by liquid scintillation spectrometry in each fraction (loading wash, lysis, EDTA wash, filter). DNA fragmentation was determined as the fraction of DNA in the loading wash fraction + lysis fraction + EDTA wash fraction relative to total DNA (loading wash + lysis + EDTA wash + filter). The results were expressed as the percentage of DNA fragmented in treated cells compared to DNA fragmented in untreated cells (background) using the formula

$$(F\text{-}F0/1\text{-}F0) \times 100$$

where F and F0 represent DNA fragmentation in treated and control cells, respectively[58].

Analysis of DNA fragmentation by agarose gel electrophoresis. To visualize oligonucleosome-sized DNA fragments, at specified times after drug treatment, cellular DNA was extracted by a salting-out procedure, as described

previously[59]. Electrophoresis was performed in 1.6% agarose gel in Tris-acetate buffer (pH 8.0). After electrophoresis, DNA was visualized by ethidium bromide staining.

DEVDase activity determination. Cytosolic extracts were prepared by washing control and transfected Namalwa cells twice by centrifugation/resuspension in 10 ml ice-cold PBS (without Ca^{++} and Mg^{++}). The cells are then homogenized at 4°C for 30 min in a lysis buffer containing 100 mM HEPES (pH 7.5), 5 mM EDTA, 5 mM DTT, 20% glycerol and 0.1% NP-40. The samples were centrifuged (13,000g for 10 min at 4°C), and supernatants collected as cytosolic extracts. Caspase activities were measured by monitoring fluorescence continuously in a dual luminescence LS 50B fluoremeter (Perkin-Elmer) at an excitation wavelength of 380 nm and an emission wavelength of 460 nm for the substrate Ac-DEVD-AMC. Reactions were carried out in cuvettes, and temperature was maintained at 37°C with a water-jacketed sample compartment. The assay mixture contained 100 mM HEPES (pH 7.5), 20% (v/v) glycerol, 5 mM DTT, 5 mM EDTA and 100 μM fluorogenic peptides. Enzyme activities were determined as initial velocities and expressed as relative intensity/min/mg[60].

Electron microscopy. The cells were centrifuged at 400g for 10 min and washed in ice-cold PBS. They were fixed in 0.1 M Millonig's phosphate buffer (pH 7.4, 292 mOsm) containing 2.5% glutaraldehyde, stained with 2% uranyl acetate, and dehydrated with several ethanol treatments. Sections (500 to 700 Å) were mounted on copper grids and stained with lead citrate[61]. The samples were examined by transmission electron microscopy using a Ziess Em10 CA microscope (JFE Enterprises, Brookeville, MD).

RESULTS

Bax-σ is a novel splice variant of Bax. Several studies have strongly suggested the presence of various Bax isoforms[7-9, 11, 12]. On SDS-PAGE (10-18% gradient) under reducing and denaturing conditions, several species of Bax protein were detected. Multiple Bax isoproteins were resolved following 2-D gel electrophoresis under reducing and denaturing conditions and immunoblotting (Fig. 1). Two Bax isoproteins possessed closed molecular weights of approximately 21-kDa and 20-kDa and similar isoelectric points, approximately between pH 6.2 and 6.8 (Fig. 1).

In a search for novel Bax variants, cDNAs were obtained by RT-PCR using

specific adapter primers of Bax-α at the ATG start codon and TGA stop codon. An amplified fragment of 540 bp was resolved from the 579-bp fragment coding for Bax-α by electrophoresis on 2% agarose gel, cloned and sequenced. Bax-σ contained exon-1, -2, -3,-4, -5 and a portion of exon-6 including sequences coding for the transmembrane domain. A 39-bp segment was spliced starting at the exon-5/exon-6 border. This deletion in a portion of exon-6 resulted in a protein that lacked amino acids 159 to 171 of Bax-α. These amino acids consisted of a small segment of Bax-α with unknown function, between the BH-2 and carboxy-terminal transmembrane domains. However, the reading frame of Bax-σ was unaffected and the C-terminal of the protein remained identical to that of Bax-α and contained the membrane anchor domain. Bax-σ also contained BH-3, BH-1, BH-2 domains and putative α-5 and α-6 helices (Fig. 2). First identified and cloned from human promyelocytic HL-60 cells, Bax-σ mRNA expression was investigated in various human cell lines and tissues by non-quantitative RT-PCR and ribonucleotidase protection assays. Table 1 summarizes the detection of Bax-α and Bax-σ in 23 human cancer cell lines and 8 normal human tissues of various origins. We found that Bax-σ had a pattern of expression almost identical to that of Bax-α.

Transient expression of Bax-σ in human B lymphoma Namalwa cells. To investigate the death-inducing activity of Bax-σ we first used a transient transfection assay in human B lymphoma Namalwa cells. Bax-σ and Bax-α cDNAs were subcloned in a modified pCEP4 expression vector that contained consensus Kosak and hemagglutinin epitope tag sequences (HA-tag)[29, 30]. As seen in Fig. 3A, the expression of both Bax-σ and Bax-α proteins can be detected 24 h after transfection. Protein expression increased 48 h post-transfection and remained stable for at least 96 h.

The kinetics of DNA fragmentation were monitored and quantitated by filter DNA elution assay. Transfection of Namalwa cells with Bax-σ and Bax-α induced DNA fragmentation in these cells as early as 24 h post-transfection, and the extent of DNA fragmentation increased with time (Fig. 3B). In cells transfected with a mock vector, no significant DNA fragmentation was observed until 48 h post-transfection (Fig. 3B). To confirm the specificity of the DNA fragmentation observed by the filter DNA elution assay, we visualized the intensity of the oligonucleosome-sized DNA fragments by agarose gel electrophoresis at specific times after transfection. Fig. 3C

shows that the DNA fragmentation pattern induced by Bax-σ and Bax-α was typical of apoptosis, with digestion of chromatin visualized as an oligonucleosome-sized DNA ladder on ethidium bromide-stained agarose gel. This oligonucleosome-sized DNA fragmentation pattern did not appear clearly in mock-transfected cells.

Activation of an apoptotic protease cascade is known to be a critical step in apoptosis induced by various death stimuli, including overexpression of pro-apoptotic proteins such as Bax-α. Several cysteine proteases, known as caspases, play a central role in the execution phase of apoptosis. In the mitochondrial pathway of caspase activation, mitochondrial volume dysregulation with matrix swelling, in response to death stimuli, leads to outer mitochondrial membrane rupture, loss of the mitochondrial voltage gradient and translocation of intermembrane proteins such as cytochrome c to the cytosol[31-35]. The release of cytochrome c allows Apaf-1/pro-caspase-9 interactions that result in the activation of caspase-9 which, in turn, activates directly effector caspase-3 and -7[36-38].

The biochemical mechanism by which Bax-α promotes cell death remains elusive. However, Bax-α may have membrane pore or ion channel activity; it induces loss of mitochondrial membrane potential, cytochrome c release and caspase activation[18, 23, 26, 35, 39]. Bax-α may act also as a docking protein by binding to and antagonizing anti-apoptotic members such as Bcl-xL and Bcl-2[40]. To elucidate the mechanism of action of Bax-σ in mediating apoptosis, caspase activity and cytochrome c release from the mitochondria to the cytosol were monitored in Bax-σ- and Bax-α–transfected Namalwa cells. The kinetics of DEVDase activity were monitored using the specific fluoropeptide derivative Ac-DEVD-AMC as substrate. Fig. 3D shows that DEVDase activity was detected rapidly in Bax-σ- and Bax-α–transfected cells. The kinetics of caspase activation correlated also with the appearance of cytochrome c in the cytosol of Namalwa cells transfected with Bax-σ and Bax-α (Fig. 3E).

Activation of caspases after death stimuli results in typical morphological changes that culminate in disassembly of the dying cell and engulfment by neighboring cells. These morphological changes are best visualized by electron microscopy and include cytoplasmic shrinkage, membrane blebbing and chromatin condensation[41]. Figure 4 presents electron micrographs of control Namalwa cells and Namalwa cells transfected by electroporation with pCEP4-HA mock vector, pCEP4-HA-Bax-σ or pCEP4-HA-Bax-α. Cells were collected and processed for electron microscopy 24 h

(micrographs not shown) and 48 h (Fig. 4) after electroporation.

In control Namalwa cells, less than 1% were apoptotic and less than 1% were necrotic; in pCEP4-HA mock vector-transfected Namalwa cells, 1% were apoptotic and 1-2% were necrotic 24 h after transfection, while 3-4% were apoptotic and 1-2% necrotic 48 h post-transfection. In contrast, in pCEP4-HA-Bax-σ-transfected Namalwa cells, 1-2% were apoptotic and 1% necrotic 24 h after transfection, whereas 35% could be visualized as apoptotic and 1% as necrotic after 48 h. Similarly, in pCEP4-HA-Bax-α-transfected cells, 4-5% were apoptotic and 1-2% necrotic after 24 h, while 25% were apoptotic and 1% necrotic 48 h after transfection.

These morphological analyses clearly demonstrate that apoptosis was the main cell death mechanism activated by Bax-σ overexpression. Moreover, as with Bax-α, Bax-σ overexpression induced cell death by promoting cytochrome c release and caspase-3-like activation.

Interactions of Bax-σ with human Ced-9-, Egl-1- and Ced-4-like family members. To investigate the selectivity of Bax-σ dimerization among various Ced-9-, Egl-1- and Ced-4-like human members, a yeast 2-hybrid system assay was used where Bax-σ was expressed as fusion protein with the LexA DNA-binding domain and putative interacting protein fused with the B42 DNA activation domain. Anchor transmembrane sequences were removed from the cDNAs to promote their nuclear translocation and to avoid toxicity of pro-apoptotic members in yeast cells.

No growth of yeast cells in selective media without tryptophan, histidine and leucine was observed in colonies expressing LexA Bax-σ together with the BH-3-only containing Egl-1 family members Bik and Bad fused to B42AD. Similarly, no interaction with Apaf-1, the human homolog of Ced-4, was detected in the yeast 2-hybrid assay (Fig. 5). However, besides its interaction with itself and other Bax isoforms, including Bax-κ, an isoform that lacks the BH-3 domain (unpublished observation), Bax-σ showed strong interaction with Ced-9 homologs containing combinations of BH-1, BH-2, BH-3 and BH-4 domains such as Bcl-xL, Bcl-xES, a BH-2- and BH-4-containing protein[42], and Bfl-1 that have anti-apoptotic activity compared to members of the family that have pro-apoptotic activities such as Bcl-xS, Bok and Bak (Fig. 5).

To study whether the yeast 2-hybrid interactions observed with anti-apoptotic members of the Ced-9/Bcl family translate into biological effects in regulating apoptosis, we investigated the relationship between Bax-σ and Bcl-xL. We used the stable transfected line Namalwa-Bcl-xL[30] to assess whether high Bcl-xL expression would impede the pro-apoptotic activity of Bax-σ. First, the kinetics of DNA

fragmentation were monitored and quantitated by filter DNA elution assay at various times after transfection of Namalwa-Bcl-xL cells with pCEP4 expression vectors alone or containing Bax-σ and Bax-α sequences. As shown in Fig. 6A and 6B, Bcl-xL impeded the activation of DNA fragmentation induced by Bax-σ and Bax-α expression. In addition, no caspase activation was detected after transfection of Bax-σ and Bax-α in these cells overexpressing Bcl-xL (Fig. 6C).

To determine if Bax-σ bound Bcl-xL in mammalian cells, co-immunoprecipitation was performed in human Namalwa cells. Consistent with the anti-apoptotic ability of Bcl-xL, HA-Bax-σ and HA-Bax-α were found to precipitate with Bcl-xL-Myc when cell extracts were immunoprecipitated either with anti-HA or with anti-Myc epitope tag antibodies (Fig. 6D). These results indicated that Bcl-xL effectively inhibited Bax-σ- and Bax-α-induced cell death.

Stable expression of Bax-σ in human B lymphoma Namalwa cells. To analyze the effect of Bax-σ expression on the drug response, we selected a stable Namalwa variant cell line that expresses Bax-σ. Five days after transfection, Namalwa cells were selected for bulk culture under hygromycin B (1.5 mg/ml) and maintained for about 3 months to obtain a stable line prior to the experiments [29, 30]. Control Namalwa cells did not survive under hygromycin B selection whereas a pCEP4-mock vector-transfected Namalwa cell line was selected under hygromycin B. Western blot analysis indicated that HA-Bax-σ protein was expressed in transfected cells, being found in subcellular mitochondrial and cytosolic fractions (Fig. 7).

To test Bax-σ activity in the selected transfected cell line, we first used the filter DNA elution assay to monitor and quantitate the kinetics of DNA fragmentation in cells undergoing apoptosis induced by several anti-cancer drugs including camptothecin, a DNA topoisomerase I inhibitor, etoposide, a DNA topoisomerase II inhibitor, cisplatin, a DNA adduct-inducing agent, vinblastine, a tubulin polymerization inhibitor, and paclitaxel (Taxol), a microtubule depolymerization inhibitor. Several studies have shown that these compounds can activate the morphological changes and internucleosomal DNA fragmentation associated with apoptosis, in several human cancer cell lines, including Namalwa cells[29, 30]. Treatment with these drugs induced apoptosis in mock-transfected Namalwa cells while low concentrations such as 0.01 μM did not trigger significant apoptosis (Fig. 8). However, Bax-σ overexpression increased the rate of cell death induced by various concentrations of these drugs, with more pronounced effects at low doses of camptothecin and vinblastine (Fig. 8). These effects were similar to those reported for

Bax-α in transfected lymphoma Namalwa cells[30], and were associated also with oligonucleosome-sized DNA fragmentation typical of apoptosis (Fig 8F). Moreover, Bax-σ variant-transfected Namalwa cells treated with camptothecin exhibited morphological changes typical of apoptosis (Fig. 9).

DISCUSSION

Bax-σ lacks amino acids 159 to 171 of Bax-α, a small segment of Bax-α with an unknown function between the BH-2 and transmembrane domains. However, Bax-σ retains all the Bcl-homolog domains BH-1, BH-2 and BH-3, the putative α-5 and α-6 helices and the anchor transmembrane domain. Although the exact mechanisms by which Bax isoproteins transduce cell death remains controversial, 3 main mechanisms have been proposed: 1. Dimerization and interference with anti-apoptotic members of the Bcl-2 family, 2. The formation of channels at the mitochondrial level, and 3. Interference with mitochondrial permeability transition pore complexes through adenine nucleotide translocator protein or voltage-dependent anion channel. Bax-σ binds to several anti-apoptotic members, including Bcl-xL, that impedes its apoptotic activity. However, the lack of amino acids 159 to 171 between the α-6 helix and the transmembrane domain may contribute to conformational changes of the protein. Such changes could modify the putative channel activity described for Bax-α, including the levels of conductance and ion selectivity. Similarly, the interaction of Bax-α to MPTP through adenine nucleotide translocator protein, or VDAC is not clearly yet understood. Whether Bax-α that lacks amino acids 159 to 171 interacts or not with MPTP remains to be elucidated, and further investigations are underway to clarify the importance of this amino acid segment for the interaction with MPTP.

Reduced Bax-α expression was associated with poor response rates to chemotherapy in women with metastatic breast adenocarcinoma[43]. Transfection studies in human breast cell lines showed that Bax-α sensitized these cells to radiation- and drug-induced apoptosis[44-46]. Others have indicated that the Bax:Bcl-2 ratio in human testicular tumors and B-cell chronic lymphocytic leukemia is related to cell susceptibility to apoptosis[11, 47, 48]. In FL5.12 cells, Bax-α can antagonize the protective effects of Bcl-xL during etoposide-induced apoptosis but is much less potent to promote cell death evoked by cisplatin[49]. However, others have reported that a mean 10-fold increased level of murine Bax expression in human SW626 cancer cells promotes cell death induced by paclitaxel[13, 50]. In the human Namalwa cell

line, Bax-α overexpression accelerated cell death by short or continuous treatments with various concentrations of camptothecin, etoposide or vinblastine but had no such effect in cisplatin- or paclitaxel-treated cells [30].

Our observations in this study indicate that Bax-σ exerted effects similar to those of Bax-α, accelerating apoptosis induced by camptothecin, vinblastine and paclitaxel but having a much less potent action with etoposide and cisplatin. Like Bax-α, the ability of Bax-σ to sensitize cells to anti-cancer drugs appears to vary, depending on the stimuli and perhaps the cell type.

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Table 1. Expression of Bax-σ and Bax-α in human tissues and cancer cells lines

Cell Types	Origin	Bax-σ	Bax-α
HL-60*	Pro-myelocytic leukemia	+	+
U-937*	Histyocytic lymphoma	+	+
Namalwa*	Burkitt's lymphoma	+	+
NL-2	Burkitt's lymphoma	a	a
Raji	Burkitt's lymphoma	+	+
Jurkatt	Acute T-cell leukemia	a	a
H9	T-cell lymphoma	+	+
CCRF CEM	Acute lymphoblastic leukemia	+	+
MCF-7	Breast adenocarcinoma	+	+
MDA-MB-468	Breast adenocarcinoma	+	+
SK-BR-3	Breast adenocarcinoma	+	+
OVCAR-3	Ovary adenocarcinoma	+	+
A-2780	Ovary adenocarcinoma	+	+
DU-145	Prostate carcinoma	a	a
PC-3	Prostate adenocarcinoma	+	+
LNCap-FGC-10	Prostate carcinoma	-	+
Calu-1	Lung carcinoma	+	+
A-431	Epidermoid carcinoma	+	+
NCI-H69	Small cell lung carcinoma	+	+
NCI-N417	Small cell lung carcinoma	+	+
Colo-205	Colon adenocarcinoma	+	+
Colo-320DM	Colon adenocarcinoma	+	+
HT-29	Colon adenocarcinoma	+	+
Spleen	Normal	+	+
Skin	Normal	±	±
Breast	Normal	+	+
Ovary	Normal	+	+
Testis	Normal	+	+
Lung	Normal	+	±
Colon	Normal	+	+
Brain	Normal	+	+

Symbols are (+) positive; (-) negative; (±) very weak; (a) atypical forms.

Note: Human Bax-σ and Bax-α expression was detected by RT-PCR from total RNA using specific primers at the ATG start codon and TGA stop codon. Electrophoresis was done in 1.6% agarose gel and the amplified fragments transferred to GeneScreen nylon membranes. Blots were hybridized with a human Bax-α cDNA probe labeled with [^{32}P]dCTP by random priming procedure. Expression of the isoforms was confirmed by RNase protection assays in selected samples (*).

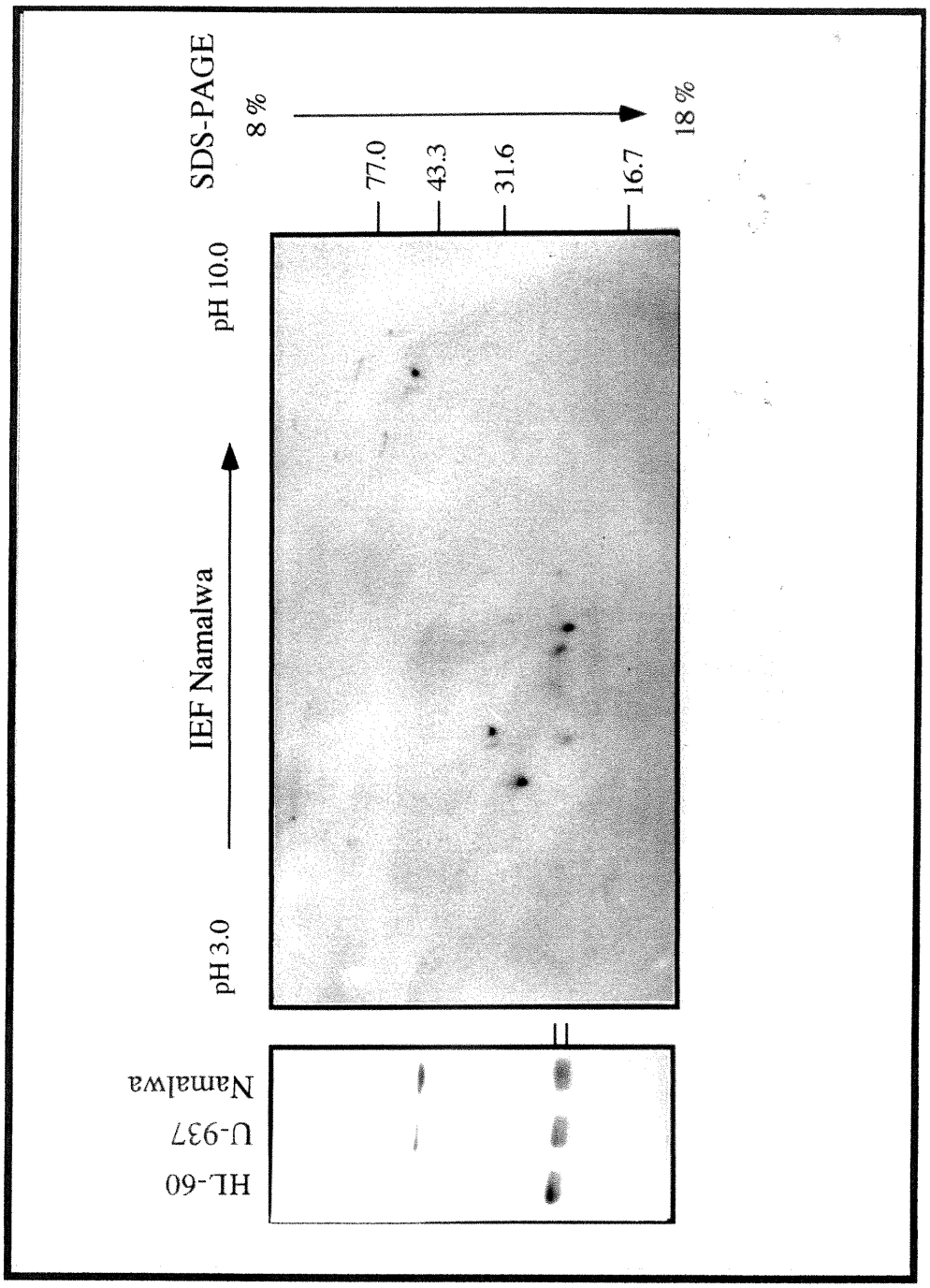
SDS-PAGE
8 %
18 %
77.0
43.3
31.6
16.7
pH 10.0
IEF Namalwa
pH 3.0
HL-60
U-937
Namalwa

Figure 1

Fig. 1. Expression of Bax variants by SDS-PAGE and 2-D gel electrophoresis.

Left panel: Cell extracts were prepared from human HL-60, U-937 and Namalwa lines. Following SDS-PAGE on a 10-18% gradient under reducing and denaturing conditions and Western blotting, Bax variants were detected using anti-Bax polyclonal antibodies (P-19). Visualization was performed by enhanced chemiluminescence (ECL) and autoradiography.

Right panel: Protein extracts were prepared from Namalwa cells, and the first dimension (IEF) was run on Immobiline DryStrip gel (pH 3-10) using Multiphor II at 3,500 V, 2.0 mA and 5 W for 8 h. Following IEF, the second dimension was run on ExcelGel SDS, gradient 8-18%. Bax variants were detected using anti-Bax polyclonal antibodies (P-20) and visualization was performed by ECL.

```
bax-α       1 atggacgggt ccggggagca gcccagaggc ggggggccca ccagctctga gcagatcatg
bax-σ       1 atggacgggt ccggggagca gcccagaggc ggggggccca ccagctctga gcagatcatg

bax-α      61 aagacagggg ccctttgct tcagggtttc atccaggatc gagcagggcg aatgggggg
bax-σ      61 aagacagggg ccctttgct tcagggtttc atccaggatc gagcagggcg aatgggggg

bax-α     121 gaggcacccg agctggccct ggacccggtg cctcaggatg cgtccaccaa gaagctgagc
bax-σ     121 gaggcacccg agctggccct ggacccggtg cctcaggatg cgtccaccaa gaagctgagc

bax-α     181 gagtgtctca agcgcatcgg ggacgaactg gacagtaaca tggagctgca gaggatgatt
bax-σ     181 gagtgtctca agcgcatcgg ggacgaactg gacagtaaca tggagctgca gaggatgatt

bax-α     241 gccgccgtgg acacagactc cccccgagag gtctttttcc gagtggcagc tgacatgttt
bax-σ     241 gccgccgtgg acacagactc cccccgagag gtctttttcc gagtggcagc tgacatgttt

bax-α     301 tctgacggca acttcaactg gggccgggtt gtcgcccttt tctactttgc cagcaaactg
bax-σ     301 tctgacggca acttcaactg gggccgggtt gtcgcccttt tctactttgc cagcaaactg

bax-α     361 gtgctcaagg ccctgtgcac caaggtgccg gaactgatca gaaccatcat gggctggaca
bax-σ     361 gtgctcaagg ccctgtgcac caaggtgccg gaactgatca gaaccatcat gggctggaca

bax-α     421 ttggacttcc tccgggagcg gctgttgggc tggatccaag accagggtgg ttgggacggc
bax-σ     421 ttggacttcc tccgggagcg gctgttgggc tggatccaag accagggtgg ttgg......

bax-α     481 ctcctctcct actttgggac gcccacgtgg cagaccgtga ccatctttgt ggcgggagtg
bax-σ     475 .......... .......... .......... ...accgtga ccatctttgt ggcgggagtg

bax-α     541 ctcaccgcct cgctcaccat ctggaagaag atgggctga
bax-σ     502 ctcaccgcct cgctcaccat ctggaagaag atgggctga
```

Figure 2A

```
bax-α       1 MDGSGEQPRG GGPTSSEQIM KTGALLLQGF IQDRAGRMGG EAPELALDPV PQDASTKKLS
bax-σ       1 MDGSGEQPRG GGPTSSEQIM KTGALLLQGF IQDRAGRMGG EAPELALDPV PQDASTKKLS

                                                      α-5 helix
                                                    ■■■■■ ■■■■■■■
                  BH-3                                BH-1
bax-α      61 ECLKRIGDEL DSNMELQRMI AAVDTDSPRE VFFRVAADMF SDGNFNWGRV VALFYFASKL
bax-σ      61 ECLKRIGDEL DSNMELQRMI AAVDTDSPRE VFFRVAADMF SDGNFNWGRV VALFYFASKL

                  α-6 helix
          ■■■■■■■    ■■■■■■■■   ■ ■■■
                                                BH-2                  TM
bax-α     121 VLKALCTKVP ELIRTIMGWT LDFLRERLLG WIQDQGGWDG LLSYFGTPTW QTVTIFVAGV
bax-σ     121 VLKALCTKVP ELIRTIMGWT LDFLRERLLG WIQDQGGW.. .......... .TVTIFVAGV

                  TM
bax-α     181 LTASLTIWKK MG
bax-σ     168 LTASLTIWKK MG
```

Figure 2B

Fig. 2. Bax-σ is a novel member of the Bax gene.

A: Nucleotide sequence alignment of Bax-σ compared to Bax-α. These sequence data are available from GenBank under accession number AF247393.

B: Amino acid sequence alignment. BH-3, BH-1, BH-2 domains, putative α-5 and α-6 helices and the COOH- terminal hydrophobic domain are indicated.

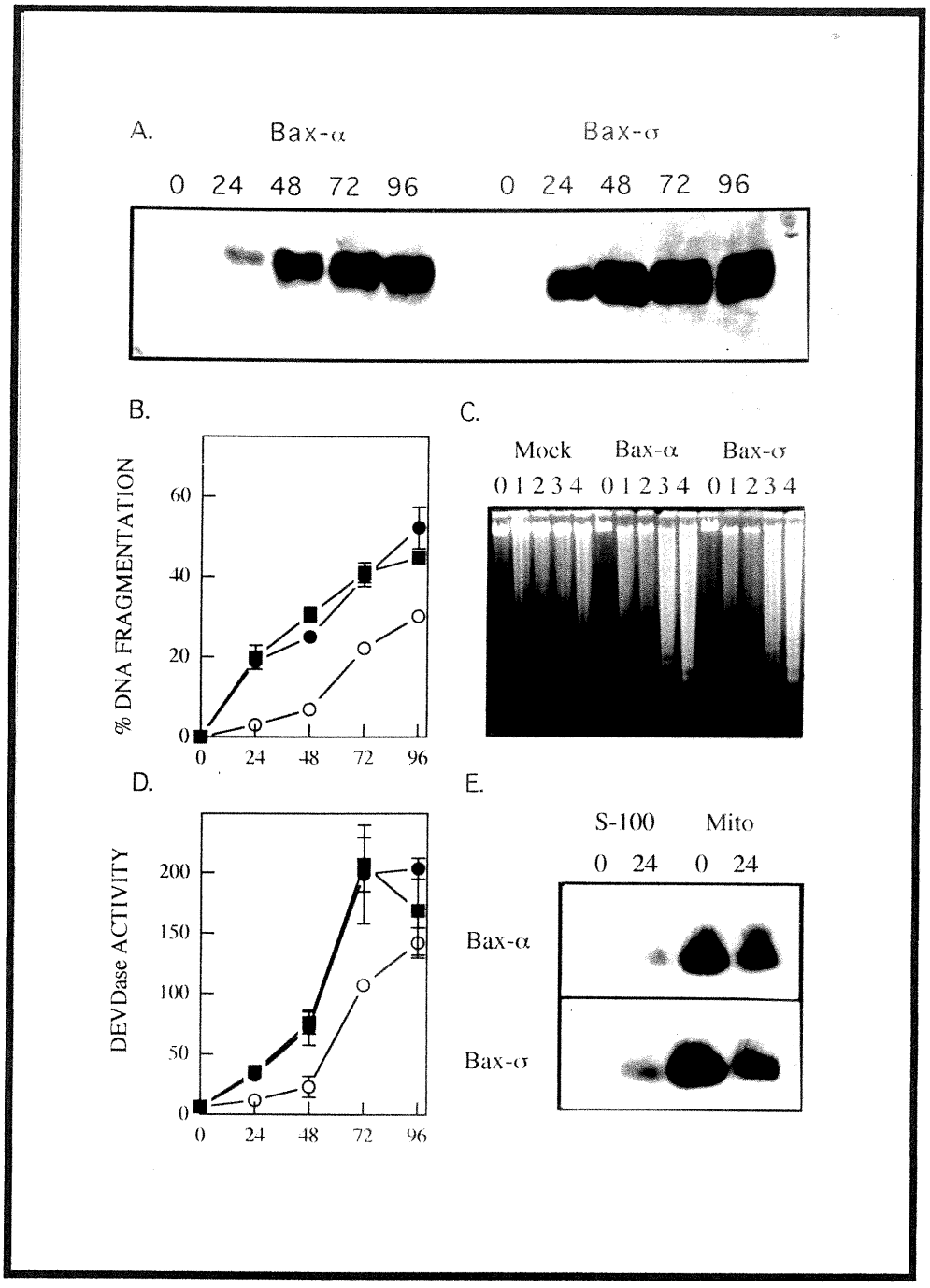
A.
Bax-α
Bax-σ
0 24 48 72 96
0 24 48 72 96
B.
% DNA FRAGMENTATION
60
40
20
0
0 24 48 72 96
C.
Mock
Bax-α
Bax-σ
0 1 2 3 4 0 1 2 3 4 0 1 2 3 4
D.
DEVDase ACTIVITY
200
150
100
50
0
0 24 48 72 96
E.
S-100
Mito
0 24 0 24
Bax-α
Bax-σ

Figure 3

Fig. 3. Transient expression of Bax-σ in human B lymphoma Namalwa cells.

A: Bax-σ and Bax-α cDNAs were inserted in frame in a modified pCEP4 vector containing Kosak and HA epitope tag sequences. After electroporation (0.27 kV, 10 μg plasmid/1.0 x 10^7 cells), Namalwa cells were grown in complete medium, and at indicated times (h) following electroporation, HA-Bax-σ and HA-Bax-α were immunoprecipitated using specific anti-HA-tag antibodies. After SDS-PAGE and transfer, proteins were revealed by anti-Bax antibodies and visualized by ECL. **B:** [^{14}C]-Thymidine-prelabeled cells were electroporated with pCEP4-HA mock vector (○), pCEP4-HA-Bax-σ (●) or pCEP4-HA-Bax-α (■) and grown in complete medium. At the times indicated (x axis; h), DNA fragmentation was determined by filter elution assays. The results are expressed as % DNA fragmentation. The points represent the means ± SE of 6 determinations. **C:** Namalwa cells were electroporated with pCEP4-HA mock vector, pCEP4-HA-Bax-σ or pCEP4-HA-Bax-α. At the days indicated following electroporation, total DNA was extracted and visualized by agarose gel electrophoresis and ethidium bromide staining. **D:** Following electroporation with pCEP4-HA mock vector (○), pCEP4-HA-Bax-σ (●) or pCEP4-HA-Bax-α (■), cytosolic extracts were prepared from cells at the times indicated (x axis; h). DEVDase activity was monitored continuously at 37°C by detecting fluorescence emission in the presence of Ac-DEVD-AMC at an excitation wavelength of 380 nm and emission wavelength of 460 nm. Caspase activities were measured as initial velocities and expressed as relative intensity/min/mg. Points represent the means ± SD of 3 independent experiments. **E:** Cytosolic S-100 extracts and mitochondrial extracts were prepared from Namalwa cells before and 24 h after electroporation with pCEP4-HA-Bax-σ or pCEP4-HA-Bax-α. Immunoblot analysis of cytochrome c was performed in both fractions using anti-cytochrome c-specific antibodies and enhanced chemiluminescence reaction.

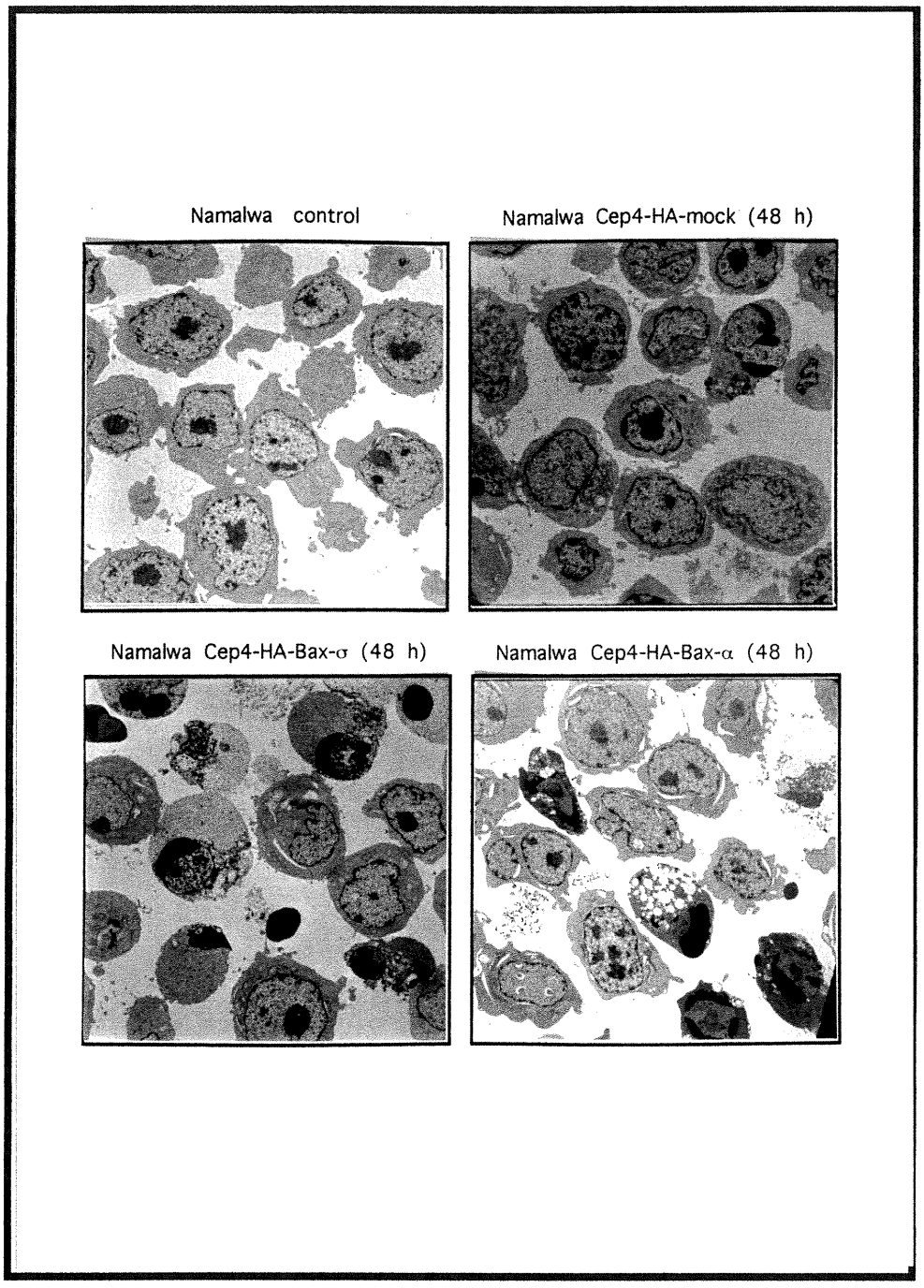
Namalwa control
Namalwa Cep4-HA-mock (48 h)
Namalwa Cep4-HA-Bax-σ (48 h)
Namalwa Cep4-HA-Bax-α (48 h)

Figure 4

Fig. 4. Cellular morphology of Namalwa cells expressing Bax-σ.

Electron micrographs of control Namalwa cells and Namalwa cells transfected by electroporation with pCEP4-HA mock vector, pCEP4-HA-Bax-σ or pCEP4-HA-Bax-α. The cells were collected and processed for electron microscopy 48 h after electroporation. In the controls, less than 1% were apoptotic and less than 1% were necrotic; in pCEP4-HA mock- transfected cells, 3-4 % were apoptotic and 1% necrotic after 48 h; in pCEP4-HA-Bax-σ-transfected cells, 35% were apoptotic and 1% necrotic after 48 h; in pCEP4-HA-Bax-α-transfected cells, 25% were apoptotic and 1% necrotic after 48 h. Approximately 250-300 cells were observed by electron microscopy.

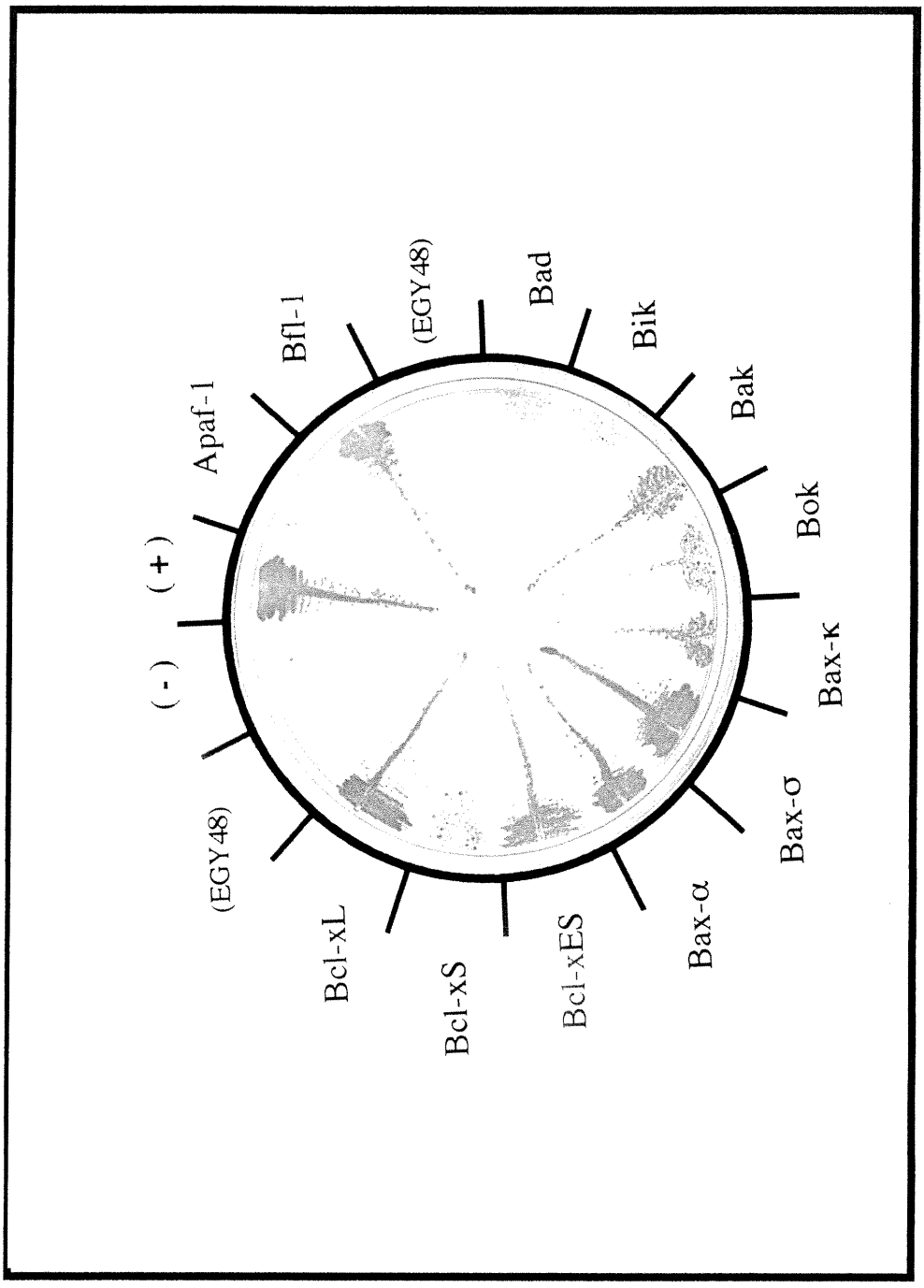
(EGY48)
Bad
Bik
Bak
Bok
Bax-κ
Bax-σ
Bax-α
Bcl-xES
Bcl-xS
Bcl-xL
(EGY48)
(-)
(+)
Apaf-1
Bfl-1

Figure 5

Fig. 5. Interaction of Bax-σ in yeast 2-hybrid assay.

EGY 48 yeast cells containing Bax-σ fused to the LexA DNA-binding domain were grown in selective medium without tryptophan, leucine and histidine in the presence of the various proteins fused to the B42 DNA activation domain as indicated. Positive controls *(+)* represent pLexA p53 and pB42 SV40 T, and negative controls *(-)* represent pLexA laminC and pB42 SV40 T. *(EGY 48)* represent untransformed yeast cells.

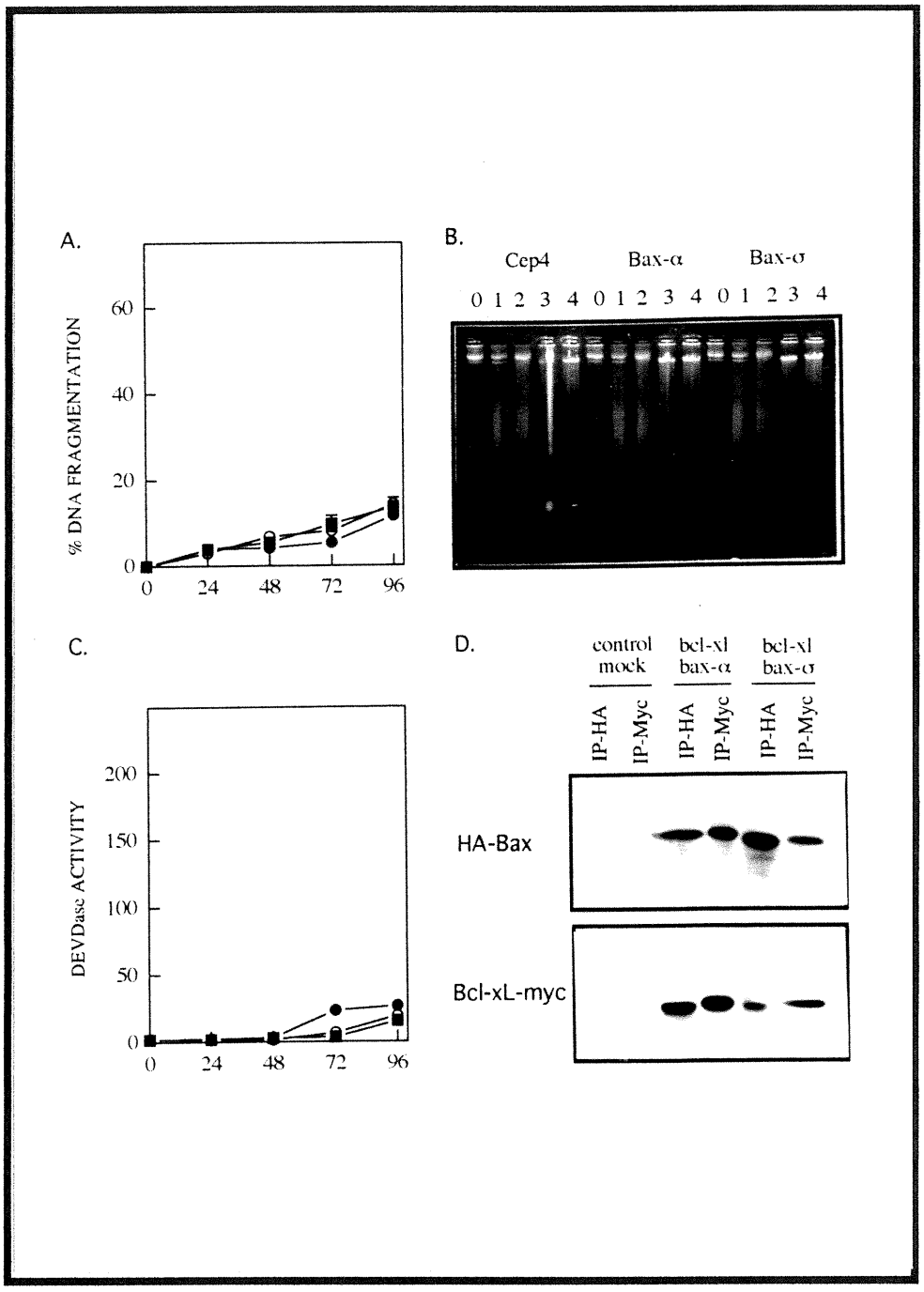
A.
% DNA FRAGMENTATION
60
40
20
0
0
24
48
72
96
B.
Cep4
Bax-α
Bax-σ
0 1 2 3 4 0 1 2 3 4 0 1 2 3 4
C.
DEVDase ACTIVITY
200
150
100
50
0
0
24
48
72
96
D.
control mock
bcl-xl bax-α
bcl-xl bax-σ
IP-HA
IP-Myc
IP-HA
IP-Myc
IP-HA
IP-Myc
HA-Bax
Bcl-xL-myc

Figure 6

Fig. 6. Transient expression of Bax-σ in human B lymphoma Namalwa cells overexpressing Bcl-xL.

A: [^{14}C]Thymidine-prelabeled Namalwa cells overexpressing Bcl-xL were electroporated with pCEP4-HA mock vector (○), pCEP4-HA-Bax-σ (●) or pCEP4-HA-Bax-α (■) and grown in complete medium. At the indicated times (x axis; h), DNA fragmentation was determined by filter elution assays. The results are expressed as % DNA fragmentation. The points represent the means ± SE of 3 determinations. **B:** Namalwa cells overexpressing Bcl-xL were electroporated with pCEP4-HA mock vector, pCEP4-HA-Bax-σ or pCEP4-HA-Bax-α. At the days indicated following electroporation, total DNA was extracted and visualized by agarose gel electrophoresis and ethidium bromide staining. **C:** Following electroporation with pCEP4-HA mock vector (○), pCEP4-HA-Bax-σ (●) or pCEP4-HA-Bax-α (■) cytosolic extracts were prepared at the times indicated (x axis; h). DEVDase activity was measured as initial velocities and expressed as relative intensity/min/mg. The points represent the means of 2 independent experiments. **D:** Control Namalwa cells were co-transfected with pCEP4-HA and pCDNA-Myc mock vectors, pCEP4-HA-Bax-σ and pCDNA-Bcl-xL-Myc vectors or pCEP4-HA-Bax-α and pCDNA-Bcl-xL-Myc vectors. After 48 h, extracts were prepared and immunoprecipitated (IP) with anti-HA and anti-Myc epitope tag antibodies. Immunocomplexes were trapped by protein A/G sepharose, and the presence of HA-Bax-σ, HA-Bax-α and Bcl-xL-Myc was detected by immunoblotting with anti-HA and anti-Myc epitope tag antibodies (as indicated in the left margin).

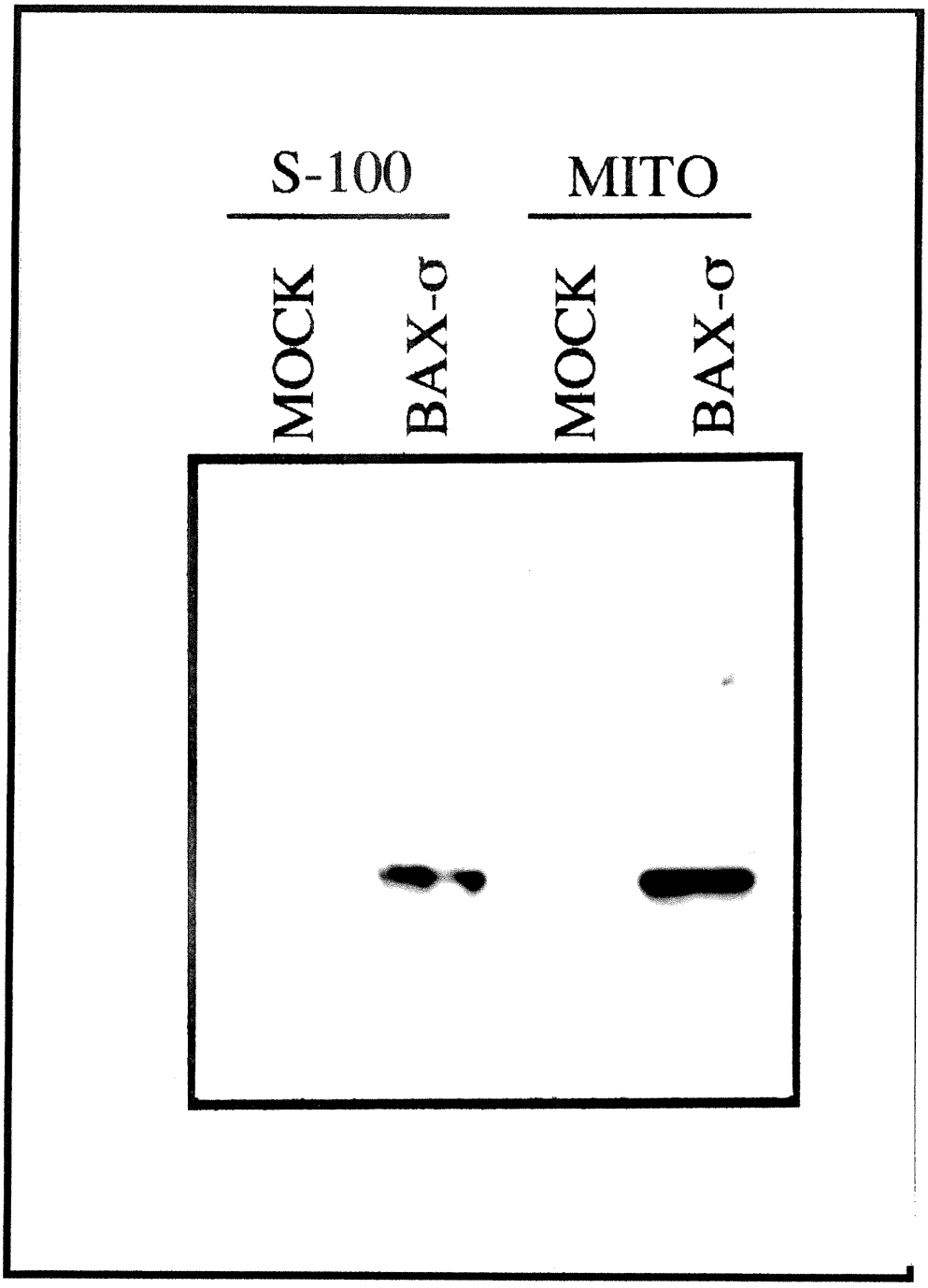

Figure 7

Fig. 7. Subcellular localization of Bax-σ in transfected Namalwa cells.

Subcellular extracts were prepared, as described in Experimental Procedures, from stable Namalwa cells transfected by electroporation with pCEP4-HA mock vector or pCEP4-HA-Bax-σ selected at 1.5 mg/ml hygromycin B. Following SDS-PAGE and electrophoretic transfer, HA-Bax-σ proteins were detected using anti-HA peptide monoclonal antibodies. Visualization was performed by enhanced chemiluminescence reaction and autoradiography.

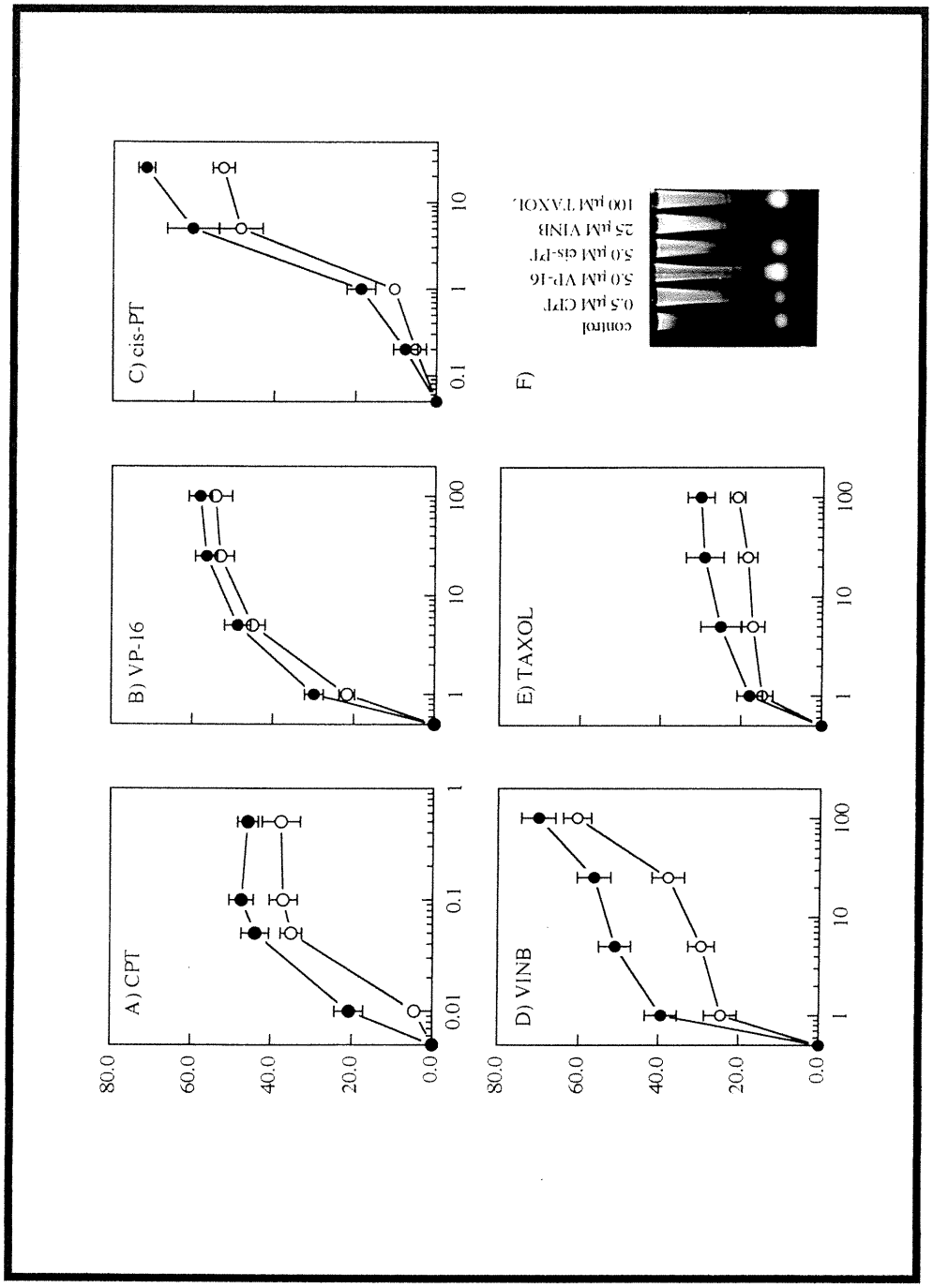
A) CPT
B) VP-16
C) cis-PT
D) VINB
E) TAXOL
F)
control
0.5 μM CPT
5.0 μM VP-16
5.0 μM cis-PT
25 μM VINB
100 μM TAXOL
80.0
60.0
40.0
20.0
0.0
0.01
0.1
1
10
100

Figure 8

Fig. 8. Kinetics of DNA fragmentation in Namalwa and Namalwa Bax-σ-transfected cells.

[^{14}C]thymidine-labeled cells were treated with various concentrations of camptothecin (CPT), etoposide (VP-16), cisplatin (cisPT), vinblastine (VINB) and paclitaxel (Taxol). DNA fragmentation was determined 18 h after drug treatments by DNA filter elution assays. The results are expressed as % DNA fragmentation. The points represent the means ± SE of 3 independent experiments performed in triplicate. The symbols in A, B, C, D and E are Namalwa cells transfected with mock vector selected at 1.5 mg/ml hygromicin (○) or Namalwa cells expressing Bax-σ and selected at 1.5 mg/ml hygromicin (●). In F, Namalwa-Bax-σ cells were treated for 24 h with various drugs, and DNA was extracted by a salting-out procedure. Following agarose gel electrophoresis, DNA was visualized by ethidium bromide staining.

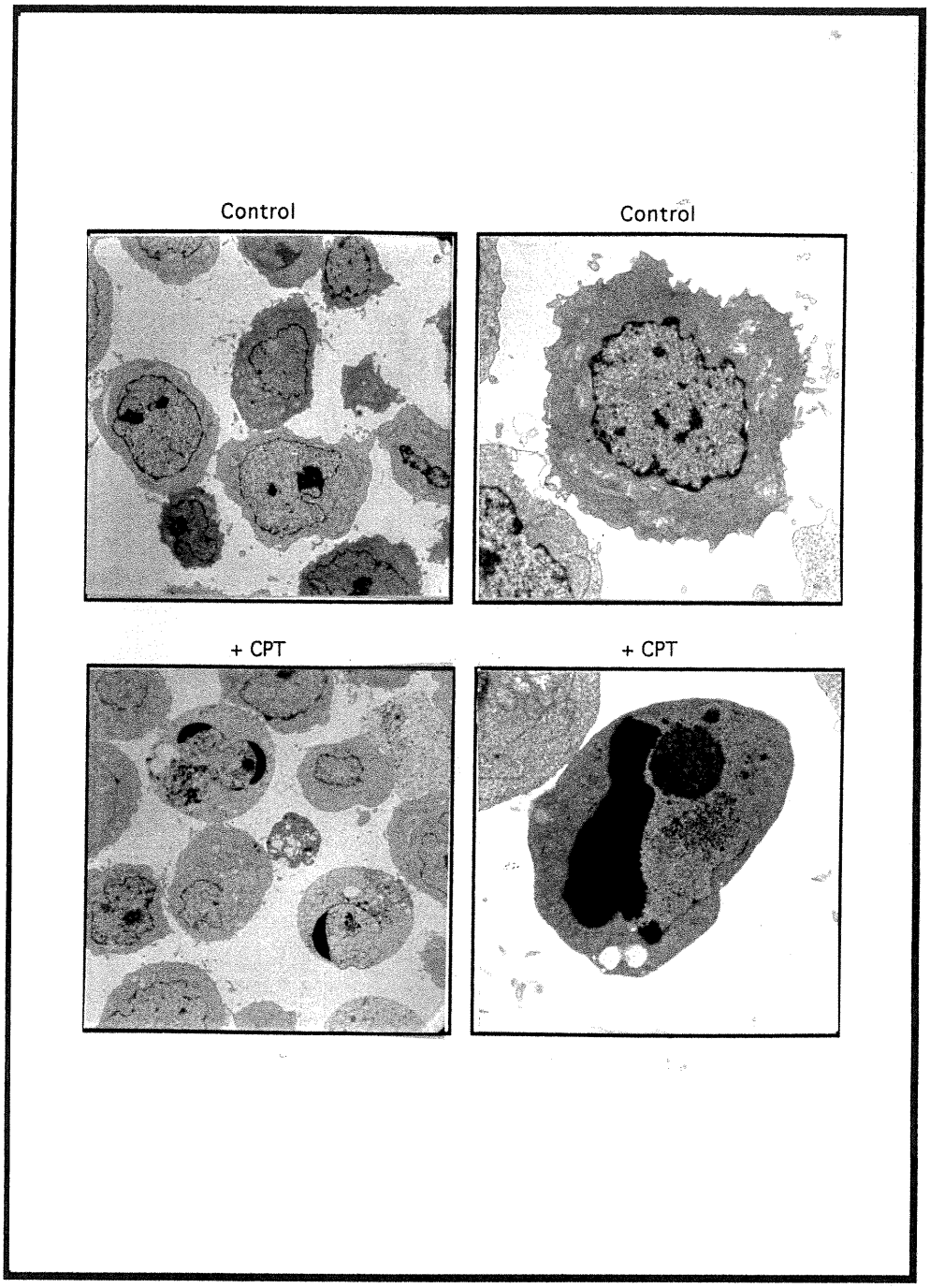

Figure 9

Fig. 9. Cellular morphology of Namalwa-HA-Bax-σ cells following camptothecin treatment.

Electron micrographs of control and CPT-treated Namalwa-HA-Bax-σ variant lines. The cells were collected and processed for electron microscopy 8 h after drug treatment.

CHAPITRE 5.

Identification and biological activity of Bcl-xES, a unique BH-4 and BH-2 containing protein that modulates cell death

(Manuscrit en préparation)

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Notre Dame Hospital, Montreal Cancer Institute
Montreal (Que.) Canada H2L 4M1

Avant propos:

Nous avons isolé une nouvelle isoforme de Bcl-x appelée Bcl-xES. La protéine Bcl-xES (extra short) possède une combinaison unique de domaines BH et contient les domaines BH-4, BH-2 ainsi que le domaine C-terminal hydrophobe. Une analyse de l'expression des messagers bcl-xES montre que les transcrits sont exprimés dans une variété de lignées cellulaires tumorales et plusieurs tissus tumoraux humains.

Ce travail consiste à étudier la fonction de Bcl-xES dans l'apoptose. L'expression de Bcl-xES dans les cellules de lymphome B Namalwa diminue la sensibilité des cellules à l'apoptose induite par les agents anticancéreux tels que la camptothécine, l'étoposide, et le cisplatine. Nous montrons que Bcl-xES est un nouveau membre anti-apoptotique de la famille des Ced-9/Bcl. Bcl-xES exerce une modulation négative de l'apoptose induite par les drogues en inhibant la libération du cytochrome c au niveau des mitochondries et l'activation subséquente des caspases.

• M. Beauchemin a mis au point le système de double-hybride dans la levure.

SUMMARY

Several Ced-9/Bcl family members that regulate either negatively or positively the apoptotic threshold of a cell have been identified in mammalians. Bcl-xES (*extra short*), a novel Bcl-x member, possesses a unique combination of BH-4 and BH-2 domains and the COOH-terminal hydrophobic transmembrane anchor domain. Bcl-xES contains sequences of the hydrophobic α-6 helices but lacks the α-5 helices' sequences suggesting that it does not have a pore channel-forming activity but a unique function as an adaptor/docking/trapping protein. mRNA expression analysis by reverse transcription-polymerase chain reaction and RNase protection assays revealed that Bcl-xES is expressed in a variety of human cancer cell lines and human tumors including bone marrow samples from patients with acute lymphoblastic leukemia. Expression of Bcl-xES is much less pronounced in some specimen of normal human tissues including breast, ovary, testis and lung. To investigate the role of Bcl-xES on apoptosis, stable transfected human B lymphoma Namalwa variant cells expressing Bcl-xES were derived. Bcl-xES has a protective effect on cell death induced by various concentrations of anticancer drugs including camptothecin, etoposide, cisplatin and by TNF-α. Its protective effect correlated with inhibition of mitochondrial cytochrome c release and caspase activation. In a yeast 2-hybrid system, Bcl-xES interacted with most Ced-9/Bcl family members, including those containing a BH-3 domain only, and with the Ced-4 homolog, Apaf-1. Its BH-2 domain is most likely responsible for binding to Ced-9/Bcl family members. Its mitochondrial localization and the presence of a BH-4 domain within Bcl-xES, suggests that the protein binds to mitochondrial voltage-dependent anion channel (VDAC), thus preventing cytochrome c release. It also suggests that Bcl-xES traps Apaf-1/caspase-9 complexes and interferes with caspase activation, thus inhibiting the apoptogenic proteolytic cascade and cell death.

Abbreviations: Ac-LEHD-AFC, Ac-Leu-Glu-His-Asp-7-amino-4-trifluoromethylcoumarin; Ac-DEVD-AMC, Ac-Asp-Glu-Val-Asp-amino-4-methylcoumarin; CPT, 20-S-camptothecin lactone; VP-16, etoposide; cisPT, cis-platinum(II) diammine dichloride; MPTP , mitochondrial permeability transition pore; $\Delta\psi m$, mitochondrial inner transmembrane potential; ANT, adenine nucleotide translocator; VDAC, voltage-dependent anion channel; RT-PCR, reverse transcriptase-polymerase chain reaction; HA-tag, hemagglutinin epitope tag sequences.

INTRODUCTION

The mammalian Ced-9/Bcl-like proteins share homology that is principally clustered within the conserved Bcl-2 homology domains (BH-1, BH-2, BH-3 or BH-4), but can also contain a long flexible loop (LOOP), a pore-forming domain (PFD) consisted of the α-5 and α-6 helices, and a carboxy-terminal hydrophobic transmembrane domain (TM) responsible for their localization to subcellular organelles, including mitochondria, endoplasmic reticulum and the nucleus (1-3). Members of this family include the anti-apoptotic Bcl-2 (4-7), Bcl-xL (8), Mcl-1(9), A1/Bfl-1 (10, 11), Bcl-w (12), Brag-1 (13), Boo/Diva (14, 15) and pro-apoptotic Bax (16), Bak (17-19), Bcl-xS (8) and Bok/Mtd (20, 21). The Egl-like subsets of Ced-9/Bcl-like that contain only BH-3 domains, no α-5 and α-6 helices, and thus possess a specific role in signaling cell death, includes pro-apoptotic Bad (22), Bik/Nbk/mBlk (23-25), Bid (26), Hrk/Dp5 (27, 28), Bim/Bod (29, 30), BNip1, BNip3 (31) and the variants Bnip3L/Nix/BNip3a(32-34). Several isoforms have been isolated for Bcl-x (Bcl-xL, -xS, -xβ, -xγ) (8, 35, 36), Bax (Bax-α, -ß, -γ, -δ, -ω, -ε, -σ) (16, 37-41), Bim/Bod (Bim-L, -EL, -S) (29) and BNip1 (BNip-1, -1a, -1b, -1c) (42), suggesting that splicing regulation could act as an important control for programmed cell death (apoptosis) (43).

The various forms of Ced-9/Bcl proteins possess unique combinations of structure-function domains that determine their capacity to interact with each other and with other proteins, depending on their localization. These protein-protein interactions participate in cell-death regulation (1-3, 44). The intracellular localization of the Bcl-related proteins and the three-dimensional structure of Bcl-xL, which revealed structural similarity with pore-forming domains of bacterial toxins (45), have suggested that Bcl-related proteins may also have pore channel-forming activity (46-48). These observations have suggested that the Ced-9/Bcl proteins function both as adaptor/docking/trapping proteins and as pore channels (1-3, 46-48).

The recent evidence implicating mitochondria in apoptosis has prompted research to understand the link between these proteins and the mitochondria (49-55). The localization of these proteins at the outer mitochondrial membrane suggests that they may influence the opening and/or closing of mitochondrial megachannels by a so-called permeability transition pore (mPTP) (56, 57) and influence release of apoptogenic protease activators from mitochondria such as apoptosis-inducing factor (AIF) (51, 58) and cytochrome c (59-61). Recent observations have revealed that some Ced-9/Bcl proteins bind to mitochondrial permeability transition pore complexes

(MPTP) and cooperate with adenine nucleotide translocator (ANT) protein to increase mitochondrial membrane permeability ($\Delta\psi m$) and potently transduce cell death (62). In reconstituted liposomes that contain the mitochondrial porin channel or voltage-dependent anion channel (VDAC), Bax-α accelerates VDAC opening in a manner that permits cytochrome c to pass directly through VDAC, while the BH-4 containing proteins Bcl-xL and Bcl-2 impede such opening (63, 64).

Recent studies have shown that protein-protein interactions of Ced-9/Bcl-like members with unrelated proteins are also important in controlling caspase activation by a trapping-like mechanism that involves multimolecular complexes between Bcl-xL / Apaf-1/ Caspase-9 (65-73). Apaf-1 binds to caspase-9 in the presence of dATP and cytochrome c and this interaction is mediated by the CARD domains found on the two proteins. The complex formation between Apaf-1 and caspase-9 zymogen leads to the cleavage and activation of caspase-9, probably through an autoproteolytic processing and caspase-9 in turn directly activates caspase -3 and -7 (70, 71). The binding of Bcl-xL to Apaf-1/caspase 9 inhibits the oligomerization and activation of caspase-9 (72, 74). The specific role of the human counterparts of Egl-1 in transducing cell death induced by various stimuli is emerging. Specific cell death stimuli mediate post-translational modifications of members of this family as Bid and Bad (75-77). These modifications mediate a subcellular redistribution of these proteins which allow their translocation to the mitochondria. At the mitochondria level, they interfere with the anti-apoptotic function of Ced-9/Bcl proteins such as Bcl-2 and Bcl-xL (75-77) and promote cytochrome c release (78).

Although the precise molecular mechanisms triggered by apoptosis-inducing agents used in cancer therapy remains unknown, many studies including ours strongly suggest that the mitochondrial pathway of caspase activation plays a central role in cell death induced by genotoxic drugs (for review (79)). The Ced-9/Bcl proteins are key regulators of caspase activation and downstream nuclear events in apoptosis triggered by a variety of drugs like camptothecin, etoposide, vinblastine, paclitaxel, cisplatin and cytarabine (41, 80-86). Overexpression of anti-apoptotic Bcl-xL and Bcl-2 antagonizes cytochrome c release from mitochondria and subsequent caspase activation triggered by anticancer drugs (59, 60, 86).

In this paper, we report the identification and characterization of Bcl-xES *(extra-short)*, a novel Bcl-x member expressed in a variety of human tissues and cancer cell lines. Bcl-xES has a unique structure compared to Bcl-xL and Bcl-2, and contains BH-4 and BH-2 domains, sequences of the α-6 helices and the

COOH-terminal hydrophobic transmembrane anchor domain but lacks the BH-1, BH-3 and flexible LOOP domains and sequences corresponding to α-5 helices.

MATERIALS AND METHODS

Chemicals. Radioactive precursors [α-^{32}P]-dCTP (> 3,000 Ci/mmol), [α-^{32}P]-dUTP (800 Ci/mmol) and [2-^{14}C]-thymidine (59 mCi/mmol) were obtained from ICN BioMedicals (Costa Mesa, CA). 20-S-camptothecin lactone, etoposide, cis-platinum(II) diammine dichloride and TNF-α were purchased from the Sigma Chemical Co. (St. Louis, MO). The fluorogenic peptide derivatives Ac-Asp-Glu-Val-Asp-7-amino-4-methylcoumarin (Ac-DEVD-AMC) and Ac-Leu-Glu-His-Asp-7-amino-4-trifluoromethylcoumarin (Ac-LEHD-AFC) were bought from Bachem Bioscience Inc. (King of Prussia, PA) and Calbiochem-Novobiochem Corporation (San Diego, CA), respectively. Hygromycin B was obtained from Roche Molecular Biochemicals (Laval, Que.). All other chemicals were of reagent grade and purchased either from Sigma and ICN or from local sources.

cDNA cloning of human Bcl-xES. Human Bcl-xES cDNA was cloned by reverse transcription-polymerase chain reaction (RT-PCR) from mRNA obtained from human HL-60 cells using specific adapter primers containing *NotI* sequences at the ATG start codon (5'-primer GCGGCCGCATGTCTCAGAGCAACCGGGAG) and TGA stop codon (complementary 3'-primer GCGGCCG CTCATTTC CGACT GAAGAGTGA). The amplified fragment was first cloned in pCRII vector (InVitrogen, Carlsbad, CA) and then subcloned at the *NotI* restriction site in the eucaryotic expression vector pCEP4 (Invitrogen) modified to include consensus Kosak and hemagglutinin epitope Tag sequences (HA-tag) (41, 84-86). All vectors were sequenced by ALF DNA sequencer (LKB-Pharmacia Biotech, Upsalla, Sweden). Restriction and modification enzymes were purchased either from Pharmacia Biotech or Gibco-BRL Life Technologies (Grand Island, NY).

Cell culture, transfection and DNA labeling. All cell lines were obtained from the American Type Culture Collection (Manassas, VA) and grown in suspension culture at 37°C in the presence of 5% CO_2 in RPMI-1640 medium supplemented with 10% heat-inactivated fetal bovine serum, 2 mM glutamine, 100 U/ml penicillin and 100 μg/ml streptomycin. Cell culture products were purchased from Gibco-BRL Life Technologies. Cesium chloride-purified pCEP4-HA-Bcl-xES and pCEP4-HA-mock vectors were transfected in Namalwa cells by electroporation at 0.27 kVolts (Gene Pulser, BioRad, Hercules, CA). To obtain stable transfected lines,

the cells were grown under hygromycin B selection at 1.5 mg/ml for 3 months prior to the experiments, as described elsewhere (41, 84-86). For DNA labeling, Namalwa cells were grown with [^{14}C]-thymidine (0.02 μCi/ml) for 24 h, then chased overnight in isotope-free medium prior to electroporation or drug treatment (41, 84-86).

Expression studies by RT-PCR and ribonuclease protection assays. Total RNA was isolated from exponentially growing cells with TRIzol® reagent according to the manufacturer's procedure (Gibco-BRL), and first strand cDNA was synthesized from 2.0 μg of RNA employing Moloney murine leukemia virus reverse transcriptase (2.5 U) (Perkin-Elmer Biosystem, Branchburg, NJ) with oligo dT(16) priming. cDNAs from normal tissues were obtained from Invitrogen, the bone marrow samples were obtained from biopsies of ALL patients (Dr. Yves Theoret, Ste-Justine Hospital, Montreal Que) and the colon adenocarcinoma samples were obtained through the US National Cancer Institute's Cooperative Human Tissue Network. Bcl-x cDNAs were amplified with 50 ng of 5'-primer ATGTCTCAGAGCAACCGGGAG and 50 ng of complementary 3'-primer TCATTTCCGACTGAAGAGTGA in a reaction mixture containing 200 μM dNTPs, 50 mM KCl, 10 mM Tris-HCl (pH 8.3) and 0.5 U Taq DNA polymerase (Perkin-Elmer Biosystem). The reaction mixtures were heated for 5 min at 95°C and amplified for 30 cycles with denaturation at 95°C for 1 min, annealing at 55°C for 45 sec and extension at 72°C for 45 sec. The amplified products were electrophoresed on a 1.6% agarose gel in Tris-acetate buffer (pH 8.0) and transferred by capillarity to GeneScreen nylon membranes (Dupont-NEM Research Products, Boston, MA). After DNA denaturation and fixation, blots were hybridized with purified human Bcl-xL cDNA probe labeled with [α -^{32}P]-dCTP by a random priming procedure. Hybridizations were undertaken overnight at 42°C in a solution containing 2x SSC, 2x Denhart, 2% (w/v) SDS, 50% (v/v) formamide and 100 μg/ml salmon sperm DNA. The blots were washed for 30 min at 42°C in 0.1x SSC and 0.1% (w/v) SDS, then for 45 min at 68°C in 0.05x SSC and 0.05% (w/v) SDS. DNA was visualized by autoradiography on Kodak X-AR film. Expression of the isoform was confirmed by RNase protection assays in some samples. Specific [α-^{32}P]-dUTP-labeled Bcl-xES cRNA probes were generated using the MAXIscript SP6 and T7 kit (Ambion, Inc., Austin, TX) using as template the *StyI* linearized pCRII plasmid containing Bcl-xES. Probe was eluted from a 5% polyacrylamide urea gel after electrophoresis, hybridized with total RNA (10 μg) for 16 h, then digested with RNase A and T1. The protected fragments were precipitated and then separated on 5% polyacrylamide urea gel. RNA

double strands were visualized by autoradiography on Kodak X-AR film. ß-actin was used as control in the RT-PCR and RNase protection assays.

Immunohistochemistry analysis Expression of HA-Bcl-xES by immunohistochemistry was visualized using anti-HA mouse monoclonal antidodies (Roche Molecular Biochemicals, Boehringer-Mannheim) as a primary antibody (10 µg/ml), a biotinylated goat anti-mouse (Sigma) as secondary antibody (10 µg/ml), followed by a steptavidin-alkaline phosphatase complex (Sigma) and colorimetric reaction using FastRed TR/Naphthol AS-MX (Sigma). Briefly, cells were spread by centrifugation on microscope slides and fixed in a solution containing 50% acetone and 50% methanol. Permeable cells were then incubated for 30 min in PBS buffered solution containing 10 % (v/v) goat serum and 5 % (w/v) bovine serum albumin (Sol A). Cells were then incubated with the primary antibody diluted in Sol A for 30 min, followed by several washes with Sol A and then incubated with the secondary antibodies diluted in Sol A. After several washes in Sol A, cells were incubated with a streptavidin-alkaline complex, washed again and incubated with a solution of FastRed TR/Naphthol AS-MX according to the manufacturer instructions (Sigma). Cell were then analyzed under an Axioscop camera- equiped microscope (Carl Zeiss, West Germany).

Subcellular fractionation and Western blot analysis. For subcellular fractionation, cells were first swelled in deionized water for 4 min on ice. The samples were then adjusted to 220 mM mannitol, 70 mM sucrose, 5 mM HEPES, (pH 7.4) and 1.0 mM EDTA and dounced briefly on ice. Nuclei (1,000g, 15 min) and mitochondria (10,000g, 15 min) were pelleted by sequential differential centrifugation. The supernatants were clarified further by centrifugation at 100,000g for 60 min (S-100 fraction). Antibodies against the peptide epitope derived from the hemagglutinin protein of human influenza virus (HA) (clone 12CA5) were obtained from Roche Molecular Biochemicals (Boehringer-Mannheim), and antibodies to cytochrome c (clone 7H8.2C12) were procured from PharMingen (San Diego, CA). Enhanced chemiluminescence (ECL) detection of peroxidase-labeled secondary antibodies was performed using Amersham Life Science reagents.

LEHDase and DEVDase activity determination. Cytosolic extracts were prepared by washing control and transfected Namalwa cells twice by centrifugation/resuspension in 10 ml ice-cold PBS (without Ca^{++} and Mg^{++}). The cells are then homogenized at 4°C for 30 min in a lysis buffer containing 100 mM HEPES (pH 7.5), 5 mM EDTA, 5 mM DTT, 20% glycerol and 0.1% NP-40. The

samples were centrifuged (13,000 x g for 10 min at 4°C), and supernatants collected as cytosolic extracts. Caspase activities were measured by monitoring fluorescence continuously in a dual luminescence LS 50B fluoremeter (Perkin-Elmer) at an excitation wavelength of 400 nm and an emission wavelength of 505 nm for the substrate Ac-LEHD-AFC, and an excitation wavelength of 380 nm and an emission wavelength of 460 nm for Ac-DEVD-AMC. Reactions were carried out in cuvettes, and temperature was maintained at 37°C with a water-jacketed sample compartment. The assay mixture contained 100 mM HEPES (pH 7.5), 20% (v/v) glycerol, 5 mM DTT, 5 mM EDTA and 500 μM Ac-LEHD-AFC or 200 μM Ac-DEVD-AMC. Enzyme activities were determined as initial velocities and expressed as relative intensity/min/mg.

Recombinant Bcl-xES(ΔTM) protein. The human Bcl-xES cDNA without the sequences coding for it carboxyl hydrophobic domain (TM) was first amplified by PCR using specific adapter primers containing an *NdeI* sequence at the ATG start codon and *BamHI* sequence at the TAA added stop codon. The PCR product was inserted in the pCRII vector (TA cloning system; Invitrogen, San Diego, CA) and then subcloned in the bacterial expression vector pET-14b(+)-His-TAG (Novagen; Madison WI) between the *NdeI* and *BamHI* sites. Vectors were sequenced by ALF DNA sequencer (LKB-Pharmacia Biotech) using both vector and internal primers. *E. Coli* BL21 (DE3) was transfected with purified plasmids and recombinant protein expression induced for up to 15 h by adding 100 μM IPTG to exponentially growing bacteria at room temperature. The bacteria were collected by centrifugation and resuspended in 5 mM imidazole, 0.5 M NaCl, 20 mM Tris-HCl (pH 7.9) and samples were sonicated on ice. Following centrifugation at 12 000 g for 20 min, bacterial lysates were then applied to a charged and equilibrated Chelating Sepharose (Pharmacia Biotech) chromatography column. The column was then washed with 10 volumes of 5 mM imidazole, 0.5 M NaCl, 20 mM Tris-HCl (pH 7.9) followed by 6 volumes of 50 mM imidazole, 0.5 M NaCl, 20 mM Tris-HCl (pH 7.9). The bound protein was eluted with 6 volumes of 1 M imidazole, 0.5 M NaCl, 20 mM Tris-HCl (pH 7.9). Individual fractions were collected and purity of recombinant His_8-Bcl-xES(ΔTM) protein preparations were determined by SDS-polyacrylamide gel electrophoresis. In some preparation, the protein was further purified by gel electroelution.

DNA filter elution assay. DNA filter elution assays were designed to monitor and quantitate DNA fragmentation associated with apoptosis (87). 5 x 10^6

[^{14}C]-Thymidine-labeled cells were loaded onto protein-adsorbing filters (vinyl/acrylic copolymer filters, Metricel membrane, 0.8-μm pore size, 25-mm diameter, Gelman Sciences Inc., Ann Arbor, MI), and then washed with 5 ml of PBS. As soon as the washing solution dripped through by gravity, lysis was performed with 5 ml of lysis solution (2% SDS, 0.04 M EDTA, pH 10.0). After the lysis solution has dripped through by gravity, the filters were washed with 5 ml of 0.02 M EDTA (pH 10.0). They were then processed as described (87). Briefly, they were placed in scintillation vials to which 1.0 ml of 1 N HCl was added. The vials were sealed and heated for 1 h at 60°C to depurinate the DNA. Then, 2.5 ml of 0.4 N NaOH was added and allowed to stand for 1 h at room temperature to release labeled DNA from the filters. Radioactivity was counted by liquid scintillation spectrometry in each fraction (loading wash, lysis, EDTA wash, filter). DNA fragmentation was determined as the fraction of DNA in the loading wash fraction + lysis fraction + EDTA wash fraction relative to total DNA (loading wash + lysis + EDTA wash + filter). The results were expressed as the percentage of DNA fragmented in treated cells compared to DNA fragmented in untreated cells (background) using the formula

$$(F\text{-}F0/1\text{-}F0) \times 100$$

where F and F0 represent DNA fragmentation in treated and control cells, respectively.

Yeast 2-hybrid system. The MatchMaker LexA 2-hybrid system (Clontech Laboratories Inc, Palo Alto, CA) was employed according to the manufacturer's instructions. Bcl-xES deleted of its transmembrane domain, Bcl-xES(ΔTM), was subcloned in the 2-hybrid plasmid pLexA vector. All other cDNAs were cloned in the 2-hybrid plasmid pB42AD, as described previously (41). Sequential transformations were conducted by the lithium acetate method in yeast strain EGY48, and the cells were plated on histidine-deficient (for pLexA marker), tryptophan-deficient (for pB42AD marker), and histidine/tryptophan/leucine-deficient (for LEU2 reporter gene assay) minimal SD induction/selection media. pLexA p53, pLex Lam, and pB42AD T from Clontech Laboratories served as positive and negative controls.

RESULTS AND DISCUSSION

Bcl-xES, a unique BH-4, BH-2 and transmembrane anchor domains containing protein. Among all the Ced-9/Bcl family members, Bcl-xES contains a unique combination of BH-4, BH-2 and transmembrane anchor domains

(TM) (Fig. 1). First identified and cloned from human promyelocytic HL-60 cells, *bcl-xES* mRNA expression was investigated in various human cell lines and tissues by non-quantitative RT-PCR and, in some specimen, by ribonucleotidase protection assay. Table 1 summarizes the detection of *bcl-xES* mRNA compared to *bcl-xL* and *bcl-xS* in a serie of human cancer cell lines and normal or cancer human tissues of various origins. *Bcl-xES* was detected in most cell lines and tissues with the exception of human normal breast, ovary, testis and lung specimens.

Expression of HA-Bcl-xES in human B lymphoma Namalwa cells. To investigate the activity of Bcl-xES on cell death, *bcl-xES* cDNA was subcloned in a modified pCEP4 expression vector that contained consensus Kosak and hemagglutinin epitope tag sequences (HA-tag) (41, 84-86). After transfection, a stable Namalwa variant line that expresses Bcl-xES was selected using hygromycin B. Control Namalwa cells did not survive under hygromycin B selection whereas a pCEP4-mock vector-transfected Namalwa cell line was also selected under hygromycin B. Immunohistochemical staining assessment of the exogenous proteins indicated that HA-Bcl-xES was expressed in all transfected cells (Fig. 2A). Subcellular fractionation and Western blot analysis indicated that HA-Bcl-xES protein was expressed primarily in the mitochondrial fraction, but also in cytosolic and nuclear fractions (Fig. 2B).

The effect of HA-Bcl-xES on kinetics of apoptosis-induced by cytotoxic agents. Previous studies showed that camptothecin (CPT), a DNA topoisomerase I inhibitor, etoposide (VP-16), a DNA topoisomerase II inhibitor, cisplatin (cisPT), a DNA adduct-inducing agent, and TNF-α induced the morphological changes and internucleosomal DNA fragmentation associated with apoptosis in Namalwa cells (41, 85, 86). To assess the effect of overexpressing Bcl-xES in these cells, kinetics of DNA fragmentation associated with apoptosis induced by these agents were monitored and quantitated by a filter DNA elution assay. Namalwa cells overexpressing bcl-xES present a significant modulation of apoptosis induced by these various cytotoxic drugs (Fig. 3). Translocation of the mitochondrial intermembrane protein cytochrome c is associated with anticancer drug-induced apoptosis. To define the effect of Bcl-xES in the mitochondrial pathway of caspase activation, cytochrome c release was monitored after CPT treatment. Bcl-xES delayed the release of cytochrome c into cytosol of transfected Namalwa cells compared to wild-type cells for at least 4 h after CPT treatment (Fig. 4A). The release of cytochrome c allows Apaf-1/procaspase-9 interaction that results in the activation of

caspase-9 which in turn, activates directly effector caspase-3 and -7 (68, 70, 71). To monitor the consequence of cytochrome c release inhibition in Namalwa-Bcl-xES cells, kinetics of caspase-9 (LEHDase activity) and caspase-3 and -7 (DEVDase activity) activities were monitored. Both LEHDase and DEVDase activities were delayed in Namalwa cells overexpressing Bcl-xES compared to wild-type cells, after CPT treatment (Fig 4B). Together, these results indicated that Bcl-xES prevented the release of cytochrome c and the subsequent activation of caspase-9, -3 and -7. To verify that Bcl-xES has no inhibitory potential on activated caspase-3 and -7, purified recombinant Bcl-xES protein was added to cell extracts containing DEVDase activities (Fig. 4 C). As expected, Bcl-xES was unable to inhibit activated caspases, indicating furthermore that its presence prevented their activation in Bcl-xES expressing cells .

Interaction of Bcl-xES with human Ced-9-, Egl-1- and Ced-4-like family members. The selectivity of Bcl-xES dimerization among various Ced-9-, Egl-1- and Ced-4-like human members, was monitored in a yeast 2-hybrid system. Transmembrane hydrophobic anchor sequences were removed from the cDNAs to promote their nuclear translocation, avoid toxicity of pro-apoptotic members in yeast cells and prevent unspecific interaction through their hydrophobic transmembrane domain. Interestingly, Bcl-xES strongly interacted with all familly members tested, including the Ced-4 homolog, Apaf-1. The absence of BH-1, BH-3 and α-5 helices may change the protein conformation from the typical globular conformation of Bcl members like Bcl-xL or Bcl-2 to a more linear structure, thus exposing the BH-2 and BH-4 domains of the protein. The affinity of Bcl-xES for most Ced-9/Bcl members and the adapter Apaf-1 protein suggests that multiple interactions within cells could explain the mode of action of Bcl-xES.

The biochemical mechanism by which Bcl-xES prevents cytochrome c release from mitochondria is yet unknow. Several hypothesis are actually investigated. First, through its BH-4 domain, Bcl-xES, as an homodimer or heterodimer, could interact with and close VDAC at the mPTP, preventing cytochrome c release. In parallel, via its BH-4 domain, Bcl-xES could potently trap Apaf-1/caspase-9 complexes, preventing caspase-9 activation. The ratio between anti- and pro-apoptotic members of the Ced-9/Bcl family is an important mechanism controlling cell death. Upon cell death stimuli, translocation of pro-apoptotic members to mitochondria may switch the mitochondrial equilibrium between pro- and anti-apoptotic proteins and may disrupt Bcl-xES interaction with VDAC at the mPTP and/or its interaction with Apaf-1/caspase-9 complexes and thus promote cell death.

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Table 1. Expression of *bcl-xES*, *bcl-xL* and *bcl-xS* mRNAs in human tissues and cancer cell lines

Cell Types	Origin	*bcl-xES*	*bcl-xL*	*bcl-xS*
HL 60*	Promyelocytic leukemia	+	+	+
U-937*	Histyocytic lymphoma	+	+	+
Namalwa*	Burkitt's lymphoma	+	+	+
NL-2	Burkitt's lymphoma	+	+	+
Raji	Burkitt's lymphoma	+	+	+
Jurkatt*	Acute T cell leukemia	+	+	+
H9	T cell lymphoma	+	+	+
CCRF CEM	Acute lymphoblastic leukemia	+	+	+
MCF 7	Breast adenocarcinoma	+/-	+	+
MDA-MB-468	Breast adenocarcinoma	+	+	+
SK-BR-3	Breast adenocarcinoma	+	+	+
OVCAR-3	Ovary adenocarcinoma	+	+	+
DU 145	Prostate carcinoma	+	+	+
PC-3	Prostate adenocarcinoma	+/-	+	+
LNCap-FGC-10	Prostate carcinoma	+	+/-	+
Calu-1	Lung carcinoma	+	+	+
A-431	Epidermoid carcinoma	+	+	+
NCI-H69	Small cell lung carcinoma	+	+	+
NCI-N417	Small cell lung carcinoma	+	+	+
Colo-205	Colon adenocarcinoma	-	-	-
Colo-320	DMColon Adenocarcinoma	-	-	-
HT-29	Colon Adenocarcinoma	+	+	+
Bone Marrow	Children with ALL (mixed)	+ (5/6)	+ (6/6)	+ (6/6)
Colon	Adenocarcinoma (mixed)	+ (7/7)	+ (7/7)	+ (7/7)
Colon	Normal (mixed)	+ (6/7)	+ (7/7)	+ (7/7)
Spleen	Normal tissue (male)	+/-	+	+
Skin	Normal tissue (male)	+/-	+	+
Breast	Normal tissue (female)	-	+	+
Ovary	Normal tissue (female)	-	+	+
Testis	Normal tissue (male)	-	+	+
Lung	Normal tissue (male)	-	+	+
Colon	Normal tissue (male)	+/-	+	+
Brain	Normal tissue (male)	+/-	+	+

Symbols are (+) positive; (-) negative; (+/-) very weak.

ALL: acute lymphoblastic leukemia.

Note: Human *bcl-xES*, *bcl-xL* and *bcl-xS* expression was detected by RT-PCR from total RNA using specific primers at the ATG start codon and TGA stop codon.

Electrophoresis was done in 1.6% agarose gel and the amplified fragments transferred to GeneScreen nylon membranes. Blot were hybridized with a human *bcl-xL* cDNA probe labeled with [^{32}P]dCTP by random priming procedure. In selected samples (*), RNAse protection assays were also performed.

```
Bclx-L     1 atgtctcaga gcaaccggga gctggtggtt gactttctct cctacaagct ttcccagaaa
Bclx-S     1 atgtctcaga gcaaccggga gctggtggtt gactttctct cctacaagct ttcccagaaa
Bclx-ES    1 atgtctcaga gcaaccggga gctggtggtt gactttctct cctacaagct ttcccagaaa

Bclx-L    61 ggatacagct ggagtcagtt tagtgatgtg gaagagaaca ggactgaggc cccagaaggg
Bclx-S    61 ggatacagct ggagtcagtt tagtgatgtg gaagagaaca ggactgaggc cccagaaggg
Bclx-ES   61 ggatacagct ggagtcagtt tagtgatgtg gaagagaaca ggactgaggc cccagaaggg

Bclx-L   121 actgaatcgg agatggagac ccccagtgcc atcaatggca acccatcctg gcacctggca
Bclx-S   121 actgaatcgg agatggagac ccccagtgcc atcaatggca acccatcctg gcacctggca
Bclx-ES  121 actgaatcgg ag..... ..... ..... ..... .....

Bclx-L   181 gacagccccg cggtgaatgg agccactggc cacagcagca gtttggatgc ccgggaggtg
Bclx-S   181 gacagccccg cggtgaatgg agccactggc cacagcagca gtttggatgc ccgggaggtg
Bclx-ES    - ..... ..... ..... ..... ..... .....

Bclx-L   241 atccccatgg cagcagtaaa gcaagcgctg agggaggcag gcgacgagtt tgaactgcgg
Bclx-S   241 atccccatgg cagcagtaaa gcaagcgctg agggaggcag gcgacgagtt tgaactgcgg
Bclx-ES    - ..... ..... ..... ..... ..... .....

Bclx-L   301 taccggcggg cattcagtga cctgacatcc cagctccaca tcaccccagg gacagcatat
Bclx-S   301 taccggcggg cattcagtga cctgacatcc cagctccaca tcaccccagg gacagcatat
Bclx-ES    - ..... ..... ..... ..... ..... .....

Bclx-L   361 cagagctttg aacaggtagt gaatgaactc ttccgggatg gggtaaactg gggtcgcatt
Bclx-S   361 cagagctttg aacag..... ..... ..... ..... .....
Bclx-ES    - ..... ..... ..... ..... ..... .....

Bclx-L   421 gtggcctttt tctccttcgg cggggcactg tgcgtggaaa gcgtagacaa ggagatgcag
Bclx-S     - ..... ..... ..... ..... ..... .....
Bclx-ES  133 ..... ..... ..... ..... ..... ....atgcag

Bclx-L   481 gtattggtga gtcggatcgc agcttggatg gccacttacc tgaatgacca cctagagcct
Bclx-S     - ..... ..... ..... ..... ..... .....
Bclx-ES  139 gtattggtga gtcggatcgc agcttggatg gccacttacc tgaatgacca cctagagcct

Bclx-L   541 tggatccagg agaacggcgg ctgggatact tttgtggaac tctatgggaa caatgcagca
Bclx-S   376 ..... ..... ....gatact tttgtggaac tctatgggaa caatgcagca
Bclx-ES  199 tggatccagg agaacggcgg ctgggatact tttgtggaac tctatgggaa caatgcagca

Bclx-L   601 gccgagagcc gaaagggcca ggaacgcttc aaccgctggt tcctgacggg catgactgtg
Bclx-S   412 gccgagagcc gaaagggcca ggaacgcttc aaccgctggt tcctgacggg catgactgtg
Bclx-ES  259 gccgagagcc gaaagggcca ggaacgcttc aaccgctggt tcctgacggg catgactgtg

Bclx-L   661 gccggcgtgg ttctgctggg ctcactcttc agtcggaaat ga
Bclx-S   472 gccggcgtgg ttctgctggg ctcactcttc agtcggaaat ga
Bclx-ES  319 gccggcgtgg ttctgctggg ctcactcttc agtcggaaat ga
```

Figure 1A

```
                        BH-4
Bcl-xL     1 MSQSNRELVV DFLSYKLSQK GYSWSQFSDV EENRTEAPEG TESEMETPSA
Bcl-xS     1 MSQSNRELVV DFLSYKLSQK GYSWSQFSDV EENRTEAPEG TESEMETPSA
Bcl-xES    1 MSQSNRELVV DFLSYKLSQK GYSWSQFSDV EENRTEAPEG TESE......

BH-3
Bcl-xL    51 INGNPSWHLA DSPAVNGATG HSSSLDAREV IPMAAVKQAL REAGDEFELR
Bcl-xS    51 INGNPSWHLA DSPAVNGATG HSSSLDAREV IPMAAVKQAL REAGDEFELR
Bcl-xES    - .......... .......... .......... .......... ..........

                                                    BH-1
Bcl-xL   101 YRRAFSDLTS QLHITPGTAY QSFEQVVNEL FRDGVNWGRI VAFFSFGGAL
BclxS    101 YRRAFSDLTS QLHITPGTAY QSFEQ..... .......... ..........
Bcl-xES   -  .......... .......... .......... .......... ..........

                                                          BH-2
Bcl-xL   151 CVESVDKEMQ VLVSRIAAWM ATYLNDHLEP WIQENGGWDT FVELYGNNAA
Bcl-xS   126 .......... .......... .......... ........DT FVELYGNNAA
Bcl-xES   45 ........MQ VLVSRIAAWM ATYLNDHLEP WIQENGGWDT FVELYGNNAA

                                    TM
Bcl-xL   201 AESRKGQERF NRWFLTGMTV AGVVLLGSLF SRK
Bcl-xS   138 AESRKGQERF NRWFLTGMTV AGVVLLGSLF SRK
Bcl-xES   87 AESRKGQERF NRWFLTGMTV AGVVLLGSLF SRK
```

Figure 1B

Fig. 1. Bcl-xES, a novel member of the Ced-9/Bcl family.

A) Nucleotide sequence alignement of Bcl-xES compared to those of Bcl-xL and Bcl-xS. The Bcl-xES sequence data are available from GenBank under accession number xxxxxx.

B) Amino acids sequence alignement of Bcl-xES. BH-4, BH-3, BH-1, BH-2 domains and COOH-terminal hydrophobic domain (TM) are indicated.

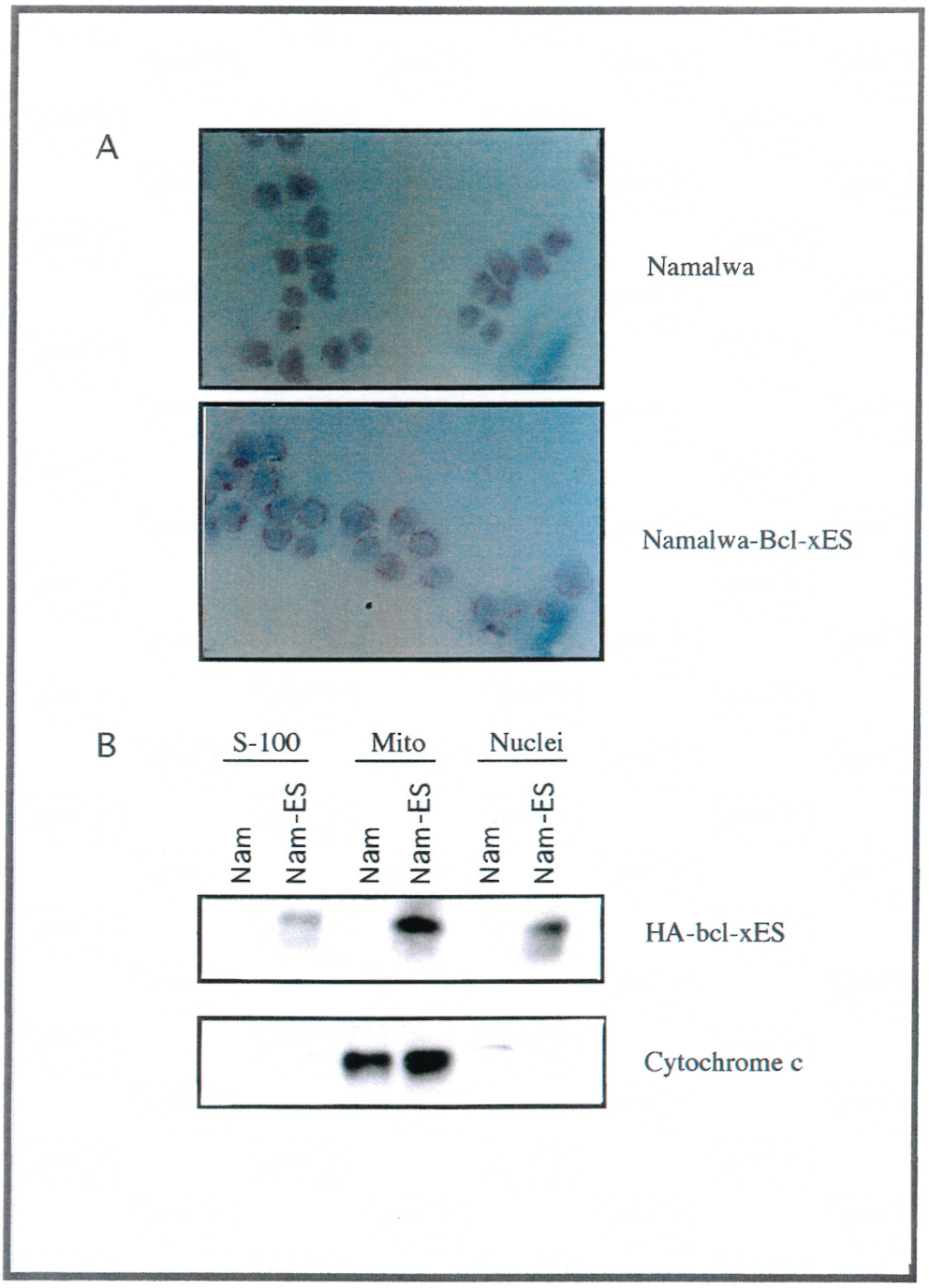
A
Namalwa
Namalwa-Bcl-xES
B
S-100
Mito
Nuclei
Nam
Nam-ES
Nam
Nam-ES
Nam
Nam-ES
HA-bcl-xES
Cytochrome c

Figure 2

Fig. 2. Stable expression of Bcl-xES in transfected Namalwa cells.

A) Immunohistochemical staining of the HA-Bcl-xES proteins in transfected Namalwa variant cells. Upper panel is control Namalwa cells and lower panel is HA-Bcl-xES transfected Nanmalwa cells. Expression of HA-Bcl-xES by immunohistochemistry was visualized using anti-HA mouse monoclonal antibodies as primary antibody, a biotinylated goat anti-mouse as secondary antibody followed by a steptavidin-alkaline phosphatase complex and colorimetric reaction using FastRed TR/Naphthol AS-MX. Cell were analyzed under an Axioscop camera- equiped microscope (Carl Zeiss, West Germany).

B) Subcellular localization of Bcl-xES in transfected Namalwa cells. Subcellular extracts were prepared from control and stable Namalwa cells transfected by electroporation with pCEP4-HA-Bcl-xES selected at 1.5 mg/ml hygromycin B. Following SDS-PAGE and electrophoretic transfer, HA-Bcl-xES protein was detected using anti-HA peptide monoclonal antibodies. Cytochrome c was monitored, as control. Visualization was performed by enhanced chemiluminescence reaction and autoradiography.

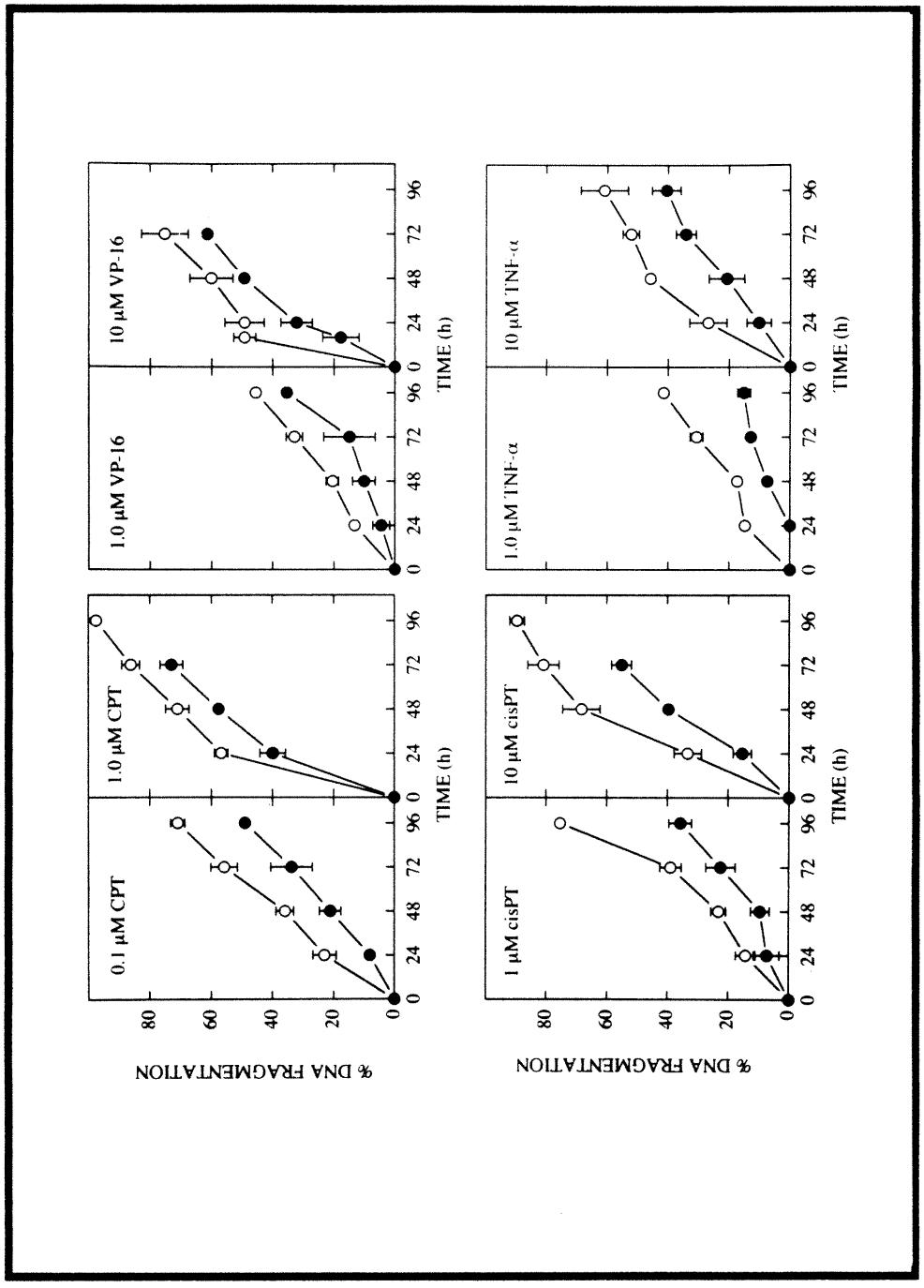
0.1 µM CPT
1.0 µM CPT
1.0 µM VP-16
10 µM VP-16
1 µM cisPT
10 µM cisPT
1.0 µM TNF-α
10 µM TNF-α
% DNA FRAGMENTATION
TIME (h)
0
20
40
60
80
0
24
48
72
96

Figure 3

Fig. 3. Kinetics of DNA fragmentation in control and Bcl-xES Namalwa cells.

[^{14}C]Thymidine-labeled cells were treated at various concentrations of camptothecin (CPT), etoposide (VP-16), cis-platinum(II) diammine dichloride and tumor necrosis factor (TNF-α). At the indicated times (x axis; hour), DNA fragmentation was determined by DNA filter elution assays. Results are expressed as percent DNA fragmentation. Points represent the means +/- SE of three independent experiments performed in triplicate. Symbols are Namalwa cells (○); Bcl-xES-transfected Namalwa cells selected at 1.5 mg/ml hygromicin (●). Mock vector-transfected Namalwa cells selected at 1.5 mg/ml hygromicin show similar sensitivity to control Namalwa cells (not shown) .

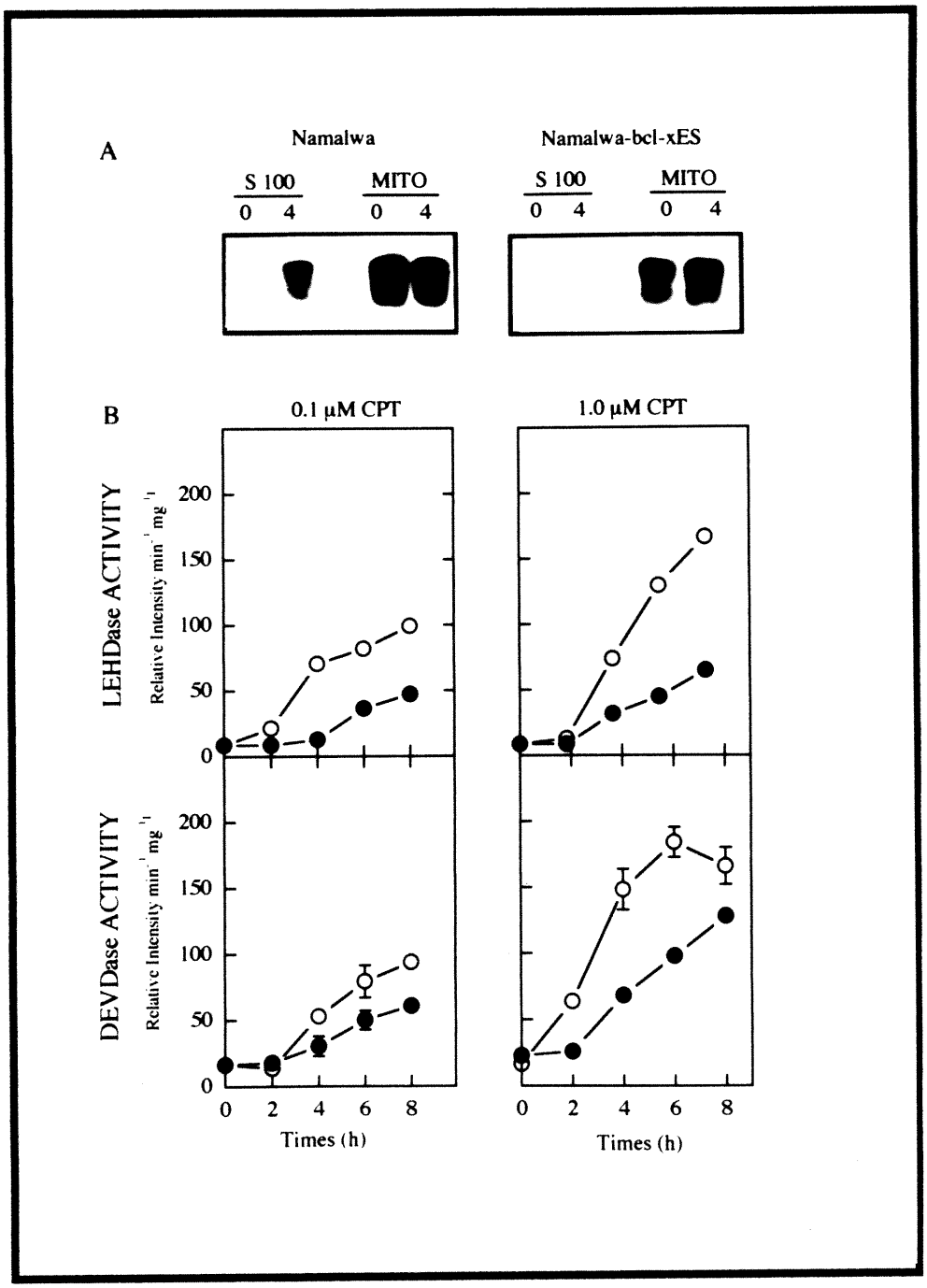
A
Namalwa
Namalwa-bcl-xES
S 100
MITO
S 100
MITO
0 4
0 4
0 4
0 4
B
0.1 μM CPT
1.0 μM CPT
LEHDase ACTIVITY
DEVDase ACTIVITY
Relative Intensity min⁻¹ mg⁻¹
200
150
100
50
0
0 2 4 6 8
Times (h)

Figure 4

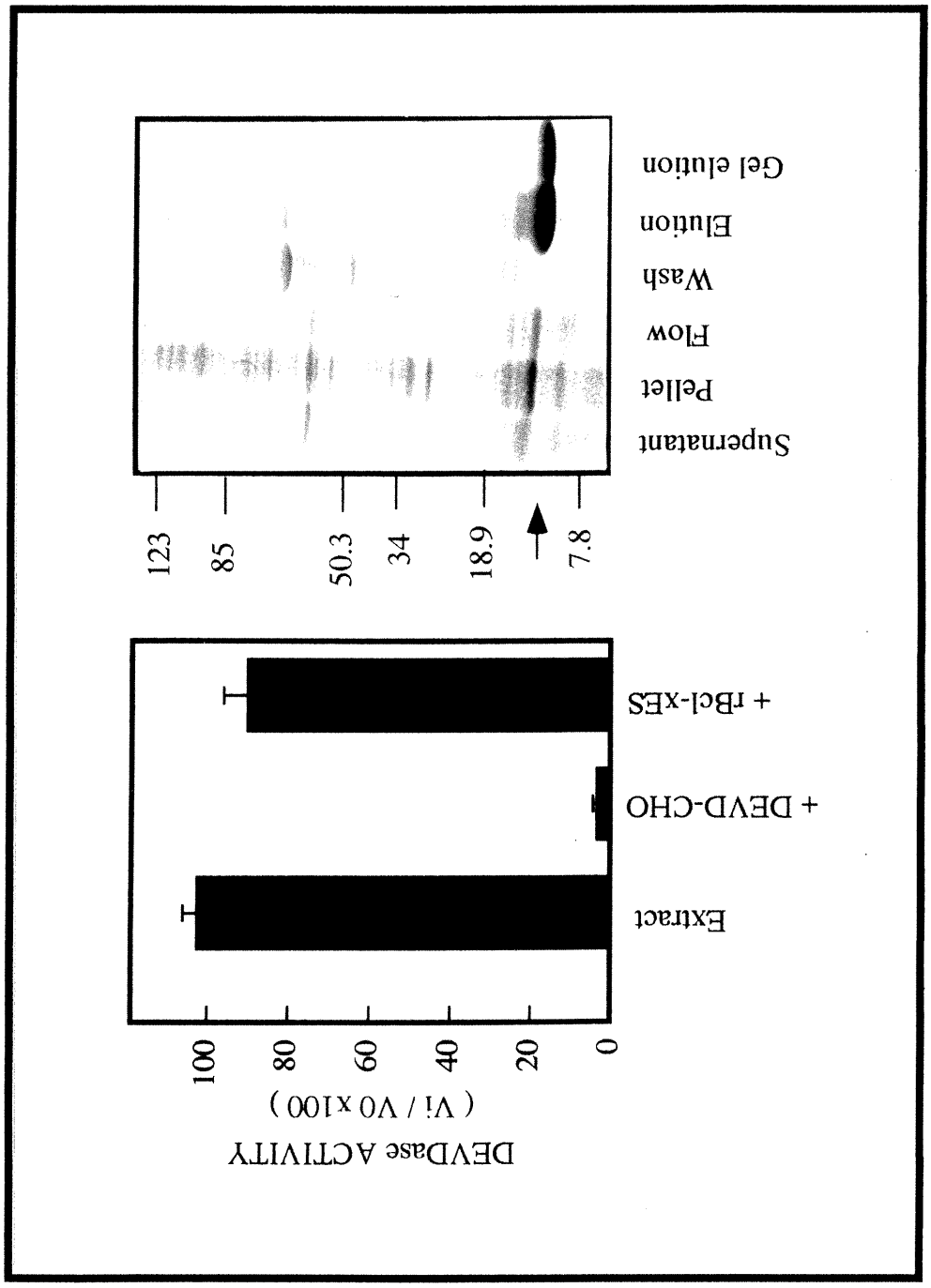
DEVDase ACTIVITY
(Vi / Vo x100)
0
20
40
60
80
100
Extract
+ DEVD-CHO
+ rBcl-xES
123
85
50.3
34
18.9
7.8
Supernatant
Pellet
Flow
Wash
Elution
Gel elution

Figure 4 c

Fig. 4. Cytochrome c release, LEHDase and DEVDase activities after CPT treatment.

A) Cytosolic S-100 and mitochondrial extracts were prepared from Namalwa and Namalwa Bcl-xES cells at the indicated times (0, 4 h) after 30 min treatment with 0.1 μM CPT. Immunoblot analysis of cytochrome c was performed using anti-cytochrome c specific antibodies and revealed by ECL.

B) Namalwa and Namalwa Bcl-xES transfected cells were treated for 30 min with 0.1 μM (left panels) or 1.0 μM CPT (righ panels). Cytosolic extracts were then prepared from Namalwa (○) and Namalwa Bcl-xES cells (●) at the indicated times following treatment. LEHDase (upper panels) and DEVDase (lower panels) activities were monitored continuously at 37°C by detecting fluorescence emission in the presence of Ac-LEHD-AFC and Ac-DEVD-AMC, respectively. Enzyme activities were measured as initial velocities and expressed as relative intensity / min / mg. Points represent the means +/- SD of three independent experiments.

C) The effect of added Bcl-xES(ΔTM) recombinant protein on DEVDase activities.

Left panel: DEVDase activity was measured by monitoring the hydrolysis of Ac-DEVD-AMC in the absence and presence of 200 μM Ac-DEVD-CHO and 20 μg of purified Bcl-xES(ΔTM). Data are expressed as percent relative activity (v_i / v_0) and represent the means +/- SE of three independent determinations. The amount of Bcl-xES(ΔTM) protein added per assay represent 10% of the total extracts (20 μg Bcl-xES(ΔTM) protein in 200 μg of extracted proteins.

Righ panel: SDS-polyacrylamide gel electrophoresis of various fractions in the purification of recombinant Bcl-xES(ΔTM) protein. From left to right, lanes are bacterial lysate supernatant (soluble fraction); bacterial pellet (insoluble fraction); column flow through; column wash, eluted bcl-xES protein and gel eluted final purification step. Gel was stained with Comassie Blue R-250.

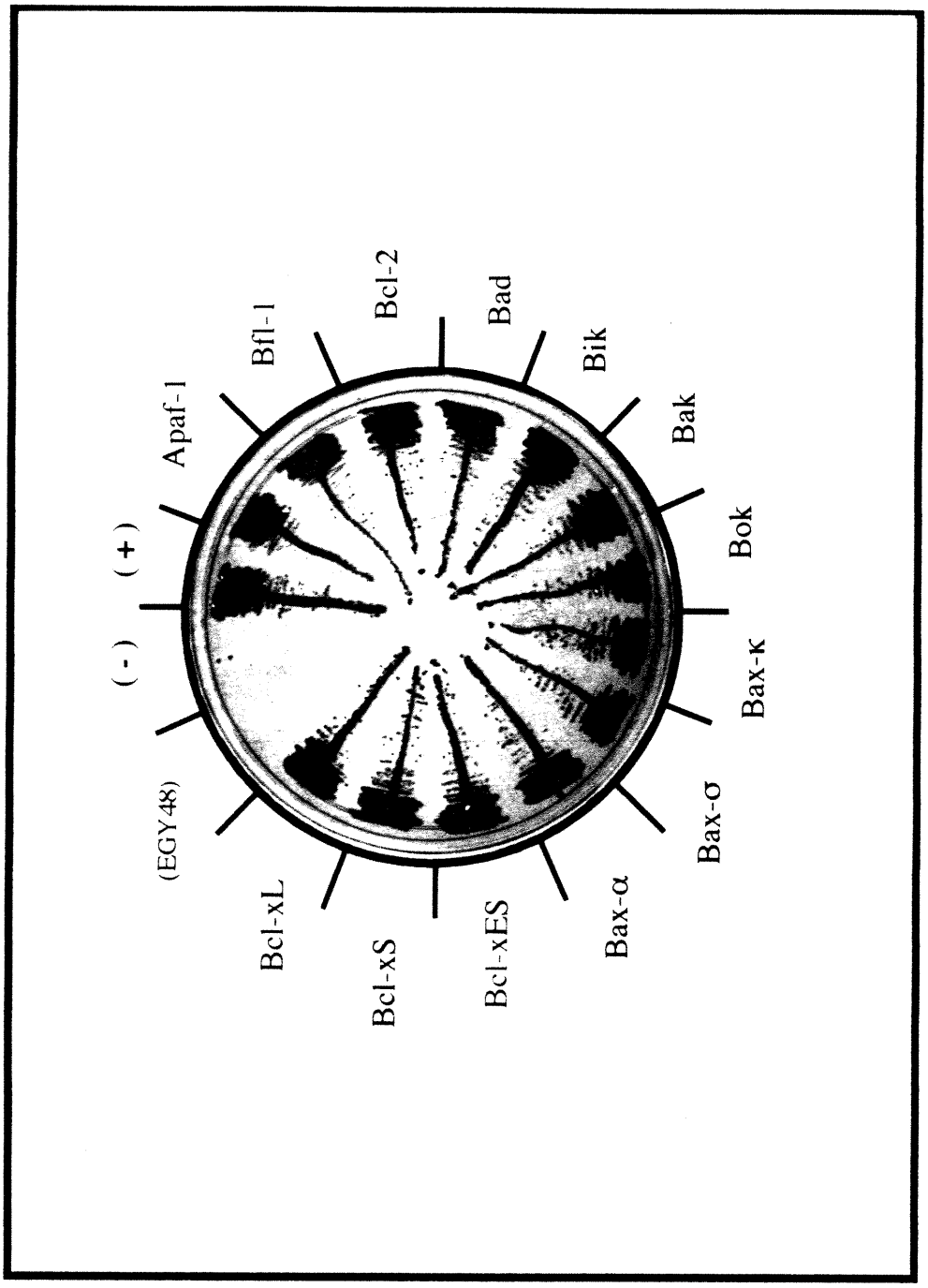
Bcl-2
Bad
Bik
Bak
Bok
Bax-κ
Bax-σ
Bax-α
Bcl-xES
Bcl-xS
Bcl-xL
(EGY48)
(-)
(+)
Apaf-1
Bfl-1

Figure 5

Fig. 5. Interaction of Bcl-xES in yeast 2-hybrid assay.

EGY 48 yeast cells containing Bcl-x-ES fused to the LexA DNA-binding domain were grown in selective medium without tryptophan, leucine and histidine in the presence of the various proteins fused to the B42 DNA activation domain as indicated. Positive controls *(+)* represent pLexA p53 and pB42 SV40 T and negative controls *(-)* represent pLexA laminC and pB42 SV40 T. Untransformed yeast cells were include (EGY 48) .

CHAPITRE 6.

Bcl-xL interacts with cdc2/cdk1-cyclin A/B1 complexes and inhibits cdc2/cdk1 kinase activity

(Manuscrit soumis pour publication)

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Avant propos:

La surexpression de Bcl-xL dans les cellules de lymphome B Namalwa protège les cellules face à l'apoptose induite par la camptothécine mais modifie également la réponse cellulaire aux lésions cytotoxiques générées par la drogue en permettant la restauration d'un point-contrôle du cycle cellulaire à la transition G2/M. Cette observation nous a conduit à étudier la relation entre la protéine anti-apoptotique Bcl-xL et la régulation du point-contrôle du cycle cellulaire à la transition G2/M.

Dans les cellules transfectées traitées avec la camptothécine, Bcl-xL exerce une activité anti-apoptotique au niveau des mitochondries en inhibant la libération du cytochrome c et l'activation subséquente des caspases. Par ailleurs, nous montrons que Bcl-xL interagit in vivo avec les complexes cdk1/cdc2-cycline A/B1 et inhibe l'activité histone H1 kinase de cdk1/cdc2 in vitro. L'ensemble de nos résultats suggère, qu'en plus de son activité anti-apoptotique au niveau des mitochondries, Bcl-xL contribue activement au point-contrôle du cycle cellulaire à la transition G2/M en réponse aux dommages induits par la camptothécine.

ABSTRACT

In response to cancer chemotherapeutic drugs, cells rapidly trigger the apoptotic program or undergo growth arrest at specific phases of the cell cycle. Mitochondrial Bcl-xL plays a central role in preventing alteration of mitochondrial functions, cytochrome c release, caspase activation and apoptosis. However, its pleitropic function depends on its subcellular localization. Here, we show that in addition to its mitochondrial effect that prevents apoptosis, nuclear bcl-xL binds to cdc2/cdk1-cyclin A/B1 complexes, in a manner similar to that expected from a cyclin-dependent kinase inhibitor during DNA damage-induced cell cycle checkpoint. Bcl-xL potently inhibits cdc2/cdk1 kinase activity. Inhibition of cdc2/cdk1 kinase activity by bcl-xL is reversible by a series of synthetic peptides derived from the flexible loop domain of bcl-xL. Our results suggest that bcl-xL is coupled to a cell cycle checkpoint induced by DNA damage. Thus Bcl-xL may have a dual role as dominant regulatory protein of apoptosis and as a component of the cell cycle control machinery.

INTRODUCTION

Tissue homeostasis requires a carefully orchestrated balance between cell proliferation and cell death. Cells proliferate through a cell cycle, tightly regulated by cyclin-dependent kinase activities (1-3). Apoptosis eliminates unwanted cells by the coordinated activity of gene products that regulate and execute cell death (4,5). Genes of the *Bcl*-related family encode proteins that antagonize or promote cell death (6). These proteins are found in the cytosol but most of them bear a carboxy-terminal hydrophobic transmembrane domain responsible for their localization to subcellular organelles, including the mitochondria, endoplasmic reticulum and nucleus (7). Mitochondrial bcl-related proteins play a central role in regulating apoptosis induced by a variety of death stimuli, including DNA damage. Alterations of mitochondrial functions, such as mitochondrial permeability transition, transmembrane potential disruption ($\Delta\Psi m$), ATP/ADP exchange and release of cytochrome c, are early events involved in the initiation step of apoptosis induced by most chemotherapeutic agents (8-15). Bcl-xL (16) and several of its homologs modulate the chemosensitivity of cells by preventing or accelerating the mitochondrial events that lead to caspase activation. However, Bcl-xL and Bcl-2 interact with several other cellular proteins whose functional link to apoptosis remains unknown and their pleiotropic activities also depend on their subcellular localizations (17-20).

Cells trigger the apoptotic program or undergo growth arrest at specific phases of the cell cycle in response to DNA damage. It is generally believed that cell cycle arrests afford cells the opportunity to repair DNA damage before progressing to the next stage of the cycle, and inapropriate progression of damaged cells through cell cycle is often associated with enhanced cytotoxicity and apoptosis (21,22). Current anticancer drugs including DNA topoisomerase inhibitors can perturb the orderly progress of DNA replication and cell division (23,24). The cell cycle checkpoints maintain the order and fidelity of events of the cell cycle in response to unreplicated or damaged DNA. The progression from G2 into mitosis is regulated by the cdc2/cdk1 serine-threonine kinase. Activation of cdc2/cdk1 requires association of the positive subunit cyclin B1 and phosphorylation of Thr-161 by the cdk-activating kinase (25-29). Before mitosis, the cyclin/kinase complex is also negatively regulated by phosphorylations at Thr-14 and Tyr-15, which are catalyzed by inhibitory protein kinases, including Wee 1, Myt 1 and Mik 1 (30-33). At the G2/M transition, dephosphorylation of Thr-14 and Tyr-15 by the cdc25c protein phosphatases trigger cdc2/cdk1-cyclinB1 activation and entry into mitosis (34, 35). Phosphorylation of

cdc25c by the chk1 kinase promotes its cytosolic sequestration by 14-3-3 (36-40). Inhibition of cdc2/cdk1-cyclinB1 kinase activity by stabilizing the complex in an hyperphosphorytated state has been shown to be involved in the G2/M cell cycle checkpoint after DNA damage (36, 41, 42).

Several observations have supported the existence of molecular connections between cell death and cell cycle regulation. In the present study, we found that cells that are protected by bcl-xL from apoptosis following anticancer drug treatment underwent a G2 arrest. Characterization of the restoration of this cell cycle checkpoint revealed that nuclear bcl-xL plays a direct role. Bcl-xL both *in vivo* and *in vitro* interacts with cdk1/cdc2-cyclinA/B1 complexes and inhibits their kinase activities in response to DNA damage. An amino acid sequence within the flexible loop domain of Bcl-xL seems to be involved in these interactions. Our observations suggest that Bcl-xL modulates cell response to DNA damage, not only by preventing apoptosis, but also by participating actively in a G2/M cell cycle checkpoint.

MATERIALS AND METHODS

Cell culture, transfection and drug treatments. The human Namalwa cell line was obtained from the American Type Culture Collection and the derivation of stable Namalwa cells transfected with pCEP4-HA-Bcl-xL or pCEP4-HA-mock vectors has been described previously (43). 20-S-camptothecin lactone and etoposide treatments were of 30-min duration followed by incubation of cells in drug-free medium. Cis-platinum (II) diamine dichloride and TNF-α treatments were continuous.

Cell death assays. The kinetics of DNA fragmentation (Fig. 1a) were determined in [2-^{14}C]-thymidine (59 mCi/mmol; ICN BioMedicals) pre-labeled cells by DNA filter elution assays, as described elsewhere (43). Points represent the means ± SE of 3 duplicate experiments. For subcellular fractionation and cytochrome c release assays (Fig. 1b and 1c), the cells were first swelled in deionized water for 4 min on ice. The samples were adjusted to 220 mM mannitol, 70 mM sucrose, 5 mM HEPES, pH 7.4, 1.0 mM EDTA and dounced on ice. Nuclei (1,000g, 15 min) and mitochondria (10,000g, 15 min) were pelleted by sequential differential centrifugation. The supernatants were clarified further by centrifugation at 100,000g for 60 min (S-100 fraction) (9). DEVDase activities (Fig. 1d and 4b) were measured by monitoring fluorescence continuously in a dual luminescence fluorometer, using, as substrate, Ac-Asp-Glu-Val-Asp-amino-4-methylcoumarin (Ac-DEVD-AMC; Bachem

Bioscience Inc.). Enzyme activities were determined as initial velocities and expressed as relative intensity/min/mg (43). Points represent the means ± SE of 3 independent experiments. For analysis of oligonucleosome-sized DNA fragmentation (Fig. 1d and 4b), DNA was extracted by a salting-out procedure and electrophoresis was done in 1.6% agarose gel with DNA being visualized by ethidium bromide staining. For electron microscopy (Fig. 1e, 1f, 4c), cells were fixed in 0.1 M Millonig's phosphate buffer containing 2.5% glutaraldehyde and stained with 2% uranyl acetate. Sections (500 to 700 Å) were mounted on copper grids and stained with lead citrate. The samples were examined by transmission electron microscopy using a Ziess Em10 CA microscope (JFE Enterprises). Approximately 200 cells per sample were observed by electron microscopy.

Flow cytometry analysis. Cell were fixed in 70% ethanol for 2 h at 4°C, treated with 150 μg/ml RNase A (30 min, 25°C), and then resuspended in 500 μl PBS. Propidium iodide (50 μg/ml) was added before cytofluorometry analysis. DNA content and cell cycle distribution were analysed using a Becton Dickinson FACStar Plus (Fig. 2a, 3j, 4a) flow cytometer.

Immunoblotting and co-immunoprecipitation. The following antibodies were used: anti-HA (clone 12CA5, Boehringer-Mannheim), anti-Bcl-xL (clone 44, PharMingen; B22630, Transduction Laboratories), anti-cytochrome c (clone 7H8.2C12, PharMingen), anti-cdc2/cdk1 (Ab-1, Oncogene Research Products; clone 17, SantaCruz Biotechnology), anti-cyclin B1 (clone GSN-1, PharMingen; clone 2H1-H6, Oncogene Research Products), anti-cyclin A (clone EG7.1 and clone BF683, Oncogene Research Products), anti-cyclin D1 (R-124, SantaCruz Biotechnology), anti-cyclin E (M-20, SantaCruz Biotechnology), anti-cdk2 (M2 and D-12; SantaCruz Biotechnology), anti-cdk4 (H-22 and C-22; SantaCruz Biotechnology), anti-cdk 6 (H-230, SantaCruz Biotechnology), and enhanced chemiluminescence detection of peroxidase-labeled secondary antibodies was performed using Amersham Life Science reagents. To prepare total proteins (Fig. 2b) cells were extracted with lysis buffer containing 50 mM Tris, pH 7.4, 120 mM NaCl, 1% Triton X-100, 1 mM sodium orthovanadate, 2 mM phenylmethylsulfonyl fluoride, 5 mM sodium pyrophosphate and a cocktail of protease inhibitors (Boehringer-Manneim). Insoluble material was discarded after centrifugation (10,000g; 20 min). Co-immunoprecipitation studies were performed on nuclei-enriched extracts prepared from 25 x 10^6 cells (Fig. 2d, f). Cells were lysed for 5 min on ice without agitation in 0.3% Nonidet P-40 prepared in 10 mM Hepes, pH 7.4, 80 mM KCl, 20 mM NaCl,

5 mM $MgCl_2$, 5 mM EGTA, 1 mM phenylmethylsulfonyl fluoride, 1 mM sodium orthovanadate, 1.0 μg/ml aprotinin, and 10% glycerol. Nuclei were harvested by centrifugation at 1,000g for 10 min and lysed at 4°C for 30 min with gentle agitation in 1% NP-40 PBS buffer containing 1% BSA, 1 mM phenylmethylsulfonyl fluoride, 1 mM sodium orthovanadate, and 1.0 μg/ml aprotinin (42). Insoluble pellets were discarded by centrifugation (10,000g; 10 min) and supernatants were incubated 3 h at 4°C with antibodies. Immune complexes were trapped by protein A/G PLUS-Agarose prior to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). These experiments were performed in triplicate. For co-immunoprecipitation studies performed on total extract, 2.5×10^6 cells were lysed directly in the 1% NP-40 PBS buffer as above (Fig. 2e).

Cdc2/cdk1 kinase assay. Cdc2/cdk1 kinase activity (Fig. 2c, Fig. 3a-h) was measured by immune complex kinase assay using purified histone H1 as substrate (42). Reactions were carried out for 10 min at 37°C in 20 mM Tris-HCl, 10 mM MgCl2, pH 7.5, 5 μM unlabeled ATP, 10 μCi [γ^{32}P]ATP (4,500 Ci/mmol) and 3 μg histone H1 with or without various concentrations of purified recombinant bcl-xL(ΔTM) or synthetic peptides. Kinase inhibition studies were performed in triplicate. Recombinant human bcl-xL(ΔTM) protein was produced and purified as described previously (44) and the synthetic peptides were obtained from Research Genetics Inc. Relative densitometry analysis of autoradiographs was based on the integrated density value using an AlphaImager 2000 scanner (Alpha Innovatech) (44).

RESULTS

Human B lymphoma Namalwa cells (a p53 double mutant line) overexpressing bcl-xL present marked resistance to apoptosis induced by various cytotoxic drugs (Fig. 1a) (43). Bcl-xL sub-localization remains stable during camptothecin (CPT) treatment (Fig. 1b), and its overexpression inhibits mitochondrial cytochrome c release (Fig. 1c), caspase activation (Fig. 1d), oligonucleosome-sized DNA fragmentation (Fig. 1d) and signs of morphological change, as observed by electron microscopy (Fig. 1e). These observations contrast with those recorded in treated control Namalwa cells that rapidly undergo apoptosis associated with cytochrome c release (Fig. 1c), caspase activation (Fig. 1d), DNA fragmentation (Fig. 1a, d) and morphological changes typical of apoptosis (Fig. 1f).

By blocking apoptosis activation in response to CPT-induced DNA damage, overexpressed bcl-xL allows Namalwa cells to undergo a cell cycle checkpoint. The

flow cytometry profiles in Figure 2a show that CPT-induced DNA damage in untransfected cells rapidly leads to the appearance of a sub-G1 population of apoptotic cells whereas Namalwa-bcl-xL cells accumulate slowly in the late S/early G2 phase of the cell cycle. Expression of cyclin A/B1 and phosphorylation level of cdc2/cdk1 are key regulators involved in control of both S phase progression and onset of mitosis during normal cell division (25,45) but also in DNA damaged cells (42 46). Accumulation of Namalwa-bcl-xL cells at the G2 boundary is associated with the appearance of the slower hyperphosphorylated inactive form of cdc2/cdk1 and increased expression of cyclin A and B1 (Fig. 2b). Cdc2/cdk1-associated histone H1 kinase activity decreases with time in CPT-treated, bcl-xL-transfected cells (Fig. 2c), consistent with DNA damage-induced cell cycle arrest.

Several studies provide some evidence that bcl-related proteins themselves can affect control of the cell cycle (47-54). Therefore, we investigated if bcl-xL participates directly in the cell cycle arrest after DNA damage. First, we asked whether enforced expression of bcl-xL modifies cdc2/cdk1 or cyclin A/B1 nuclear localization after DNA damage, a parameter that plays critical role in promoting G2/M transition (55). The nuclear and cytosolic distribution patterns of cdc2/cdk1 and cyclin A/B1 were unaffected in cells overexpressing bcl-xL (data not shown). However, co-immunoprecipitation with either anti-cdc2/cdk1 or anti-cyclin B1 or anti-cyclin A antibody revealed the presence of bcl-xL within the immune complex (Fig. 2d, e). Bcl-xL preferentially interacts with cdc2/cdk1-cyclin A/B1 compared to other cdk-cyclin complexes after CPT treatment (Fig. 2e). Moreover, the amount of bcl-xL co-precipitated with cdc2/cdk1 increased with time as the cells underwent the cell cycle checkpoint after DNA damage (Fig. 2f). To confirm these results, reciprocal experiments were performed in which bcl-xL was immunoprecipitated. Analysis of the immune complexes with either a rabbit anti-cdc2/cdk1 antibody or a mouse anti-cdc2/cdk1 antibody, revealed an enhancement of cdc2/cdk1 co-precipitated with bcl-xL after DNA damage (Fig. 2f).

These results demonstrate a physical interaction between bcl-xL and the cdc2/cdk1-cyclin A/B1 complexes, in a manner similar to that expected from a cdk inhibitor during a DNA damage-induced cell cycle checkpoint. Although a series of cdk inhibitors has been found associated with the inhibition of other cdk-cyclin complexes involved in the G1 checkpoint after DNA damage, they are clearly weakly responsible for the inhibition of cdc2/cdk1-cyclin A/B1 complexes after DNA damage (56). Attempts to identify known cdk inhibitors responsible for the G2 arrest in these

cells after CPT treatment were also unsuccessful. However, the enhanced interaction between bcl-xL and cdc2/cdk1 in G2 arrested cells suggests that bcl-xL modulates cdc2/cdk1 kinase activity after CPT-and VP-16-induced DNA damage. To address this question, we performed an *in vitro* kinase assay of immunoprecipitated cdc2/cdk1 kinase complex incubated with histone H1 and various amounts of purified recombinant human Bcl-xL(ΔTM) protein. Bcl-xL(ΔTM) protein inhibited cdc2/cdk1 activity dose-dependently (Fig. 3a) whereas the buffered solution used in the purification protocol of bcl-xL did not (Fig. 3b). A series of peptides covering the entire amino acid sequence of bcl-xL(ΔTM) was then used to confirm these observations. None of these peptides inhibited cdc2/cdk1 kinase activity significantly (Fig. 3c). However, a peptide (P-03) corresponding to a fragment of the flexible loop within bcl-xL potently reversed the inhibitory effect of bcl-xL on cdc2/cdk1 kinase activity (Fig. 3d), in a dose-response manner (Fig. 3e). P-03-L and P-03-S were then synthesized to confirm these results. None of the P-03 derivative peptides inhibited cdc2/cdk1 kinase activity (Fig. 3f) but both were potent in reversing the inhibitory effect of bcl-xL on cdc2/cdk1 kinase activity (Fig. 3g, h). Together, these results reveal that bcl-xL binds to cdc2/cdk1-cyclin A/B1 complexes and potently inhibits cdc2/cdk1 kinase activity. The interaction of bcl-xL with the cdk complexes appears to involve a small amino acid sequence or binding domain located within the flexible loop of bcl-xL (Fig. 3i). Whether bcl-xL interacts directly with cdc2/cdk1-cyclin A/B1 complexes or whether their association is mediated or enhanced by other cellular proteins is yet unknown. To confirm the importance of the flexible loop domain of bcl-xL, we monitored the effect of overexpressed bcl-xES on cell cycle arrest after CPT treatment. Bcl-xES is a BH-4, BH-2 and carboxy-terminal hydrophobic transmembrane domain containing isoform of *bcl-x*, that lacks the flexible loop domain of bcl-xL (E. Schmitt. and R. Bertrand, manuscript in preparation). In Namalwa cells, HA-Bcl-xES overexpression delayed significantly the occurrence of apoptosis after CPT treatment (Fig. 3j). However, the Namalwa Bcl-xES cells did not accumulate at the G2/M phase boundary of the cell cycle after CPT treatment (Fig 3j). Attempts to co-immunoprecipitate bcl-xES with cdc2/cdk1 or cyclin A or B1 were also unsuccesful (data not shown). Together, these experiments indicated that the flexible loop domain is required to confer bcl-xL ability to contribute in G2 checkpoint after CPT treatment.

Following a cell cycle checkpoint, cells arrest transiently to allow DNA repair, arrest permanently and enter a senescent state, or die. Following the cell cycle arrest,

many Namalwa-bcl-xL cells appear in a sub-G1 peak, 72 to 96 h after induction of DNA damage, while others may reinitiate cell cycle progression (Fig. 4a). Caspase activation and oligonucleosomic DNA fragmentation typical of apoptosis are detected after G2 arrest (Fig. 4b). However, morphological change analysis shows 25 to 30% apoptotic cells, 20 to 25% necrotic cells, and the remaining with a normal morphology (Fig. 4c).

DISCUSSION

This study suggests that beyond its apoptosis inhibitory function at the mitochondrial level following DNA damage, bcl-xL acts directly on the cell cycle machinery. By binding to and inhibiting cdc2/cdk1 kinase activity, bcl-xL contributes to restoration of the G2 cell cycle checkpoint. The similarity between the apoptotic phenotype and that of mitotic catastrophe resulting from unscheduled cdc2/cdk1 activation, suggests that cdc2/cdk1 may be an effector of apoptosis. According to this hypothesis, activation of cdc2/cdk1 kinase was showed to be a critical determinant of apoptosis induced by various stimuli, including fragmentin-2, staurosporine, taxol, Fas and DNA damage (57-60). Thus, the cdc2/cdk1 kinase inhibition mediated by bcl-xL may contribute to suppression of this apoptotic trigger and in addition, participate directly in the establishment of a G2 checkpoint after DNA damage.

Several observations have supported the existence of molecular connections between cell death and cell cycle regulation. Defective control of cell cycle progression can act as an effective trigger of apoptosis. Inappropriate cyclin/kinase activation, which can be regulated by p53, Bcl-2 or Bax, increased expression of some cyclins or dysregulated expression of cdk inhibitors could trigger programmed cell death (53, 57, 60-64). Beyond its effect on cdk activity in the nucleus, the cdk inhibitor p21/CIP1/WAF1 also interacts with procaspase 3 at the level of mitochondria, and inhibits its activation (65). Apoptotic regulatory proteins, such as bcl-2, bcl-xL and bax, also influence the control of cell cycle entry (47-54) and others have reported that bcl-2 and bcl-xL binds to the checkpoint protein Rad9, which contains a BH3-like domain (66). More recently, specific caspase activities were also reported to play a role for T cell proliferation (67, 68). Our observations suggest that bcl-xL modulates cell response to chemotherapeutic agents, not only by preventing mitochondrial dysfunction associated with apoptosis, but also by contributing in the restoration of a G2/M cell cycle checkpoint.

The importance of the flexible loop domain within bcl-xL is highlighted in this study. Althoug not necessary for bcl-xL antiapoptotic activity, our results suggest that

this domain may play a significant role during cell cycle progression and G2/M checkpoint after DNA damage. Further characterization of this unstructured domain, by site-directed mutagenesis, is under investigation. Cell cycle-dependent phosphorylation of bcl-2, within its unstructured loop domain (69-75) and its degradation by an ubiquitin/proteosome pathway were reported recently (76).

This study demonstrates a biochemical interaction between bcl-xL and cdc2/cdk1-cyclin A/B1 complexes coupled to a functional biological effect in response to DNA damage. Conceivably, our observation could imply that bcl-xL also plays a dominant role in a context of a physiological cell cycle checkpoint during development. Mouse *Bcl-x* $^{-/-}$ phenotype was reported to be severe with mice dying at embryonic day 13 with extensive cell death in postmitotic immature neurons in the developing brain, and with the life-span of immature lymphocytes severely shortened (77). These abnormalities were similar to those observed in c-*abl*- and *rb*-deficient mice, genes coding for proteins involved in cell cycle control (77). The possibility of a functional link between the abnormalities in the *bcl-x*-deficient mice and the loss of a physiological cell cycle checkpoint during development remains to be elucidated.

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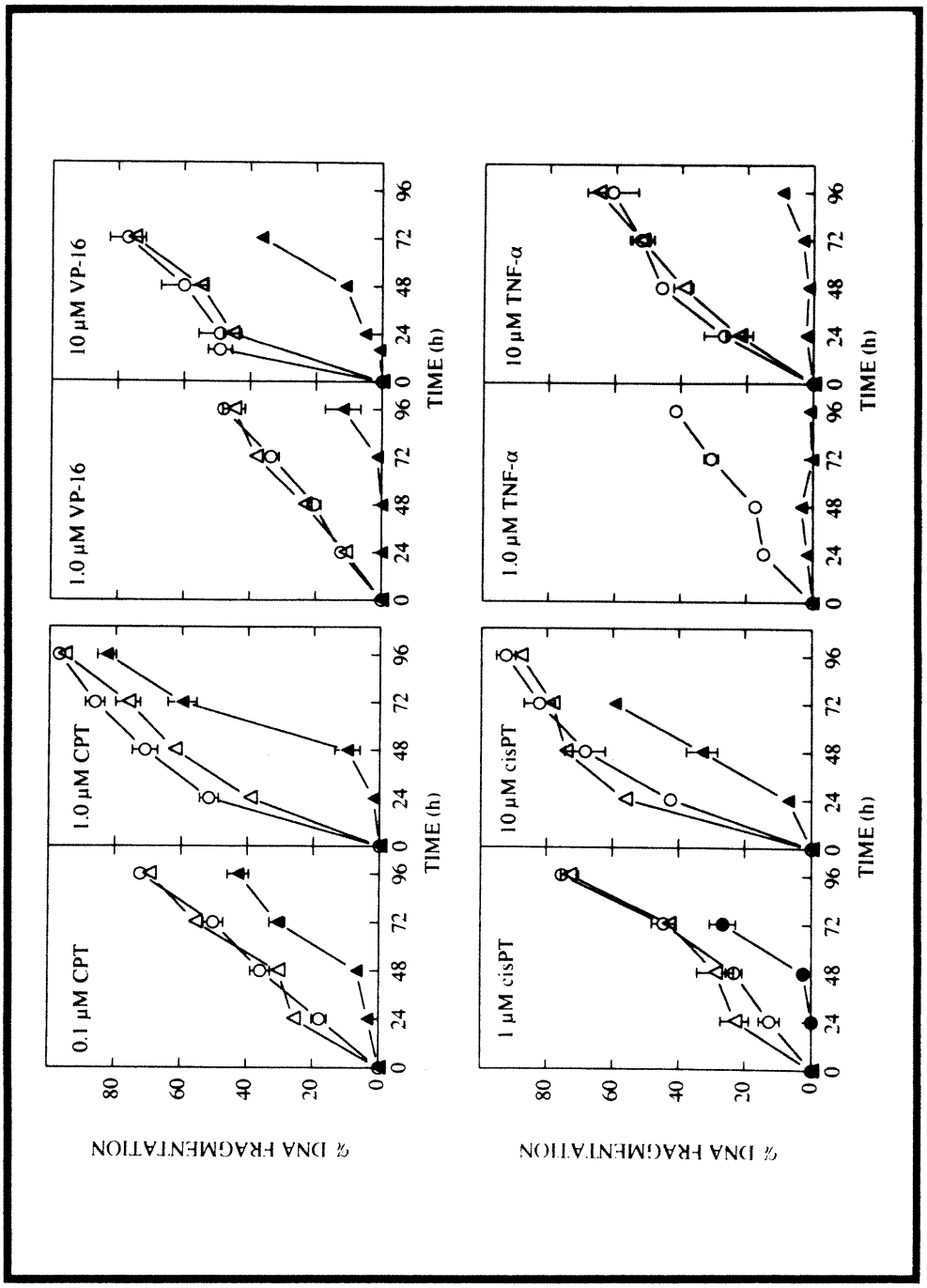

0.1 μM CPT
1.0 μM CPT
1 μM cisPT
10 μM cisPT
1.0 μM VP-16
10 μM VP-16
1.0 μM TNF-α
10 μM TNF-α
% DNA FRAGMENTATION
TIME (h)
0
20
40
60
80
24
48
72
96

Figure 1a

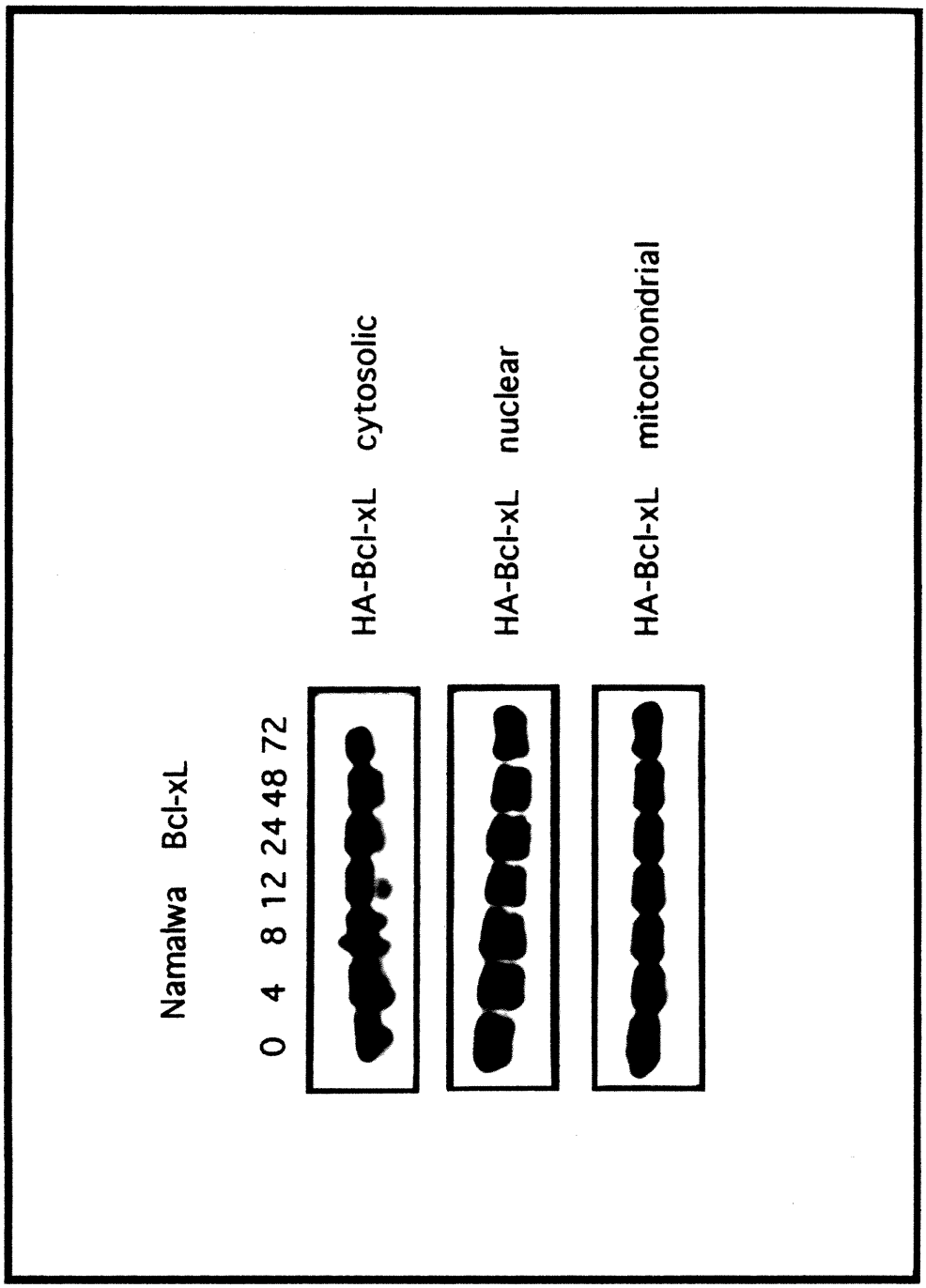

Namalwa Bcl-xL
0 4 8 12 24 48 72
HA-Bcl-xL cytosolic
HA-Bcl-xL nuclear
HA-Bcl-xL mitochondrial

Figure 1b

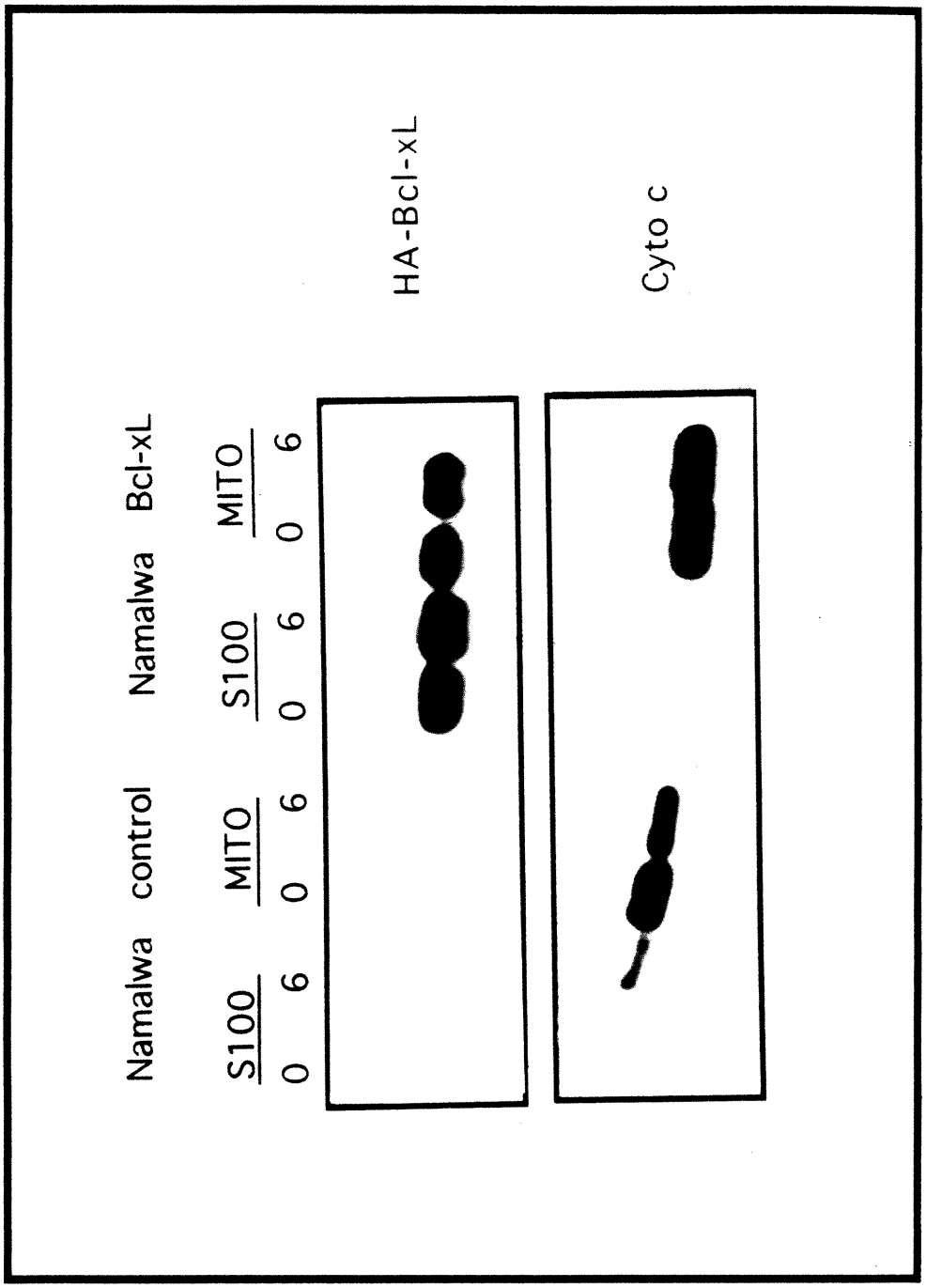

Namalwa control
S100 0 6
MITO 0 6
Namalwa Bcl-xL
S100 0 6
MITO 0 6
HA-Bcl-xL
Cyto c

Figure 1c

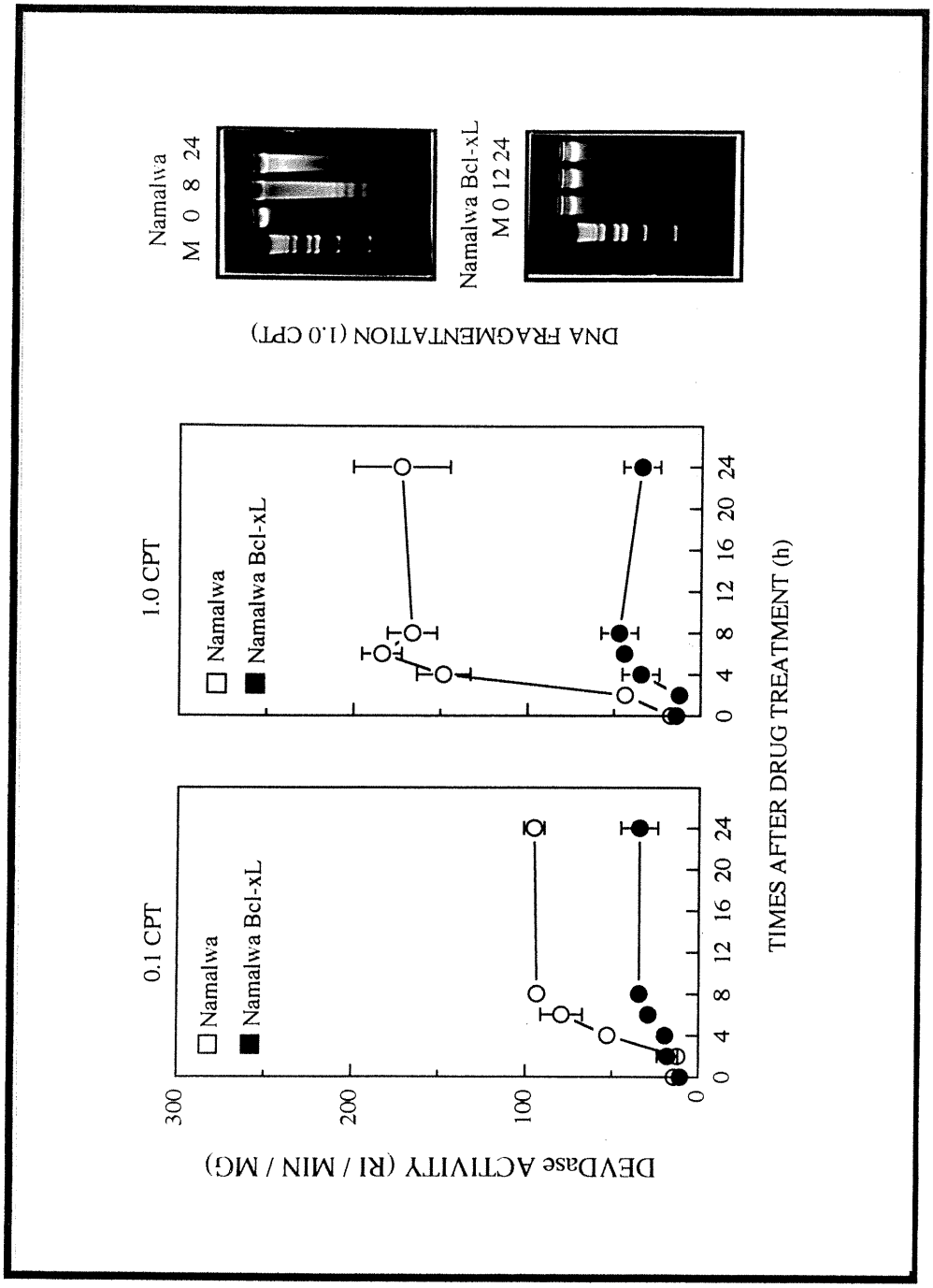

Namalwa
M 0 8 24
Namalwa Bcl-xL
M 0 12 24
DNA FRAGMENTATION (1.0 CPT)
1.0 CPT
Namalwa
Namalwa Bcl-xL
0.1 CPT
Namalwa
Namalwa Bcl-xL
DEVDase ACTIVITY (RI / MIN / MG)
300
200
100
0
0 4 8 12 16 20 24
TIMES AFTER DRUG TREATMENT (h)

Figure 1d

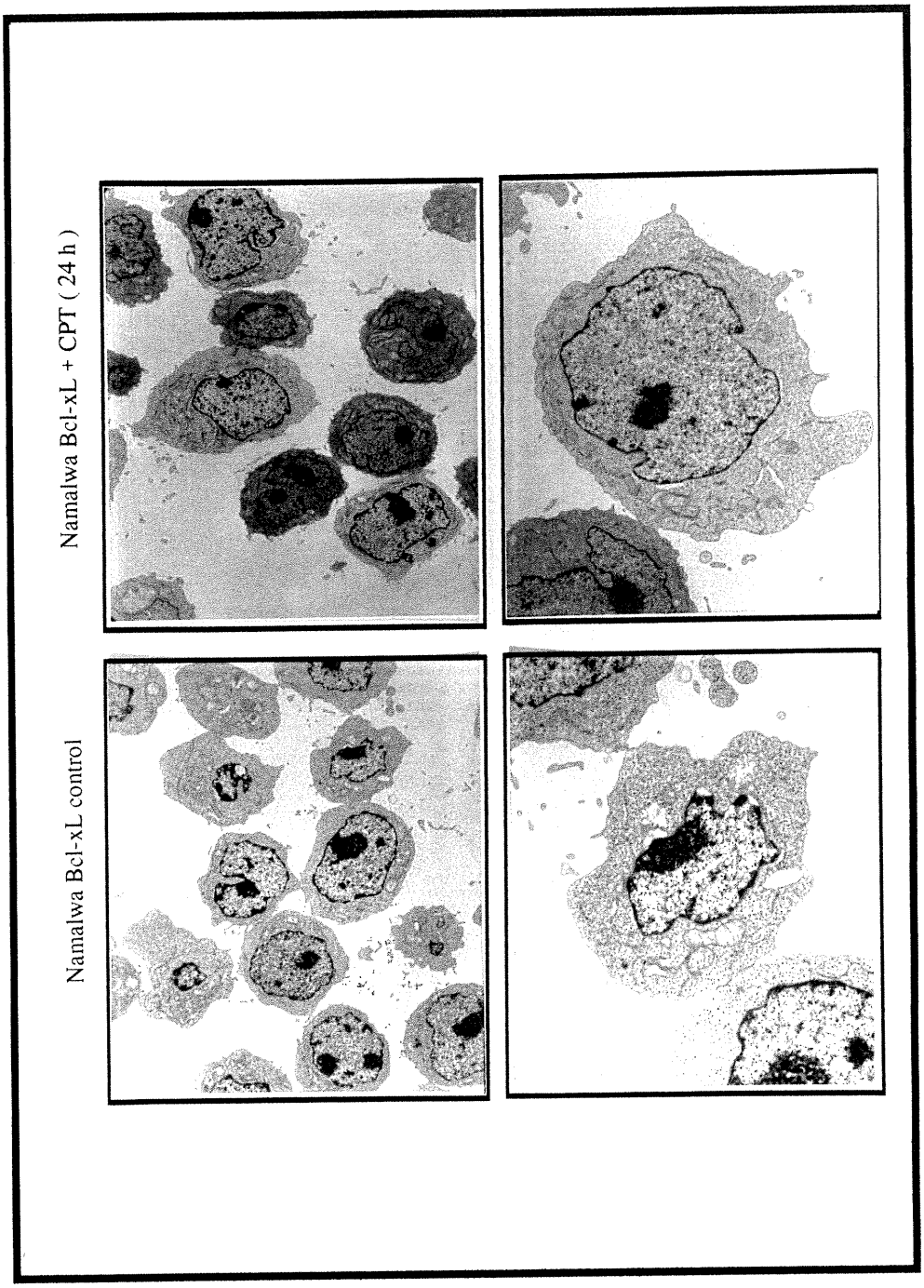


Figure 1e

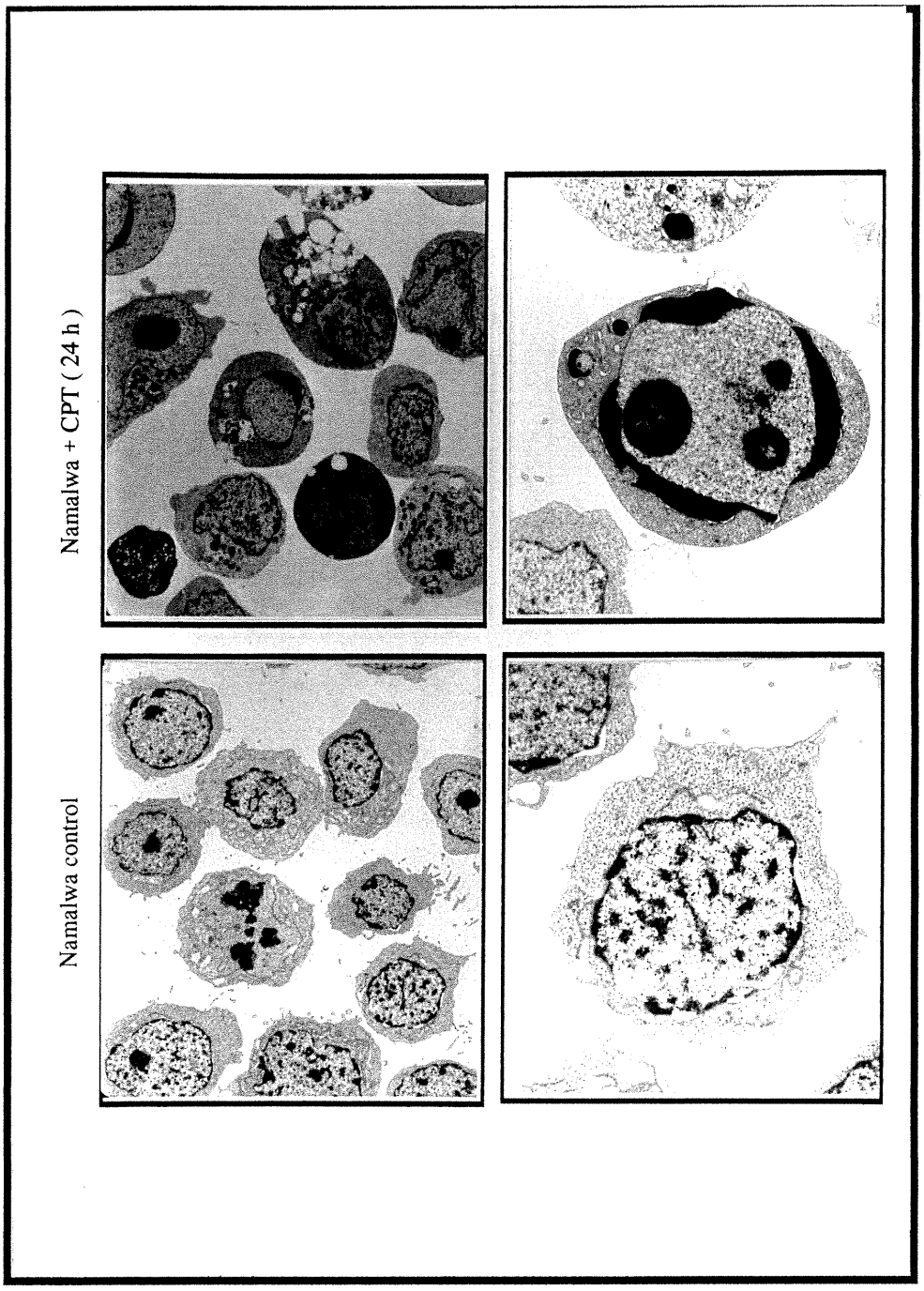


Figure 1f

Figure 1. Effect of bcl-xL overexpression on cell death in the Namalwa cell line.

a, The kinetics of DNA fragmentation were determined after treatments with various concentrations of camptothecin (CPT), etoposide (VP-16), cisplatin (cisPT) and TNF-α in untransfected (○), mock-transfected (Δ) and bcl-xL-transfected (▲) Namalwa cells. **b,** Stable expression and sub-localization of bcl-xL during CPT treatment in Namalwa-Bcl-xL cells. **c,** Bcl-xL prevented cytochrome c release from mitochondria to cytosol after CPT treatment. **d,** Cytochrome c release was associated with caspase activation and oligonucleosome-sized DNA fragmentation in CPT-treated Namalwa cells; its retention in the mitochondria of CPT-treated Namalwa-Bcl-xL cells correlated with non-activation of caspase and oligonucleosome-sized DNA fragmentation. **e,** Cellular morphology of control and CPT-treated Namalwa-Bcl-xL cells; less than 1% of cells were apoptotic or necrotic. **f,** Electron micrograph of control and apoptotic (35-40%) CPT-treated Namalwa cells.

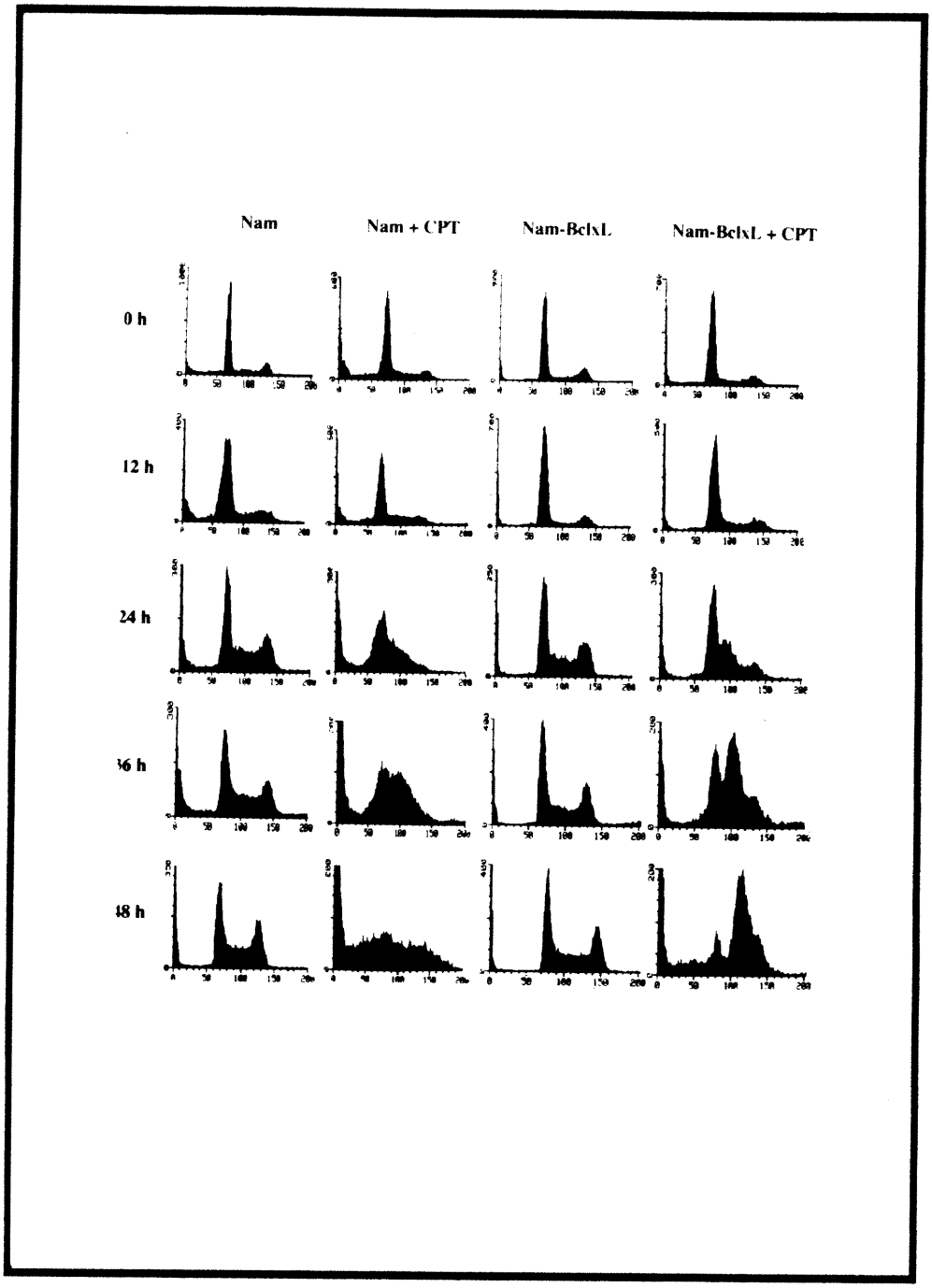


Figure 2a

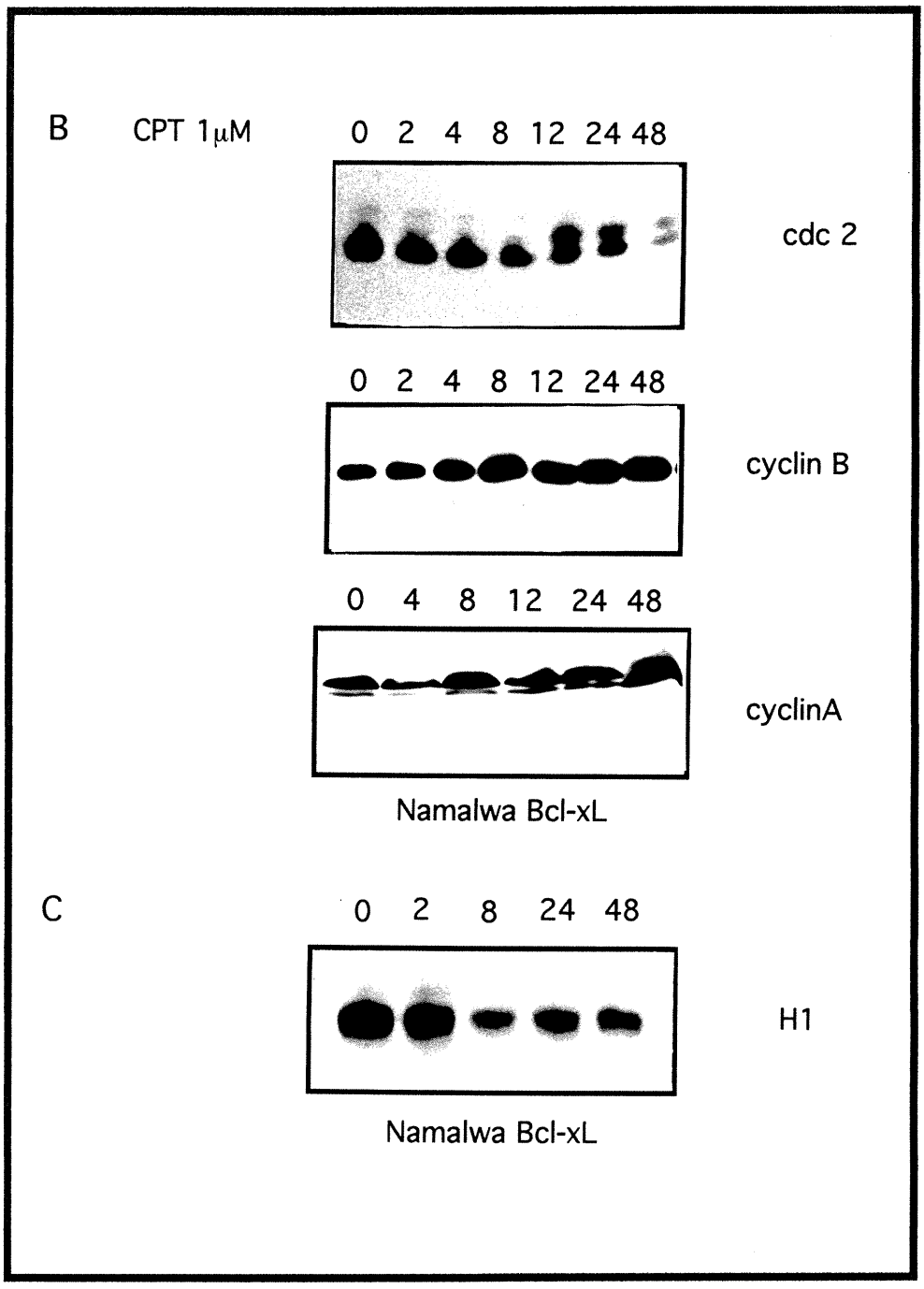

B
CPT 1μM
0 2 4 8 12 24 48
cdc 2
0 2 4 8 12 24 48
cyclin B
0 4 8 12 24 48
cyclinA
Namalwa Bcl-xL
C
0 2 8 24 48
H1
Namalwa Bcl-xL

Figure 2b-c

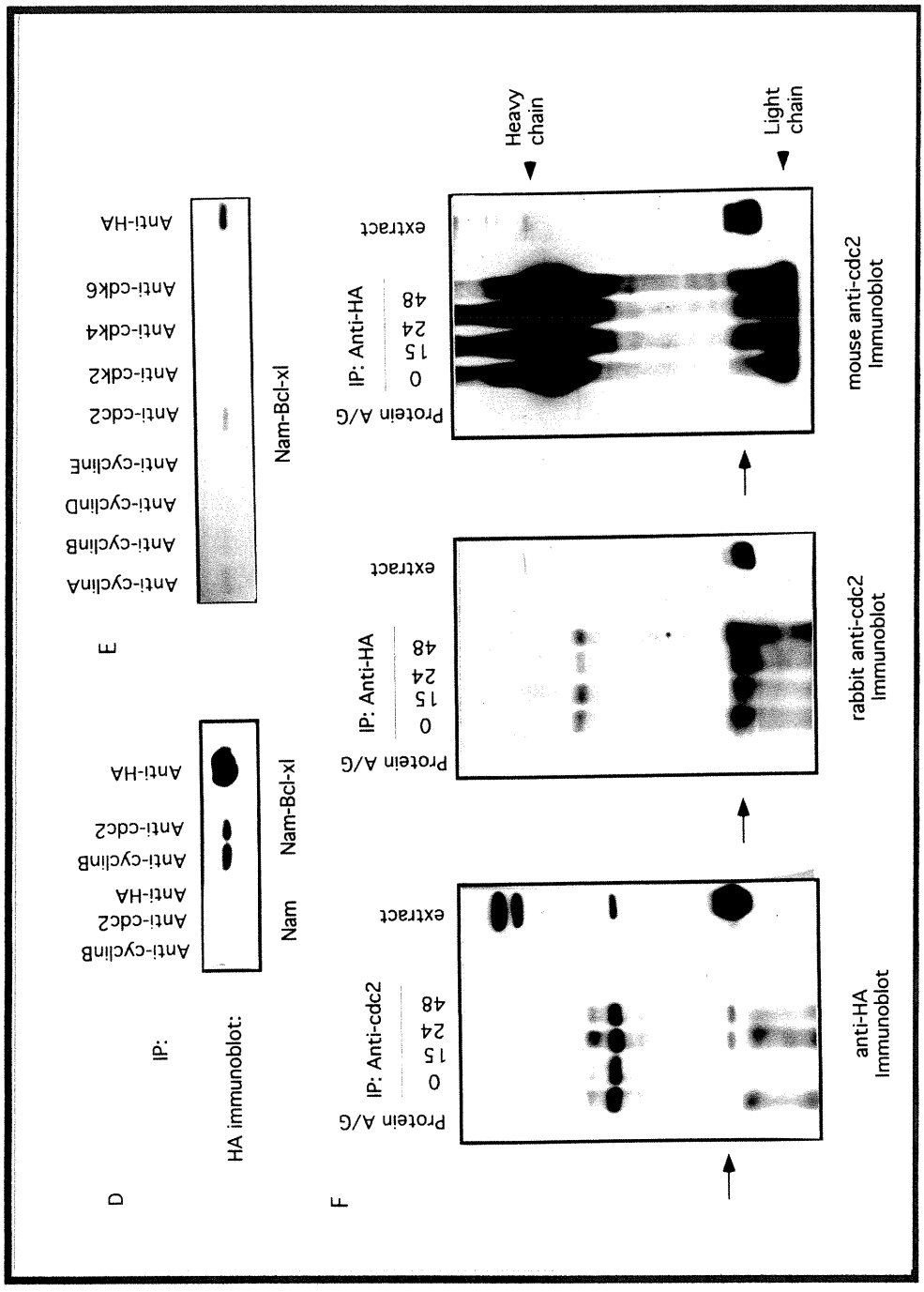

D
IP:
HA immunoblot:
Anti-cyclinB
Anti-cdc2
Anti-HA
Anti-cyclinB
Anti-cdc2
Nam
Nam-Bcl-xl
E
Anti-cyclinA
Anti-cyclinB
Anti-cyclinD
Anti-cyclinE
Anti-cdc2
Anti-cdk2
Anti-cdk4
Anti-cdk6
Anti-HA
Nam-Bcl-xl
F
Protein A/G
IP: Anti-cdc2
0
15
24
48
extract
anti-HA
Immunoblot
Protein A/G
IP: Anti-HA
0
15
24
48
extract
rabbit anti-cdc2
Immunoblot
Protein A/G
IP: Anti-HA
0
15
24
48
extract
mouse anti-cdc2
Immunoblot
Heavy chain
Light chain

Figure 2d-f

Figure 2. Effect of bcl-xL overexpression on cell cycle progression in CPT- treated cells and its interaction with cdc2/cdk1 and cyclin A/B1 complexes.

a, Flow cytometry analysis of cell cycle distribution in control and CPT-treated cells (1.0 μM; 30 min). Bcl-xL- overexpressing cells arrested in the late S/early G_2 phase of the cell cycle after CPT treatment, whereas untransfected, treated cells died and appeared in the sub-G1 peak. **b,** Cell cycle arrest in CPT-treated Namalwa-Bcl-xL cells was associated with the appearance of the hyperphosphorylated form of cdc2/cdk1 protein and increased expression of cyclin B1 and A. **c,** Cdc2/cdk1 kinase activity decreased rapidly in CPT-treated, bcl-xL-overexpressing cells. **d,** Bcl-xL protein co-immunoprecipitated with cyclin B1 and cdc2/cdk1 after CPT treatment (from nuclei-enriched fraction). **e,** Bcl-xL interaction with cdc2/cdk1-cyclin A/B1 after CPT treatment compared to other cdk-cyclin complexes (from total extract). **f,** Reciprocal co-immunoprecipitation experiments indicated that bcl-xL interaction with cdc2/cdk1 increased with time as CPT-treated cells underwent the cell cycle checkpoint (from nuclei-enriched fraction).

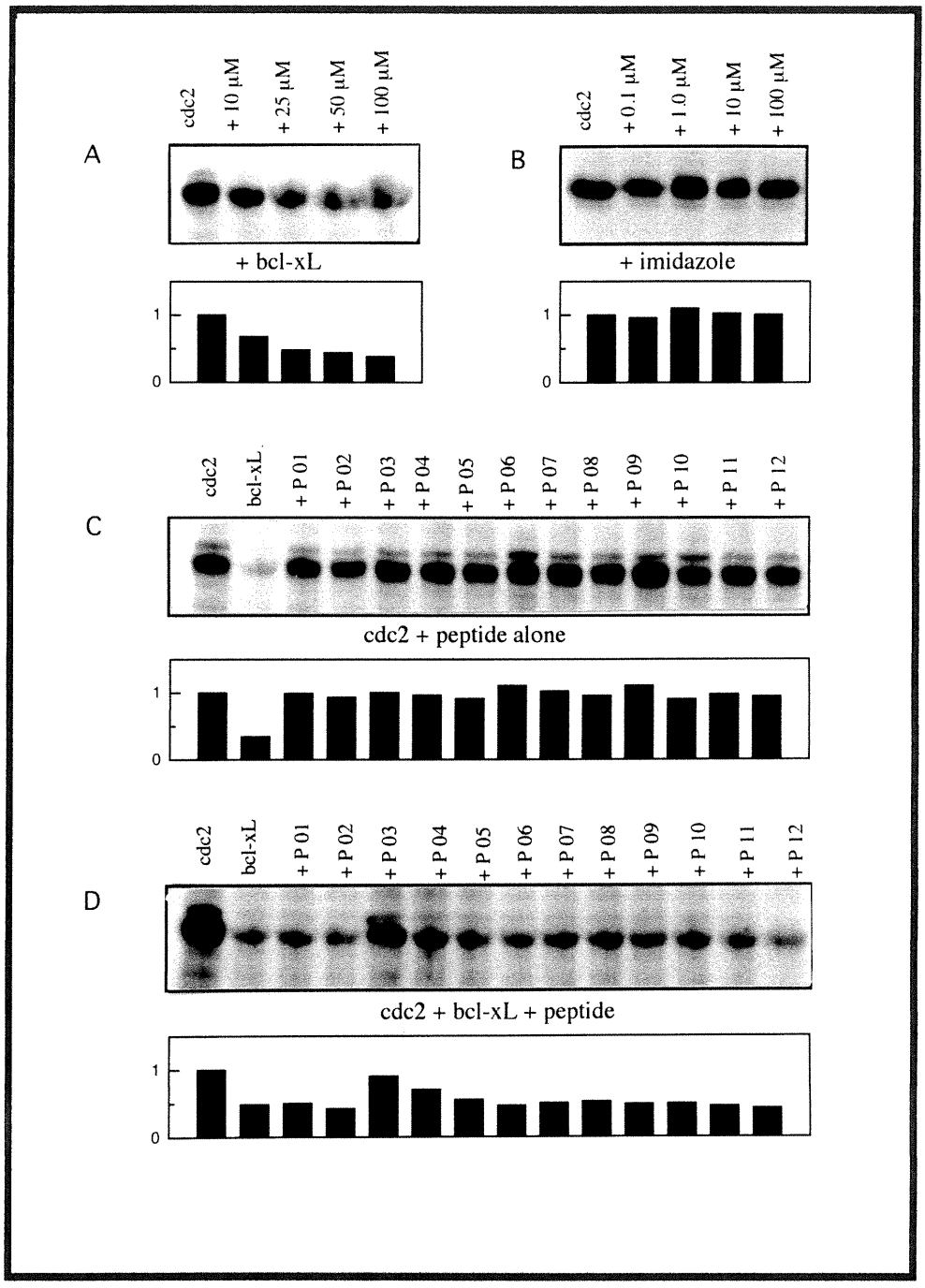
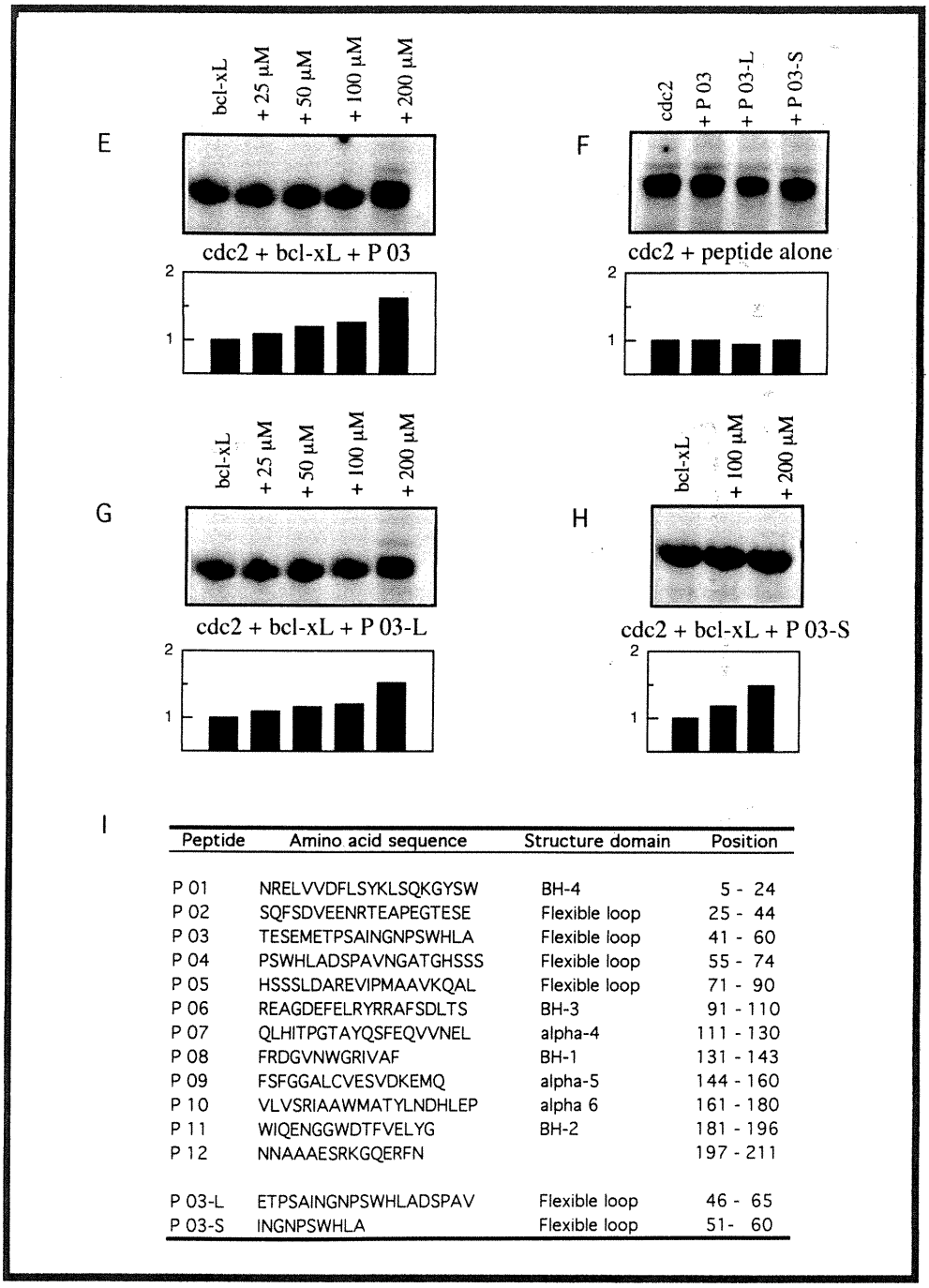

A
cdc2
+ 10 µM
+ 25 µM
+ 50 µM
+ 100 µM
+ bcl-xL
1
0
B
cdc2
+ 0.1 µM
+ 1.0 µM
+ 10 µM
+ 100 µM
+ imidazole
1
0
C
cdc2
bcl-xL
+ P 01
+ P 02
+ P 03
+ P 04
+ P 05
+ P 06
+ P 07
+ P 08
+ P 09
+ P 10
+ P 11
+ P 12
cdc2 + peptide alone
1
0
D
cdc2
bcl-xL
+ P 01
+ P 02
+ P 03
+ P 04
+ P 05
+ P 06
+ P 07
+ P 08
+ P 09
+ P 10
+ P 11
+ P 12
cdc2 + bcl-xL + peptide
1
0

Figure 3a-d



I

Peptide	Amino acid sequence	Structure domain	Position
P 01	NRELVVDFLSYKLSQKGYSW	BH-4	5 - 24
P 02	SQFSDVEENRTEAPEGTESE	Flexible loop	25 - 44
P 03	TESEMETPSAINGNPSWHLA	Flexible loop	41 - 60
P 04	PSWHLADSPAVNGATGHSSS	Flexible loop	55 - 74
P 05	HSSSLDAREVIPMAAVKQAL	Flexible loop	71 - 90
P 06	REAGDEFELRYRRAFSDLTS	BH-3	91 - 110
P 07	QLHITPGTAYQSFEQVVNEL	alpha-4	111 - 130
P 08	FRDGVNWGRIVAF	BH-1	131 - 143
P 09	FSFGGALCVESVDKEMQ	alpha-5	144 - 160
P 10	VLVSRIAAWMATYLNDHLEP	alpha 6	161 - 180
P 11	WIQENGGWDTFVELYG	BH-2	181 - 196
P 12	NNAAAESRKGQERFN		197 - 211
P 03-L	ETPSAINGNPSWHLADSPAV	Flexible loop	46 - 65
P 03-S	INGNPSWHLA	Flexible loop	51- 60

Figure 3e-i

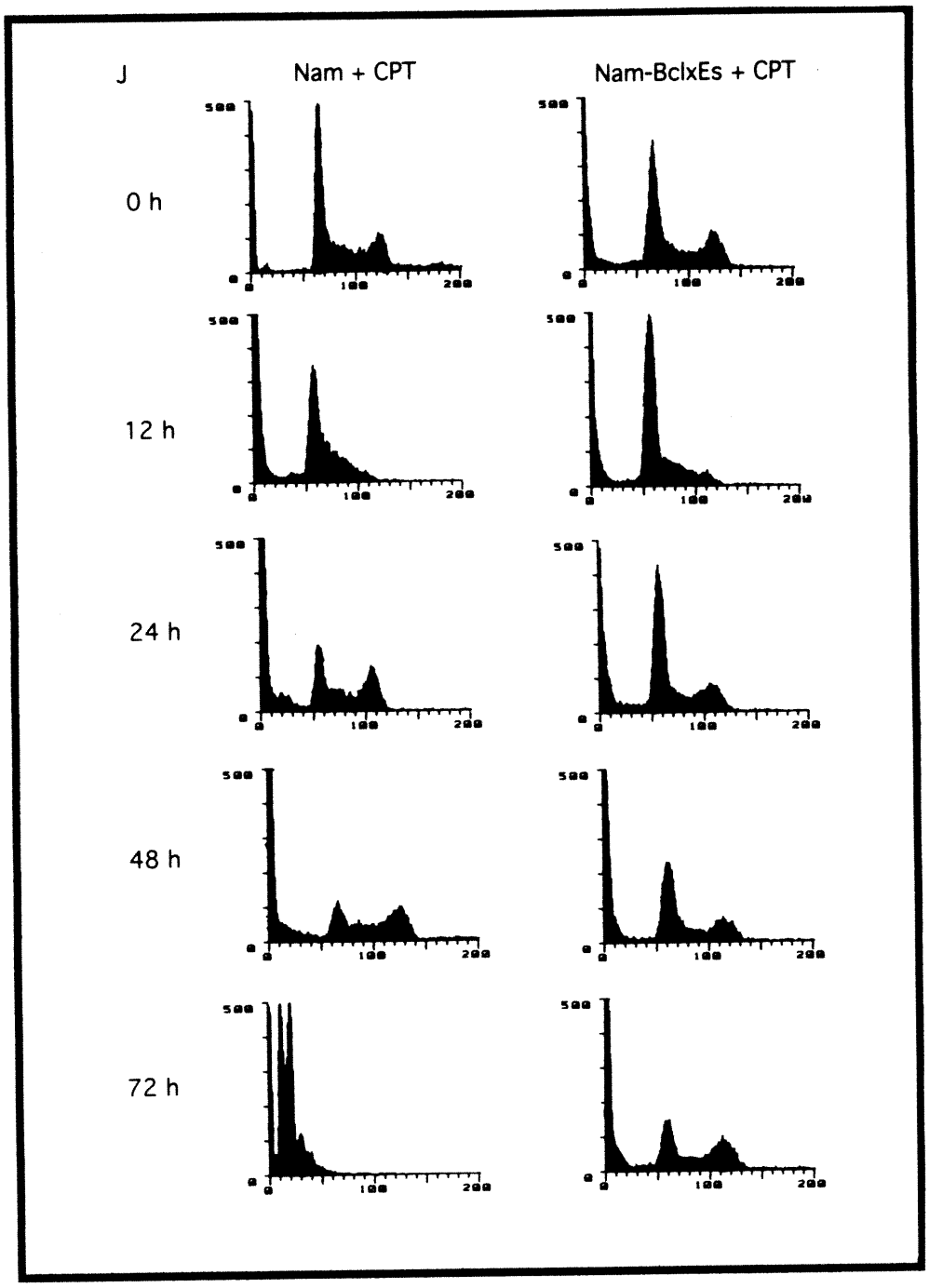


Figure 3j

Figure 3. Bcl-xL inhibits cdc2/cdk1 kinase activity.

a, Histone H1 phosphorylation mediated by cdc2/cdk1 activity was inhibited by the addition of various concentrations of human recombinant bcl-xL(ΔTM) protein; the histograms represent relative densitometry analysis of the corresponding autoradiograph. **b,** Although the preparations of recombinant bcl-xL(ΔTM) protein were dialysed extensively after purification, the effect of various concentrations of imidazole was tested on cdc2/cdk1 kinase activity. Imidazole had no inhibitory effect on the rate of histone H1 phosphorylation. **c,** A series of peptides that covers all the amino acid sequence of human bcl-xL(ΔTM) was synthesized. None of these peptides (200 μM) had a direct effect on cdc2/cdk1 kinase activity. **d**, The potency of these peptides (200 μM) in blocking the inhibitory effect of bcl-xL(ΔTM) (25 μM) on cdc2/cdk1 kinase activity was tested. Peptide 03 was the most potent in reversing the inhibitory effect of bcl-xL(ΔTM) on cdc2/cdk1 kinase activity. **e,** Reversal of bcl-xL(ΔTM)-mediated inhibition of cdc2/cdk1 activity by various concentrations of P-03. **f,** P-03 and two additional peptides, P-03-L and P-03-S (200 μM each), had no direct effect on cdc2/cdk1 kinase activity. **g,** P-03-L reversed the inhibitory effect of bcl-xL(ΔTM) on cdc2/cdk1 kinase activity. **h,** P-03-S, the smaller peptide used in this study, potently reversed the effect of bcl-xL(ΔTM) on cdc2/cdk1 kinase activity. **i,** Amino acid sequence, structure/function domain and position within bcl-xL protein of all the peptides used. **j,** Effect of enforced bcl-xES overexpression on cell cycle progression in CPT- treated cells (1.0 μM; 30 min). Bcl-xES overexpressing cells delayed the occurrence of apoptosis but did not accumulate in G2/M after CPT treatment.

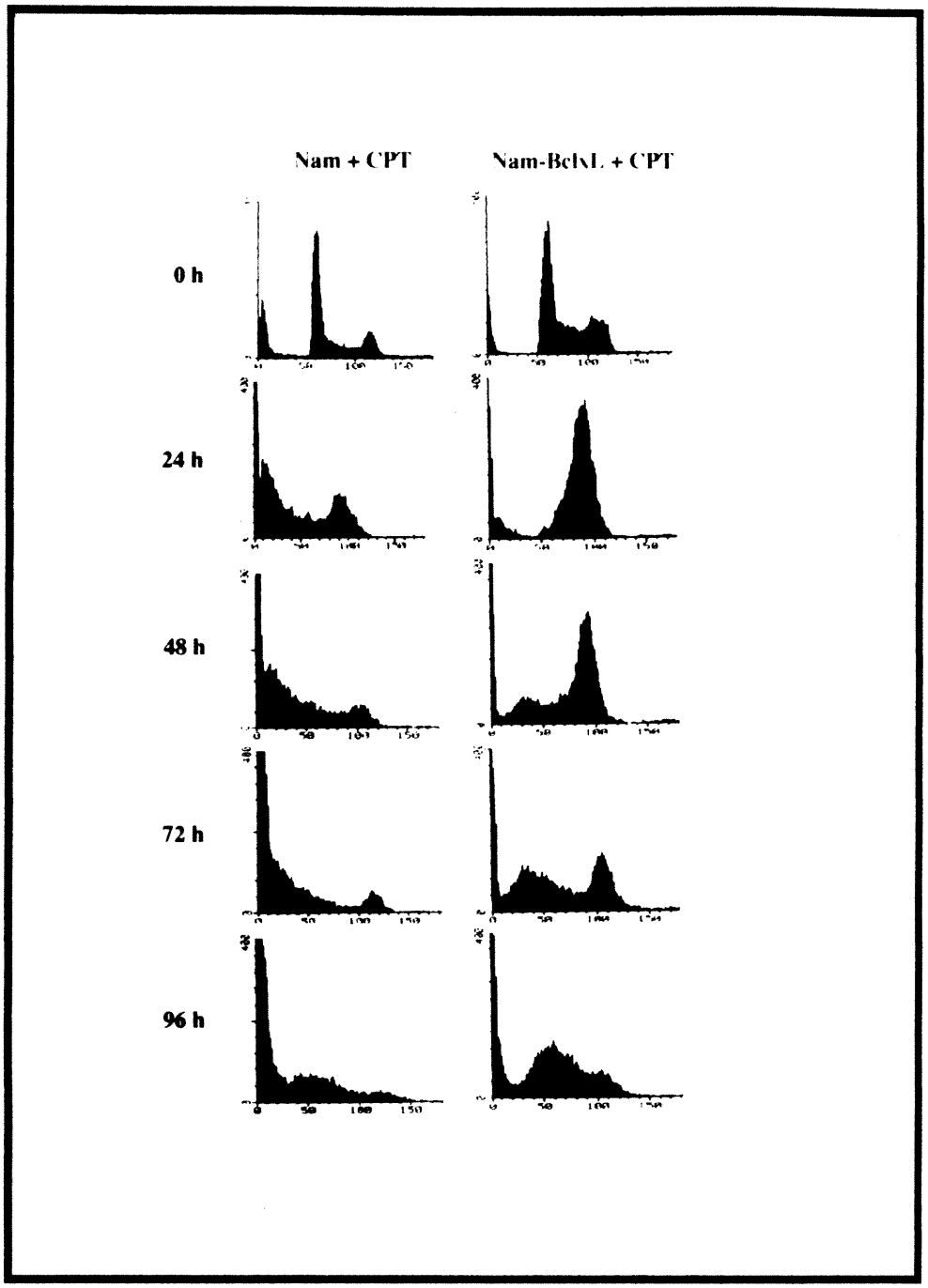


Figure 4a

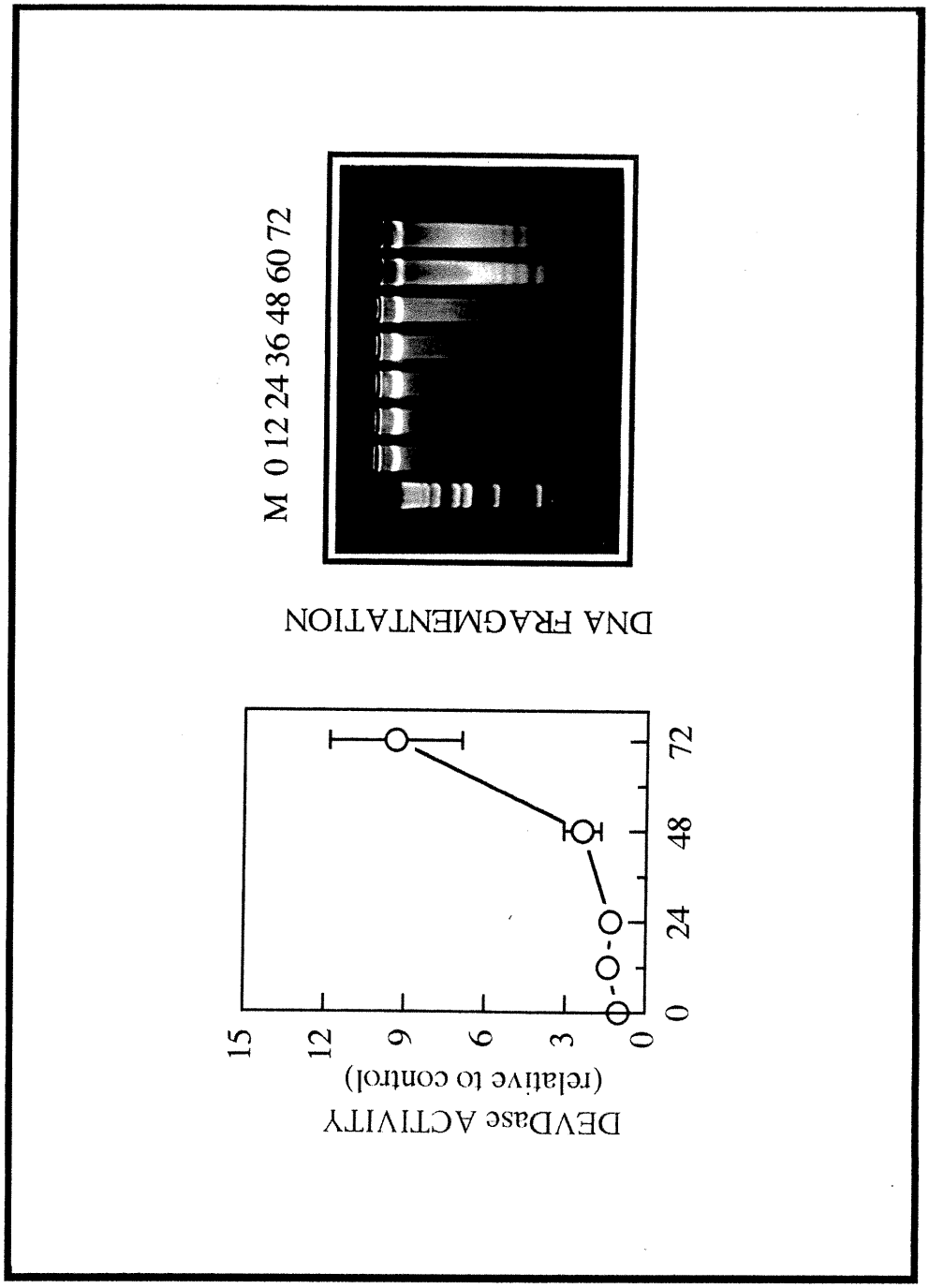

M 0 12 24 36 48 60 72
DNA FRAGMENTATION
DEVDase ACTIVITY
(relative to control)
15
12
9
6
3
0
0
24
48
72

Figure 4b

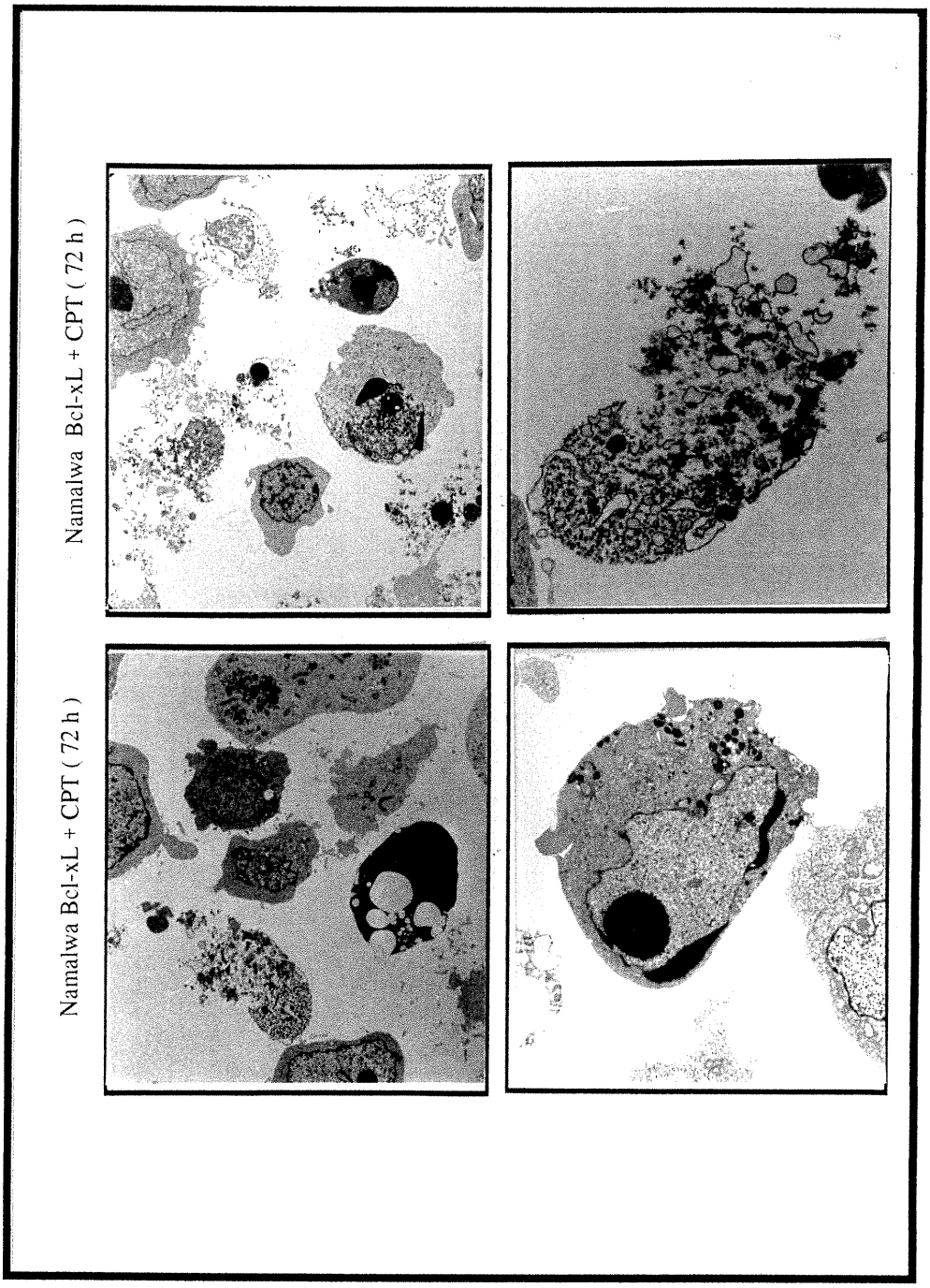


Figure 4c

Figure 4. Fate of bcl-xL-overexpressing cells after the cell cycle checkpoint.

a, Flow cytometry analysis of cell cycle distribution in CPT-treated cells (1.0 μM; 30 min). Bcl-xL overexpression prevented the rapid occurrence of apoptosis and mediated a cell cycle arrest for at least 48 h. After the cell cycle checkpoint, many cells accumulated in the sub-G1 peak. **b,** Caspase activation and occurrence of oligonucleosome-sized DNA fragmentation correlated with the observed accumulation of cells in the sub-G1 peak, 72 h post-treatment. **c,** Cellular morphology observed by electron microscopy revealed that 25-30% of the cells underwent apoptosis while 20-25% showed a necrotic mode of cell death, 72 h after CPT treatment.

CHAPITRE 7. DISCUSSION

En oncologie, l'intérêt porté à l'apoptose provient des observations indiquant que l'apparition et la croissance des tumeurs ne sont pas uniquement la conséquence d'une prolifération cellulaire excessive mais sont le résultat d'un déséquilibre entre la prolifération et la mort cellulaire. Des dérégulations de la mort cellulaire programmée qui augmentent le seuil de déclenchement de l'apoptose dans les cellules sont impliquées dans la carcinogénèse et la progression des cellules tumorales (7, 201, 202). Les radiations ionisantes ainsi que la plupart des drogues utilisées dans les thérapies anticancéreuses induisent la mort des cellules tumorales par apoptose. Ainsi, un dérèglement de l'apoptose ou de son contrôle représente une nouvelle forme de résistance aux traitements (223-225). Les mécanismes d'activation de l'apoptose par les drogues anticancéreuses sont encore peu connus et font actuellement l'objet de recherches intenses. Afin d'améliorer l'efficacité des agents cytotoxiques utilisés dans le traitement des cancers ou de développer de nouvelles stratégies thérapeutiques, il est essentiel d'élucider les mécanismes moléculaires impliqués dans la mort cellulaire programmée.

L'existence d'un contrôle génétique de l'apoptose a été mis en évidence chez le nématode *Caenorhabditis elegans* avec l'identification des gènes *ced-3, ced-4, ced-9* et *egl-1* (21-25). *Bcl-2* fut le premier gène répresseur de l'apoptose identifié dans les cellules humaines au niveau de la translocation chromosomique t(14;18) présente dans les lymphomes folliculaires de type B (41). La fonction anti-apoptotique de Bcl-2 a été démontrée dans des lignées cellulaires hématopoiétiques ou neuronales privées de facteurs de croissance (46, 128). D'autres études ont montré que la surexpression de Bcl-2 protège les cellules de l'apoptose induite par les radiations ionisantes et différentes drogues anticancéreuses telles que le cisplatine, la camptothécine ou l'étoposide (44, 45, 298-300, 302). En 1993, les gènes *bcl-x* et *bax* furent clonés. A l'époque, deux isoformes de fonction opposée, codées par *bcl-x* furent identifiées (*bcl-xL et bcl-xS*). L'isoforme longue (Bcl-xL) inhibe la mort cellulaire induite par la privation de l'IL-3 alors que l'isoforme courte (Bcl-xS) bloque l'activité anti-apoptotique de Bcl-2 (48). De manière analogue à Bcl-xS, la surexpression de Bax-α sensibilise les cellules à l'apoptose induite par la privation de l'IL-3, de plus, la protéine Bax-α dimérise avec Bcl-2 et neutralise sa fonction anti-apoptotique (55). Des études d'inactivation *in vivo* des gènes *bcl-x* et *bax* ont mis en évidence le rôle joué par ces gènes dans la mort cellulaire programmée au cours du développement. Les

souris déficientes du gène *bcl-x* présentent des défauts dans la maturation du système nerveux et hématopoiétique qui conduisent à une mort embryonnaire précoce à environ 13 jours de gestation. L'invalidation du gène *bax* ne génère pas un phénotype léthal mais les souris $bax^{-/-}$ présentent, entre autre, des hyperplasies des thymocytes et des cellules B suggérant qu'une inactivation de Bax joue un rôle dans le développement des tumeurs (220, 303). Des analyses d'expression des protéines Bcl-xL et Bax-α chez la souris ont révélé une distribution ubiquitaire des protéines. Cependant, Bcl-xL et Bax-α semblent exprimées préférentiellement dans certaines lignées cellulaires, soit certaines cellules neuronales, hématopoiétiques, épithéliales et germinales (304-306).

Au cours de nos travaux, plusieurs études ont également démontré qu'une surexpression des protéines Bcl-xS et Bax-α sensibilise les cellules tumorales en culture à l'apoptose induite par différentes drogues cytotoxiques, alors qu'une surexpression de Bcl-xL exerce l'effet opposé (228, 307-311). Dans la même lancée, des études d'expression des protéines Bax-α et Bcl-xL dans des tumeurs humaines ainsi que des modèles de souris transgéniques et de souris nues ou scid, ont suggéré que ces protéines régulatrices de l'apoptose participent à la carcinogénèse, la progression tumorale et modulent la sensibilité des cellules tumorales *in vivo* aux traitements anticancéreux (216-218, 232, 312-314).

L'ensemble de ces travaux a mis en évidence l'activité anti- ou pro-apoptotique des protéines Bcl-2, Bcl-xL et Bax-α et a suggéré que leur niveau d'expression module la sensibilité des cellules face à l'apoptose induite par les agents cytotoxiques utilisés en chimiothérapie. Néanmoins, la fonction et le mode d'action de ces protéines dans la cascade apoptotique, ainsi que leurs relations moléculaires avec les autres familles de protéines impliquées dans l'apoptose, notamment la famille des Ced-3/ICE/caspases, demeuraient méconnus.

7.1 Le niveau d'expression des protéines Bcl-xL et Bax-α module la sensibilité des cellules tumorales à l'apoptose induite par les agents anticancéreux

Les études présentées dans les chapitres 2 et 3, montrent que le niveau d'expression des protéines Bcl-xL et Bax-α modulent la réponse cellulaire face aux drogues anticancéreuses. La surexppression de Bcl-xL dans les cellules promyélocytiques humaines U937, module les cinétiques d'activation de l'apoptose induite par plusieurs drogues ayant des mécanismes d'action différents, telles que la

camptothécine (CPT, un inhibiteur de la topoisomérase I), l'étoposide (VP-16, un inhibiteur de la topoisomérase II), la vinblastine (un inhibiteur de la polymérisation de la tubuline), le paclitaxel (taxol, un inhibiteur de la dépolymérisation des microtubules) et le cisplatine (un agent formant des ponts covalents dans l'ADN). En revanche, la surexpression de Bax-α sensibilise les lymphomes B humains Namalwa à l'effet cytotoxique de ces drogues. Il est important de noter que les concentrations de drogues utilisées dans ces études sont du même ordre que celles utilisées en clinique. De plus, ces lignées cellulaires sont p53-déficientes et constituent d'excellents modèles d'activation de l'apoptose p53-indépendants. La surexpression de Bcl-xL protège les cellules de la cytotoxicité de toutes les drogues testées, durant une période de temps plus ou moins longue selon les concentrations et le type de drogue utilisées (Chap. 2, Fig 3 et 4). Alors que l'effet anti-apoptotique de Bcl-xL est très évident, l'effet pro-apoptotique de Bax-α, mesuré dans les cellules Namalwa, est plus nuancé (Chap. 3, Fig. 4 et 5). En effet, si la surexpression de Bax-α sensibilise les cellules à l'apoptose induite par la camptothécine, l'étoposide et la vinblastine, elle ne modifie pas la réponse cellulaire au paclitaxel et au cisplatine aux concentrations utilisées dans cette étude. Ces résultats suggèrent que la protéine Bax-α est impliquée dans des voies spécifiques de signalisation de l'apoptose dans les cellules Namalwa. Dans l'apoptose induite par la camptothécine, l'effet pro-apoptotique de Bax-α est plus prononcé à de faibles concentrations de drogue (0.01 μM et 0.05 μM). Lorsque les concentrations de camptothécine utilisées sont plus élévées (0.1 μM et 0.5 μM), les différences entre les cellules transfectées et les cellules témoins sont moins significatives. L'effet pro-apoptotique relativement modéré de Bax-α, observé dans les cellules Namalwa, peut s'expliquer par le fait que le niveau d'expression de la protéine exogène est relativement faible. Etant donné le caractère apoptogène de Bax-α, il est souvent difficile, voir même impossible, dépendamment des lignées cellulaires utilisées, de sélectionner des populations de transfectants stables qui expriment des niveaux substantiellement élévés de la protéine.

Les résultats présentés dans les chapitres 2 et 3 montrent une corrélation directe entre le niveau d'expression des protéines Bcl-xL et Bax-α et la sensibilité des cellules face à une série d'agents anticancéreux et sont en accord avec d'autres études qui suggèrent que le niveau d'expression des protéines Ced-9/Bcl pro-apoptotiques par rapport aux protéines Ced-9/Bcl anti-apoptotiques détermine la sensibilité des cellules à un stimulus de mort cellulaires (55, 61, 100, 315).

7.2 Les protéines Bcl-xL et Bax-α n'interfèrent pas avec les mécanismes d'action primaire de la camptothécine, un inhibiteur de la topoisomérase I

Nous avons montré que la surexpression des protéines Bcl-xL et Bax-α protège ou sensibilise les cellules à l'apoptose suite aux effets cytotoxiques de plusieurs drogues qui exercent des mécanismes d'action différents. De plus, ces protéines modulent également la réponse des cellules à des stimuli physiologiques de mort cellulaire tels que la privation de sérum et la stimulation des récepteurs de mort cellulaire TNF-R1 ou CD95/Apo-1/Fas. Ces résultats suggèrent que Bcl-xL et Bax-α modulent la réponse cellulaire aux agents anticancéreux mais n'interfèrent pas avec le mécanisme d'action primaire des composés. Pour vérifier davantage cette hypothèse, des mesures de la réduction du taux de synthèse de l'ADN, ainsi que la quantification des complexes de clivage stabilisés par la camptothécine ont été réalisées dans les cellules témoins et les cellules transfectées, traitées avec différentes concentrations de camptothécine.

La camptothécine est un inhibiteur spécifique de la topoisomérase à ADN de type I (topoisomérase I) (316). Les topoisomérases à ADN sont des enzymes essentielles au métabolisme de l'ADN durant la transcription, la réplication et la mitose. Ces enzymes sont également impliquées dans les mécanismes de réparation et de recombinaison de l'ADN (317). Trois types de topoisomérases à ADN (topoisomérases I, II et III) ont été identifiés chez les eucaryotes. La topoisomérase I est une enzyme très active lors de la réplication et la transcription, qui permet la relaxation de l'ADN superenroulé en catalysant une brisure simple-brin transitoire par une réaction de transestérification qui lie de façon covalente un résidu tyrosine de l'enzyme à l'extrémité 3' du brin d'ADN clivé (316, 317). Ces complexes covalents topoisomérase-ADN, sont appelés "complexes de clivage" et représentent des intermédiaires transitoires de la réaction de topoisomérisation. La camptothécine stabilise les complexes de clivage catalysés par la topoisomérase I, lesquels peuvent être détectés et mesurés dans la cellule par les techniques de l'élution alkaline ou de la précipitation des fragments d'ADN après dénaturation des protéines au sodium dodecyl sulfate *(KCl-SDS precipitation assay)* (318-322). Les complexes de clivage stabilisés par la camptothécine sont réversibles en l'absence de drogue, suggérant que les lésions primaires sont converties en lésions secondaires qui peuvent mener à l'activation des voies de signalisation de l'apoptose (254, 323-330). De plus, des inhibiteurs de la synthèse de l'ADN tels que l'aphidicoline abolissent l'effet toxique de

la camptothécine. De ces observations est né le modèle suggérant qu'une collision entre la fourche de réplication de l'ADN et les complexes de clivage stabilisés par la CPT, est à l'origine de brisures double-brins et d'un arrêt de la synthèse de l'ADN (317, 328, 331-334).

Les mesures des complexes de clivage stabilisés par la camptothécine et des taux d'inhibition de la synthèse de l'ADN ont révélé que ni Bcl-xL ni Bax-α, exprimés à différents niveaux dans les cellules, n'interfèrent avec le mécanisme d'action primaire de la camptothécine (Chap. 2, Tableau 1 et 2; Chap. 3, Tableau 1). Ces résultats démontrent que Bcl-xL et Bax-α agissent en aval des lésions cytotoxiques induites par les agents anticancéreux.

7.3 Les protéines Bcl-xL et Bax-α contrôlent l'activation des caspases et des facteurs impliqués dans la fragmentation de l'ADN au cours de l'apoptose

Les signaux de mort cellulaire qui émergent des lésions cytotoxiques générées par les agents anticancéreux ne sont pas bien compris. On avait, cependant, de bonnes raisons de penser que ces signaux apoptotiques convergent vers un tronc commun effecteur de la mort cellulaire constitué par les protéases à cystéines ou caspases (151, 335, 336). D'autres études ont également mis en évidence l'implication de protéases à sérine dans le processus apoptotique (14, 337-342). Plusieurs travaux ont par la suite montré que l'activation de la caspase-3 joue un rôle critique dans l'apoptose induite par les drogues génotoxiques telles que les inhibiteurs de topoisomérases (343-346). Afin d'évaluer l'effet de Bcl-xL sur l'activité des caspases dans les cellules U937 traitées avec la camptothécine, nous avons mesuré la cinétique d'hydrolyse de substrats fluorescents spécifiques des caspases-1 et -3 (DABCYL-YVADAPV-EDANS et Ac-DEVD-AMC). Dans les cellules U937 témoins traitées à la camptothécine, les caspases apparentées à la caspases-3 sont très rapidement activées. En revanche, dans les cellules qui surexpriment la protéine Bcl-xL et montrent un délai dans la fragmentation de l'ADN, l'activation de ces capases induite par la camptothécine est retardée. Dans ce système, les caspases apparentées à la caspase-1 ne semblent pas impliquées dans le processus apoptotique (Chap. 2, Fig. 5). L'addition de la protéine recombinante Bcl-xL(ΔTM) dans un extrait cytosolique actif provenant de cellules U937 en apoptose, n'inhibe pas l'activité DEVDase mesurée, suggérant que Bcl-xL agit en amont de l'activation des caspases (Chap. 2, Fig. 5).

L'utilisation d'un système acellulaire reconstitué a permis d'analyser davantage les facteurs impliqués dans la fragmentation de l'ADN et de situer l'activité de la protéine Bcl-xL dans la cascade apoptotique induite par la camptothécine. Nos résultats montrent que des activités inhibées par l'Ac-DEVD-CHO (un inhibiteur spécifique des caspases apparentées aux caspases-3) et le N-tosyl-L-phenylalanylchloromethyl ketone (TPCK, un inhibiteur de protéases à sérine) sont impliquées dans la fragmentation de l'ADN lors de l'apoptose induite par la camptothécine. En accord avec l'effet anti-apoptotique de Bcl-xL observé dans les cellules, l'activité de ces facteurs est détectée plus tardivement dans les extraits cytosoliques provenant de cellules qui surexpriment Bcl-xL. Par ailleurs, l'addition de la protéine recombinante Bcl-xL(ΔTM) dans des extraits cytosoliques actifs, n'inhibe nullement la fragmentation des noyaux exogènes. Ces résultats montrent que Bcl-xL agit en amont des caspases et des facteurs TPCK-sensibles impliqués dans la fragmentation de l'ADN induite par la camptothécine et inhibe, à tout le moins temporairement, leur activation (Chap. 2, Fig. 6).

L'utilisation du système acellulaire a permis de mettre en évidence l'existence de deux activités distinctes impliquées dans la promotion de la fragmentation de l'ADN. La première, qui est inhibée par l'aldéhyde Ac-DEVD-CHO, correspond aux caspases, notamment aux caspases apparentées à la caspases-3. La seconde n'est pas encore identifiée, mais sa sensibilité au TPCK suggère qu'elle appartient à la famille des protéases à sérine (Chap.2, Fig. 6). L'inhibition des caspases *in vivo* inhibe totalement la fragmentation de l'ADN (15). En revanche, nous montrons que l'inhibition des caspases avec l'Ac-DEVD-CH0 *in vitro,* dans un extrait de cellules en apoptose, n'atténue que légèrement l'apparition des fragments internucléosomiques de l'ADN. Cette observation suggère que l'Ac-DEVD-CH0 prévient l'accumulation de facteurs promoteurs de la fragmentation de l'ADN mais n'inhibe par ces mêmes facteurs déjà actifs dans l'extrait. D'autres études effectuées dans notre laboratoire ont montré que la digestion de l'ADN lors de l'apoptose induite par la camptothécine s'effectue en deux étapes (15, 257, 342). Dans un premier temps l'ADN est clivé en fragments de haut poids moléculaire (45-50 kpb) et cette première étape permet une digestion supplémentaire de l'ADN en fragments internucléosomiques. Dans ces études il est montré que les caspases sont impliquées dans la fragmentation de haut poids moléculaire de l'ADN alors que les activités TPCK-sensibles sont impliquées dans la fragmentation internucléosomique (15) (Annexe I).

La cinétique d'activation des caspases a également été mesurée dans les cellules Namalwa témoins et les cellules transfectées avec Bax-α, traitées avec la camptothécine et l'étoposide. Contrairement à Bcl-xL, la surexpression de Bax-α accélère l'activation

des caspases apparentées aux caspases-3 et promouvoit la fragmentation de l'ADN ainsi que le clivage de PARP, un substrat des caspases-3 et -7 (Chap.3, Fig 6, 7 et 8). D'autres études, en accord avec les nôtres, ont montré que les protéines Bcl-2 et Bcl-xL bloquent l'apoptose induite par différents stimuli de mort cellulaires incluant un grand nombre d'agents génotoxiques et plusieurs travaux suggèrent que Bcl-2 et Bcl-xL agissent en amont des caspases et inhibent leur activation. (347-351). L'activation des caspases apparentées aux caspases-3, dans la mort cellulaire induite par Bax-α, a également été mise en évidence et Bax-α accroît la sensibilité des cellules à l'apoptose induite par les drogues (308, 352-354). Nos résultats suggèrent que Bcl-xL ainsi que Bax-α assurent un point de contrôle dans l'apoptose induite par les drogues génotoxiques en contrôlant l'activation des caspases, les protéases effectrices de l'apoptose (355) (Annexe II).

7.4 Les protéines Bcl-xL et Bax-α régulent l'activation des caspases en contrôlant la libération du cytochrome c au niveau de la mitochondrie

La mitochondrie a pris une place considérable dans la régulation de la mort cellulaire programmée au cours de ces dernières années (19, 121). L'engagement dans l'apoptose s'accompagne de profondes modifications affectant les mitochondries telles que l'ouverture des mégacanaux mitochondriaux, la perte du potentiel de membrane ($\Delta\Psi m$) et la translocation de protéines apoptogéniques mitochondriales, telles que le cytochrome c, dans le cytoplasme. Ces événements mitochondriaux aboutissent à l'activation des caspases et sont régulés positivement ou négativement par les protéines de la famille des Ced-9/Egl-1/Bcl (39, 40, 90, 111, 123, 177-181). Le relargage du cytochrome c par les mitochondries permet la formation du complexe cytochrome c/Apaf-1/procaspase-9 qui induit l'activation de la caspase-9. La caspase-9 active, va à son tour activer les caspases effectrices -3 et -7 et déclencher ainsi la phase de dégradation de l'apoptose (32, 36, 163, 171). Dans nos travaux, nous montrons que la protéine Bax-α accélère l'activation des caspases en favorisant la libération du cytochrome c (Chap. 4, Fig. 3). En revanche, Bcl-xL s'oppose à l'activation des caspases en inhibant la libération du cytochrome c (Chap. 6, Fig. 1). Le rôle déterminant des mitochondries dans la phase d'engagement de l'apoptose induite par les agents anticancéreux a été démontré dans plusieurs études (pour revue voir Schmitt et al., Annexe III (229)).

7.5 Caractérisation de Bax-σ et Bcl-xES, des nouvelles isoformes de Bax et Bcl-x

Plusieurs isoformes de Bax (Bax-α, Bax-β, Bax-γ, Bax-δ, Bax-ω et Bax-ε) ont été identifiées (55, 77-79). Nous avons identifié et cloné une isoforme supplémentaire de *bax*, appelée *bax-σ*. La protéine Bax-σ possède une stucture très semblable à celle de Bax-α mais présente une délétion de 13 acides aminés localisée entre le domaine BH-2 et le domaine C-terminal hydrophobe. Ainsi, Bax-σ possède les domaines BH-1, 2 et 3, le domaine transmembranaire ainsi que les hélices α-5 et α-6 qui constituent le domaine PFD *(pore forming domain)*. Une analyse de l'expression des messagers *bax-σ* et *bax-α* dans une variété de lignées cellulaires tumorales et de tissus sains, montre que l'expression des ARNms codant les deux isoformes est largement distribuée et suggèrent que les protéines Bax-α et Bax-σ sont souvent co-exprimées (Chap. 4, Tableau 1). La légère différence de séquence entre les protéines Bax-α et Bax-σ ainsi que leur poids moléculaire quasi-identique, rend difficile la détection de la protéine endogène Bax-σ dans des extraits cellulaires fractionnés par électrophorèse sur gel de polyacrylamide-SDS (Chap. 4, Fig. 1). Cependant, une électrophorèse bidimentionnelle sur gel a permis d'identifier plusieurs isoformes de Bax dont deux qui possèdent un poids moléculaire respectif d'environ 21 et 20 kDa et des points isoélectriques très rapprochés, suggérant quelles pourraient correspondre à Bax-α et Bax-σ, respectivement (Chap. 4, fig. 1). Dans le but d'étudier la fonction de Bax-σ nous avons exprimé la protéine de façon transitoire dans les cellules Namalwa, et dérivé une population de transfectants stable. Nos résultats montrent que Bax-σ exerce une fonction pro-apoptotique analogue à celle de Bax-α. En effet, dans les transfectants transitoires, la surexpression de Bax-σ induit la libération du cytochrome c, l'activation des caspases, la fragmentation de l'ADN et les changements morphologiques caractéristiques de l'apoptose (Chap. 4, Fig. 3 et 4). Dans les transfectants stables, la surexpression de Bax-σ accroit légèrement la sensibilité des cellules à l'apoptose induite par certaines drogues anticancéreuses telles que la camptothécine, la vinblastine et le taxol (Chap. 4, Fig. 7, 8 et 9).

Le mécanisme d'action de la protéine pro-apoptotique Bax-σ n'est pas encore clairement défini mais plusieurs hypothèses sont actuellement envisagées: 1) La titration des Ced-9/Bcl anti-apoptotiques par dimérisation (55, 100); 2) La formation de canaux ioniques au niveau des membranes intracellulaires, notamment au niveau des membranes mitochondriales (117); 3) La modulation de l'activité des mégacanaux mitochondriaux par son interaction avec les protéines VDAC et/ou ANT (110-112,

188).

Dans un système de double-hybride chez la levure, en plus de former des homodimères, l'isoforme Bax-σ semble interagir préférentiellent avec les homologues Ced-9/Bcl qui exercent une activité anti-apoptotique tels que Bcl-xL, Bfl-1 et Bcl-xES (Chap. 4, Fig. 5). De plus, la surexpression de Bcl-xL dans les cellules Namalwa abolit l'activité pro-apoptotique de Bax-σ et des expériences de co-immunoprécipitations montrent que Bax-σ interagit avec Bcl-xL dans les cellules (Chap. 4, Fig. 6). Ces résultats suggèrent que le modèle de dimérisation est également valide pour Bax-σ. Nous n'avons pas encore déterminé si Bax-σ possède la capacité de former des canaux ioniques dans les membranes artificielles. Si tel est le cas, on peut imaginer que la délétion des acides aminés 159 à 171 localisés entre l'hélice α-6 du domaine PFD et le domaine transmembranaire, contribue à un changement de conformation de la protéine qui confère des caractéristiques spécifiques aux canaux formés par Bax-σ. Compte tenue de la présence du domaine BH-3, l'activité pro-apoptotique de Bax-σ pourrait également impliquer des interactions fonctionnelles avec les protéines VDAC et ANT qui composent les mégacanaux mitochondriaux. De plus, plusieurs résidus sérine, thréonine et tyrosine se trouvent dans la portion délétée de Bax-σ. L'absence de ces résidus, potentiellement phosphorylés, suggère un mécanisme d'action différent entre Bax-α et Bax-σ. Afin de clarifier davantage le mode d'action de Bax-σ, il est nécessaire de poursuivre nos investigations.

Nous avons également identifié et cloné une nouvelle isoforme de *bcl-x*, appelée *bcl-xES (extra short)*. L'ADN complémentaire qui code *bcl-xES* a une séquence identique à celle de *bcl-xL* mais présente une délétion de 342 nucléotides, sans bris du cadre de lecture. La protéine Bcl-xES possède les domaines BH-4 et BH-2 ainsi que le domaine C-terminal hydrophobe mais les domaines BH-1 et -2, ainsi que le domaine PFD et le domaine flexible sont absents. Nous avons analysé l'expression des messagers *bcl-xES* dans différentes lignées cellulaires tumorales et dans des tissus sains et tumoraux d'origine variée. Dans les cellules tumorales, le patron d'expression des ARNm *bcl-xES* est semblable à celui de *bcl-xL* et *bcl-xS*. En revanche, *bcl-xES* semble moins exprimé dans les tissus sains (Chap. 5, Tableau 1). Un plus grand échantillonnage et une quantification rigoureuse s'avèrent néanmoins nécessaire avant d'en tirer des conclusions. Afin d'étudier la fonction de Bcl-xES, nous avons dérivé des cellules Namalwa qui surexpriment la protéine. Dans les cellules transfectées, Bcl-xES est préférentiellement exprimée au niveau de la mitochondrie, mais la protéine est

également détectée au niveau du noyau et dans le cytosol (Chap. 5, Fig 2). La protéine Bcl-xES exerce une fonction anti-apoptotique analogue à celle de Bcl-xL et diminue la sensibilité des cellules à l'apoptose induite par la camptothécine, l'étoposide, le cis-platine et le TNF (Chap. 5, Fig. 3). Dans l'apoptose induite par la camptothécine, Bcl-xES retarde la libération du cytochrome c et l'activation des caspases-9 et -3 (Chap. 5, Fig. 4). Compte tenu de l'homologie de séquence entre Bcl-xL et Bcl-xES et des domaines BH présents dans Bcl-xES, trois hypothèses concernant le mécanisme d'action de Bcl-xES peuvent être proposées 1) La titration des Ced-9/Egl-1/Bcl pro-apoptotiques par dimérisation; 2) La séquestration du complexe cytochrome c/Apaf-1/procaspase-9 au niveau des mitochondries; 3) La modulation de l'activité du canal anionique voltage-dépend (VDAC).

Les interactions de la protéine Bcl-xES avec plusieurs membres de la famille des Ced-9/Bcl/Egl-1 dans un système de double-hybride chez la levure, suggèrent que Bcl-xES peut moduler l'activité de ces protéines *in vivo* (Chap. 5, Fig. 5). L'absence des domaines BH-1,-3 et des hélices α-5 et α-6 doit modifier considérablement la structure de Bcl-xES comparativement à celle de Bcl-xL et exposer davantage les domaines BH-2 et BH-4 de la protéine. Nos résultats montrent également que Bcl-xES interagit avec Apaf-1 dans la levure et inhibe l'activation de la procaspase-9 dans les cellules traitées à la camptothécine. Ces résultats suggèrent que Bcl-xES inhibe l'activation des caspases en séquestrant le complexe cytochrome c/Apaf-1/procaspase-9 au niveau de la membrane mitochondriale. Des expériences de cinétique de co-immunoprécipitation des protéines Bcl-xES, Apaf-1 et procaspase-9, dans les cellules transfectées traitées avec la camptothécine, vont permettre ou non de confirmer cette hypothèse. La protéine Bcl-xES possède le domaine BH-4. Ce domaine est responsable de l'inhibition de l'activité du canal anionique voltage-dépendant (VDAC) par Bcl-xL (114). L'inhibition de la libération du cytochrome c par Bcl-xES pourrait impliquer des interactions fonctionelles entre Bcl-xES et la protéine VDAC des mégacanaux mitochondriaux. La localisation et proximité de toutes ces protéines au niveau de la mitochondrie peut également suggérer la présence d'un mégacomplexe de contrôle de l'apoptose impliquant Bcl-xES/VDAC/Apaf-1/procaspase-9.

7.6 Un couplage fonctionnel entre Bcl-xL et le contrôle du cycle cellulaire

La réponse cellulaire face aux lésions cytotoxiques induites par les drogues anticancéreuses peut être variée et comprend 1) Des arrêts dans la progression du

cycle cellulaire; 2) L'activation des mécanismes de réparation de l'ADN; 3) L'engagement des voies de signalisation de l'apoptose; 4) La mort cellulaire par nécrose (226, 254, 356). Nous montrons que la surexpression de Bcl-xL dans les cellules Namalwa protège les cellules face à l'apoptose induite par la camptothécine mais modifie également la réponse des cellules aux dommages induits par la camptothécine en permettant la restauration d'un point-contrôle en phase G2 du cycle cellulaire. Cette observation nous a conduit à étudier la relation entre la protéine Bcl-xL et la régulation de la progression S/G2 et la transition G2/M du cycle cellulaire.

La surexpression de la protéine Bcl-xL dans les cellules Namalwa modifie de façon très significative la sensibilité des cellules face à l'effet cytotoxique de différentes drogues (Chap. 6, Fig. 1). Dans les cellules Namalwa traitées avec la camptothécine, Bcl-xL exerce une activité anti-apoptotique au niveau des mitochondries en inhibant la libération du cytochrome c et l'activation subséquente des caspases, la fragmentation de l'ADN et les changements morphologique typiques de l'apoptose (Chap. 6, Fig. 1). Par ailleurs, nous montrons que la surexpression de Bcl-xL dans les cellules transfectées permet la restauration d'un arrêt du cycle cellulaire en phase G2 alors que les cellules Namalwa témoins engagent rapidement le programme apoptotique (Chap. 6, Fig. 2). L'accumulation en phase G2 des cellules qui surexpriment Bcl-xL s'accompagne d'une diminution de l'activité histone H1 kinase des complexes cdc2-cdk1/cycline A/B1 impliqués dans la régulation de la fin de la phase S / début G2 et la transition G2/M et se traduit par une augmentation de l'expression des cyclines A et B1 et l'apparition de la forme hyperphosphorylée inactive de la kinase cdc2/cdk1 (Chap. 6, Fig. 2). Des études récentes suggèrent que certaines protéines de la famille des Ced-9/Egl-1/Bcl peuvent affecter le contrôle du cycle cellulaire (293-296, 357, 358). Nous montrons que la protéine Bcl-xL surexprimée, interagit avec les complexes cdc2/cdk1-cycline A/B1 dans les cellules Namalwa. Les interactions potentielles entre Bcl-xL et d'autres cdks et cyclines ont également été évaluées et Bcl-xL semble interagir préférentiellement avec les complexes cycline-kinases impliqués dans la régulation de la phase G2 et la transition G2/M. De façon intéressante, l'interaction Bcl-xL-cdc2/cdk1-cycline A/B1 augmente lorsque les cellules s'accumulent en phase G2, suggérant que Bcl-xL participe activement au point-contrôle du cycle cellulaire à la transition G2/M, en modulant l'activité de la kinase cdc2/cdk1 après des dommages à l'ADN induits par la camptothécine (Chap. 6, Fig. 2). L'inhibition de façon dose-dépendante de l'activité kinase des complexes cdc2/cdk1-cycline A/B1 par la protéine recombinante Bcl-xL(ΔTM), *in vitro*, appuie cette hypothèse. Toutefois, les concentrations de protéine recombinante utilisées pour inhiber l'activité de cdc2/cdk1

sont relativement élevées et suggèrent que la protéine Bcl-xL nécéssite la présence d'un cofacteur cellulaire et/ou des modifications post-traductionnelles pour inhiber cdc2/cdk1 *in vivo*.

Des expériences de compétition avec des peptides correspondant à des séquences spécifiques de la protéine Bcl-xL ont permis de définir un domaine d'interaction de Bcl-xL avec les complexes cdc2/cdk-1-cycline A/B1 (Chap.6, Fig. 3). Ce domaine contient cinq sites potentiels de phosphorylation et se situe dans la région flexible de la protéine, localisée entre les domaines BH-4 et BH-3. Bien que certaines études suggèrent que la région flexible de Bcl-xL et Bcl-2 est un domaine de régulation négative de l'activité anti-apoptotique des protéines, la fonction de cette région a été peu étudiée et reste à être définie (148, 150). Nos résultats suggèrent que le domaine flexible de Bcl-xL, notamment la séquence d'acides aminés correspondant au peptide P-03, est impliqué dans la régulation du point-contrôle du cycle cellulaire à la transition G2/M après un dommage à l'ADN induit par la camptothécine. Afin de confirmer l'importance de la région flexible de Bcl-xL dans l'arrêt en phase G2, nous avons évalué, dans le même contexte, l'effet d'une surexpression de la protéine Bcl-xES, une isoforme de *bcl-x* qui ne possède pas de domaine flexible. Nous montrons que les cellules Namalwa qui surexpriment Bcl-xES sont temporairement protégées de l'apoptose induite par la camptothécine mais n'effectuent pas d'arrêt en phase G2 du cycle cellulaire (Chap. 6, Fig. 3). De plus, des expériences de co-immunoprécipitations montrent que Bcl-xES n'interagit pas avec les complexes cdc2/cdk1cycline A/B1.

Plusieurs études ont mis en évidence une connection entre la mort cellulaire programmée et le cycle cellulaire (202, 278). Un contrôle défectif de la progression du cycle cellulaire aboutit souvent à l'activation de l'apoptose (279, 282, 283, 295, 359). Inversement, des travaux suggèrent que certaines protéines régulatrices de l'apoptose telles que les protéines Bcl-2 et Bax-α peuvent moduler la progression du cycle cellulaire (293-296, 357, 358). Une étude très récente a montré que les protéines Bcl-xL et Bcl-2 interagissent avec la protéine Rad-9 impliquée dans le point-contrôle du cycle cellulaire à la transition G2/M (280). Les résultats présentés dans le chapitre 6 suggèrent qu'en plus de son activité anti-apoptotique au niveau de la mitochondrie, Bcl-xL participe au point-contrôle du cycle cellulaire en réponse à des dommages à l'ADN, en interagissant avec les complexes cdk1/cdk1-cycline A/B1 et en inhibant l'activité kinase de cdc2/cdk1.

7.7 Conclusions

Une régulation défectueuse de l'apoptose semble jouer un rôle central dans le développement de cancers et dans la réponse des cellules tumorales aux agents anticancéreux utilisés en chimiothérapie. Les gènes *ced-9/egl-1/bcl* codent des protéines impliquées dans la phase d'engagement de l'apoptose et influencent la décision d'une cellule de mourir ou survivre suite à un stimulus apoptotique. Les recherches intensives effectuées récemment dans le domaine de l'apoptose ont permis de mieux comprendre la fonction et le mode d'action des protéines de la famille de Ced-9/Egl-1/Bcl dans la régulation de l'apoptose induite par les agents anticancéreux.

Dans nos travaux nous montrons que le niveau d'expression des protéines de la famille des Ced-9/Egl-1/Bcl module la sensibilité des cellules tumorales face à l'apoptose induite par les agents anticancéreux. Nous montrons que la protéine Bcl-xL protège les cellules tumorales face à l'apoptose induite par diverses drogues telles que la camptothécine, l'étoposide, le cisplatine, la vinblastine et le taxol, alors que Bax-α sensibilise les cellules à l'effet cytotoxique de certaines de ces drogues. Nous avons également identifié et caractérisé deux nouveaux membres de la famille des Ced-9/Egl-1/Bcl, soit Bcl-xES et Bax-σ. Bcl-xES exerce une fonction anti-apoptotique analogue à celle de Bcl-xL et diminue la chimiosensibilité des cellules. Bax-σ exerce une fonction pro-apoptotique et potentialise l'apoptose induite par certaines drogues.

Dans les cellules traitées avec la camptothécine, nous montrons que Bcl-xL et Bax-α n'interfèrent pas avec le mécanisme d'action primaire de la drogue. En revanche, les protéines Bcl-xL, Bcl-xES, Bax-α et Bax-σ modulent les événements mitochondriaux impliqués dans l'initiation de la cascade apoptotique. Nous montrons que Bcl-xL et Bcl-xES s'opposent à l'activation de l'apoptose induite par la camptothécine en inhibant la libération du cytochrome c et l'activation subséquente des caspases et des facteurs impliqués dans la fragmentation de l'ADN. Les protéines pro-apoptotiques Bax-α et Bax-σ, exercent l'effet opposé et potentialisent l'apoptose en favorisant la libération du cytochrome c et l'activation des caspases (Un modèle schématique de l'activation de l'apoptose par la camptothécine est présenté à la figure 6).

Les réponses des cellules aux altérations générées par les agents anticancéreux sont complexes et les cellules peuvent rapidement déclencher le programme apoptotique, mourir par nécrose, ou activer des points-contrôle du cycle cellulaire.

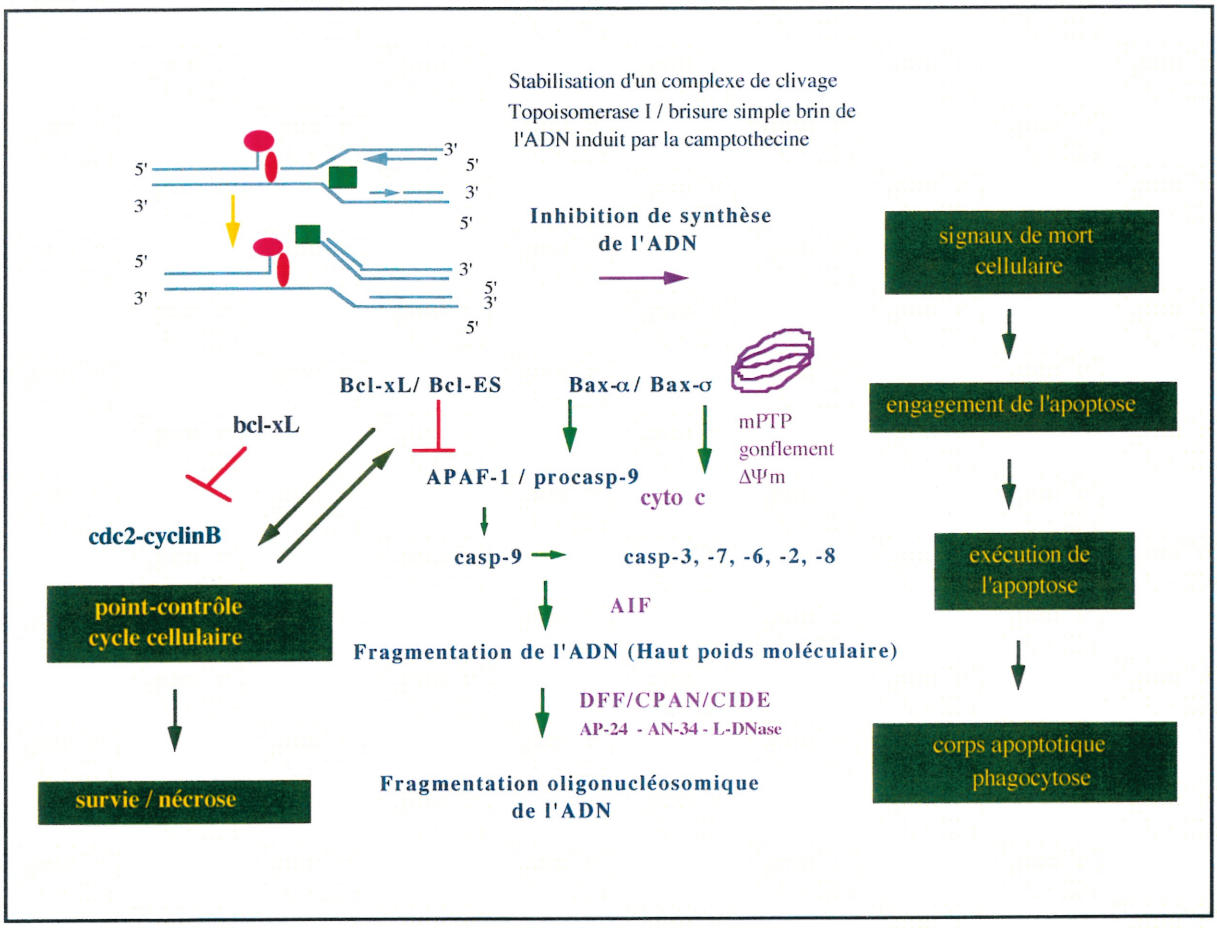


Figure 6: Modèle schématique de l'apoptose induite par la camptothécine.

→ activation

⊣ inhibition

mPTP: mitochondrial permeability transition pore

ΔΨm: mitochondrial inner transmembrane potential

AIF: facteur impliqué dans la fragmentation de haut poids moléculaire de l'ADN

DFF/CPAN/CIDE, AP-24, AN-34, L-DNase: facteurs impliqués dans la fragmentation oligonucléosomique de l'ADN

Nous montrons que la surexpression de Bcl-xL dans les cellules Namalwa modifie la réponse des cellules aux dommages induits par la camptothécine, d'une part, en inhibant l'initiation de la cascade apoptotique au niveau des mitochondries et d'autre part, en permettant la restauration d'un point-contrôle du cycle cellulaire à la transition G2/M. Les résultats présentés dans le chapitre 6 suggèrent que Bcl-xL participe activement à l'arrêt en phase G2 en interagissant directement avec les complexes cdc2/cdk1-cycline A/B1 et en inhibant l'activité kinase de cdc2/cdk1. Ces résultats mettent en évidence un lien fonctionnel direct entre la protéine anti-apoptotique Bcl-xL et les protéines régulatrices du cycle cellulaire et suggèrent la possibilité d'une nouvelle fonction des protéines Ced-9/Egl-1/Bcl dans la régulation du cycle cellulaire en réponse aux dommages à l'ADN.

7.8 Perspectives

En ce qui concerne les modes d'action des isoformes Bax-σ et Bcl-xES, tel que discuté précédemment, il serait important de compléter ces études pour définir et clarifier davantage leurs effets au niveau de l'apoptose induite par les agents anticancéreux, soit:

1) Définir dans un système acellulaire et cellulaire, les propriétés de canal ionique de Bax-σ, notamment sa capacité de modifier la distribution des ions calcium ou de petites molécules comme le glutathione.

2) Etudier la modulation de l'activité des mégacanaux mitochondriaux induite par Bax-σ et Bcl-xES, notamment par leur interaction avec les protéines VDAC et/ou ANT et étudier l'effet de Bax-σ et Bcl-xES sur le relâchement de protéines apoptogéniques telles que l'AIF et le cytochrome c.

3) Etudier les cinétiques de relocalisation intracellulaire de ces protéines en cours d'apoptose, en lien avec leurs interactions avec d'autres membres de la famille des Ced-9/Egl-1/Bcl.

4) Analyser les cinétiques d'interaction de Bcl-xES avec le complexe Apaf-1/procaspase-9 en lien avec l'inactivation/activation de la procaspase-9.

5) Amorcer des études pour évaluer le rôle et la fonction de Bcl-xES au cours

du développement et sur l'incidence de la carcinogénèse en générant des souris transgéniques.

6) Amorçer des études pour évaluer le niveau de phosphorylation de ces protéines en fonction du cycle cellulaire et en lien avec l'activation ou l'inhibition de l'apoptose.

Nous avons montré que la surexpression de la protéine Bcl-xL dans les cellules Namalwa module la réponse des cellules aux drogues anticancéreuses d'une part, en inhibant les altérations mitochondriales impliquées dans la phase d'engagement de l'apoptose et d'autre part, en permettant la restauration d'un point-contrôle du cycle cellulaire. Nos résultats suggèrent que Bcl-xL contribue activement à l'arrêt en phase G2 induit par la camptothécine, en interagissant avec les complexes cdc2/cdk1-cycline A/B1 et en inhibant l'activité kinase de cdc2/cdk1. Afin de démontrer le rôle physiologique de Bcl-xL dans le contrôle du cycle cellulaire suite à des dommages à l'ADN nous proposons:

1) D'étudier les interactions de Bcl-xL avec les complexes cdc2/cdk1-cycline A/B1 dans d'autres lignées cellulaires tumorales non transfectées, traitées avec différents agents anticancéreux. Pour ce faire, nous avons déterminé des conditions dans lesquelles les cellules Namalwa, Jurkat, U937 et HL-60 s'arrêtent spécifiquement en phase G2 ou en phase M du cycle cellulaire suite à des traitements avec différentes drogues (étoposide, taxol, nocodazol). L'étude des interactions de la protéine Bcl-xL endogène avec les complexes cdc2/cdk1-cycline A/B1, par des expériences de co-immunoprécipitations ainsi que la mesure de l'activité kinase de cdc2/cdk1 dans les lignées non traitées ou traitées avec les différentes drogues, devraient permettre de confirmer et d'élargir les observations effectuées dans les cellules Namalwa qui surexpriment Bcl-xL.

2) De confirmer l'implication du domaine flexible de Bcl-xL dans l'interaction avec les complexes cdc2/cdk1-cycline A/B1. Les protéines recombinantes Bcl-xL (Δloop), Bcl-xL(ΔP-03), Bcl-xL(ΔP-03L), Bcl-xL(ΔP-03S) ainsi que la protéine Bcl-xL(ΔP-05), qui servira de contrôle négatif, seront générées. Ces protéines seront testées *in vitro* pour leur capacité d'inhiber l'activité histone H1 kinase de cdc2/cdk1. Ces protéines seront également exprimées dans les cellules Namalwa afin d'étudier la réponse des cellules suite aux dommages induits par la camptothécine et leurs interactions avec les complexes cdc2/cdk1-cycline A/B1 *in vivo*.

3) De définir les acides aminés essentiels à l'interaction de Bcl-xL avec les complexes cdc2/cdk1-cycline A/B1. Pour ce faire, nous allons, dans un premier temps, générer une série de peptides P-03 présentant des mutations au niveau de résidus spécifiques. Ces peptides seront testés *in vitro* pour leur capacité de renverser l'effet inhibiteur de Bcl-xL sur la kinase cdc2/cdk1 et permettrons d'identifier les acides aminés impliqués dans l'interaction Bcl-xL-cdc2/cdk1-cycline A/B1. Des protéines recombinantes Bcl-xL mutées au niveau des résidus identifiés seront générées. Ces protéines seront testées pour leur capacité d'inhiber l'activité histone H1 kinase de cdc2/cdk1 *in vitro*. Ces mêmes protéines seront exprimées dans les cellules Namalwa afin d'étudier la réponse des cellules suite aux dommages induits par la camptothécine et leurs interactions avec les complexes cdc2/cdk1-cycline A/B1 *in vivo*.

4) Finalement, ces études suggèrent que d'autres membres de la famille des Ced-9/Bcl contenant un domaine flexible, soit Bcl-2 et Mcl-1, pourraient potentiellement interagir avec d'autres complexes cdk-cycline tels que cdk4-cycline D, cdk6-cycline D, cdk2-cycline A ou cdk2-cycline E et moduler leur activité. Il serait intéressant d'étudier ces interactions en lien avec d'autres points-contrôle du cycle cellulaire, notamment la transition G1/S.

Plusieurs études mettent en évidence une connection entre l'apoptose et le cycle cellulaire et certaines d'entre elles suggèrent une implication des protéines de la famille des Ced-9/Bcl dans la régulation du cycle cellulaire. L'élucidation de la fonction et des mécanismes d'action des protéines de la famille des Ced-9/Egl-1/Bcl devrait permettre de mieux comprendre la régulation de l'apoptose et le lien apoptose:cycle cellulaire et permettre le développement de stratégies novatrices pour le traitement des cancers.

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ANNEXE I

The CrmA- and TPCK-sensitive pathways that trigger oligonucleosome-sized DNA fragmentation in camptothecin-induced apoptosis: relation to caspase activation and high molecular weight DNA fragmentation

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Abstract: In human B lymphoma Namalwa variant cells expressing the serpin-like CrmA protein, the kinetics of oligonucleosome-sized DNA fragmentation was retarded compared with that of control Namalwa cells following camptothecin treatment. However, no difference in the kinetics of high molecular weight DNA fragmentation was observed between the two lines after camptothecin treatment. Similar delay and inhibition of the oligonucleosome-sized DNA fragmentation was observed in human B lymphoma Namalwa and monocytic-like leukemia U-937 cells coincubated in the presence of various concentrations of *N*-tosyl-L-phenylalanyl chloromethylketone and camptothecin. The effect of *N*-tosyl-L-phenylalanyl chloromethylketone was similar to that of CrmA and did not prevent the appearance of high molecular weight DNA fragments. Similar suppression of camptothecin-induced internucleosomal DNA fragmentation was also observed in a cell-free system when cytosolic extracts obtained from camptothecin-treated Namalwa and U-937 cells were coincubated with untreated nuclei in the presence of *N*-tosyl-L-phenylalanyl chloromethylketone. Futhermore, *N*-tosyl-L-phenylalanyl chloromethylketone had no significant effects on caspase-3-like activities in camptothecin-treated Namalwa and U-937 cells. Hydrolysis of Ac-Asp-Glu-Val-Asp-amino-4-methylcoumarin, a fluorogenic substrate with caspase-3-like activities, was detected in extracts prepared from camptothecin-treated Namalwa and U-937 cells with no apparent difference in the time courses of caspase-3-like activation in the absence or presence of *N*-tosyl-L-phenylalanyl chloromethylketone. Similarly, *N*-tosyl-L-phenylalanyl chloromethylketone was a weak inhibitor of caspase-3-like activities in vitro. Taken together, these observations suggest that the pathway sensitive to *N*-tosyl-L-phenylalanyl chloromethylketone is involved in camptothecin-induced oligonucleosome-sized DNA fragmentation. Futhermore, inhibition of this pathway had no effect on caspase-3-like activation and on the occurrence of high molecular weight DNA fragmentation.

Key words: CrmA, *N*-tosyl-L-phenylalanyl chloromethylketone, camptothecin, DNA fragmentation, apoptosis.

Résumé : Dans les lymphomes B humains Namalwa qui expriment la protéine virale CrmA, semblable à une serpine, la cinétique de la fragmentation oligonucléosomique de l'ADN était retardée par rapport aux cellules Namalwa témoins après traitement à la camptothécine. Cependant, aucune différence dans la cinétique du morcellement de l'ADN en fragments de masse moléculaire élevée n'était observée entre les deux lignées cellulaires suite au traitement à la camptothécine. Un délai similaire dans l'apparition de la fragmentation oligonucléosomique de l'ADN a également été observé dans les cellules Namalwa et les cellules monocyto-leucémiques U-937 coincubées en présence de diverses concentrations de *N*-tosyl-L-phenylalanyl chlorométhylcétone et de camptothécine. La *N*-tosyl-L-phénylalanyl chlorométhylcétone ne modifiait pas le morcellement de l'ADN en fragments de masse moléculaire élevée et semblait avoir un effet similaire à celui de la protéine CrmA. Cette inhibition de la fragmentation internucléosomique de l'ADN a aussi été observée lorsque des extraits cytosoliques de cellules Namalwa ou U-937 traitées à la camptothécine ont été coincubés avec des noyaux de cellules témoins en présence de *N*-tosyl-L-phénylalanyl chlorométhylcétone. De plus la *N*-tosyl-L-phénylalanyl chlorométhylcétone ne semblait pas affecter l'activité de caspase 3 dans les cellules Namalwa et U-937 traitées à la camptothécine. La cinétique d'hydrolyse du substrat fluorigène de la caspase 3, l'Ac-Asp-Glu-Val-Asp-amino-4-méthyl coumarine, a été mesurée dans des extraits cytosoliques de cellules Namalwa et U-937 traitées à la camptothécine et l'activité de caspase 3 ne semblait pas

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Abbreviations: CPT, camptothecin lactone; FASR, CD95/Fas receptor; PARP, poly(ADP-ribose) polymerase; TNFR1, tumor necrosis factor type 1 receptor; TAPFE, transverse alternating pulsed field electrophoresis; TPCK, *N*-tosyl-L-phenylalanyl chloromethylketone.

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modifiée en présence ou en absence de *N*-tosyl-L-phénylalanyl chlorométhylcétone. In vitro également, la *N*-tosyl-L-phénylalanyl chlorométhylcétone n'était qu'un faible inhibiteur de l'activité de caspase 3. Ces observations suggèrent que la voie d'activation sensible à la *N*-tosyl-L-phénylalanyl chlorométhylcétone intervient dans la fragmentation internucléosomique de l'ADN induite par la camptothécine et que l'inhibition de cette voie n'affecte pas le morcellement de l'ADN en fragments de masse moléculaire élevée et l'activation des enzymes semblables à la caspase 3.

Mots clés : CrmA, *N*-tosyl-L-phénylalanyl chlorométhylcétone, camptothécine, fragmentation de l'ADN, apoptose.

Introduction

Programmed cell death (apoptosis) is a pivotal biological process regulating cell survival and cell death (Ellis et al. 1991; Raff et al. 1994; Thompson 1995; Wyllie 1987, 1992). Apoptosis plays a central role during embryonic development, differentiation of the lymphoid cells, and cell homeostasis. Defective control of apoptosis is associated with various human disorders such as cancer development and neurodegenerative and immunosuppressive diseases (Ameisen et al. 1995; Carson and Ribeiro 1993; Korsmeyer 1995; Pantaleo and Fauci 1995; Reed 1994; Thompson 1995). A variety of physiological and nonphysiological signals trigger apoptosis in sensitive cells. Signals emerging from cell death receptors such as the CD95/Fas (FASR) and tumor necrosis factor type 1 receptors (TNFR1) can trigger apoptosis in a variety of cell types following binding of their respective ligands (Dhein et al. 1995; Golstein et al. 1995; Nagata and Golstein 1995; Suda and Nagata 1994; Tewari and Dixit 1995). Cytotoxic T-lymphocytes can also mediate apoptosis via secretion of serine proteases such as granzyme B (Berke 1995; Greenberg 1996). Anticancer drugs like DNA topoisomerase I and II inhibitors also induce apoptosis in various human cancer cell lines (Barry et al. 1990; Bertrand et al. 1991, 1993; Del Bino and Darzynkiewicz 1991; Jaxel et al. 1988; Kaufmann 1989; Solary et al. 1993; Walker et al. 1991).

CrmA (cytokine response modifier activity) is a Cowpox virus protein that modifies the host inflammatory response by decreasing interleukin-1β production (Ray et al. 1992) through its inhibitory effect on caspase 1 activity (Ray et al. 1992; Yuan et al. 1993). More recently, it has been observed that CrmA is also a potent inhibitor of apoptosis activated via FASR and TNFR1 as well as apoptosis induced by granzyme B (Quan et al. 1995; Tewari and Dixit 1995). CrmA inhibits with high affinity the enzymatic activity of caspase 8 (Boldin et al. 1996; Fernandez-Alnemri et al. 1996; Muzio et al. 1996; Zhou et al. 1997) that binds FASR and TNFR1 receptors via FADD and TRAFF adapter proteins (Nagata 1997) and potently inhibits the serine protease granzyme B (Quan et al. 1995). Although the mechanisms by which signals emerging from DNA-damaging agents such as DNA topoisomerase inhibitors are translated into activation of apoptosis have not yet been elucidated, several pieces of evidence now indicate that trypsin-like, caspase 3 (Fernandes-Alnemri et al. 1994; Nicholson et al. 1995; Tewari et al. 1995), and caspase 6 activities (Fernandes-Alnemri et al. 1995) are involved in the cell death machinery following treatment with DNA topoisomerase I and II inhibitors (Bruno et al. 1992; Dubrez et al. 1996; Ghibelli et al. 1995; Hara et al. 1996; Martins et al. 1997; Schmitt et al.[2,3]; Shimizu and Pommier 1996; Solary et al. 1996; Weaver et al. 1993; Yoshida et al. 1996). The trypsin-like protease activities detected in apoptosis following treatment with DNA topoisomerase inhibitors have not yet been identified. Early studies showed that a series of serine protease inhibitors blocked the low molecular weight DNA fragmentation associated with apoptosis induced by DNA topoisomerase inhibitors (Bruno et al. 1992; Weaver et al. 1993). More recently, active serine proteases that trigger low molecular weight DNA fragmentation in a cell-free system have been detected in extracts obtained from cells treated with DNA topoisomerase inhibitors. From these studies, it has been suggested that DNA topoisomerase inhibitors activate serine proteases that in turn activate a pathway involved in low molecular weight DNA fragmentation (Ghibelli et al. 1995; Hara et al. 1996; Shimizu and Pommier 1996; Solary et al. 1996; Yoshida et al. 1996). It has also been reported that a dichloroisocoumarin-sensitive pathway may lead to low molecular weight DNA fragmentation in U-937 cells following VP16 (Dubrez et al. 1996), while a trypsin-like protease activity was proposed to activate apoptosis following camptothecin (CPT) treatment in a hepatoma cell line (Adjei et al. 1996). Others have reported the purification of a novel serine protease AP24 that is also activated in leukemic cells undergoing apoptosis (Wright et al. 1994).

In this study, we show that expression of CrmA in human B lymphoma Namalwa cells delays the appearance of oligonucleosome-sized DNA fragmentation following short CPT treatment without interfering with the occurrence of high molecular weight DNA fragmentation. The effect of CrmA is similar to that observed using a low concentration of *N*-tosyl-L-phenylalanyl chloromethylketone (TPCK) in two cell lines, suggesting the presence of CrmA- and TPCK-sensitive pathways involved in the oligonucleosome-sized DNA fragmentation following CPT treatment. Neither CrmA nor TPCK inhibits the activation of caspase 3-like in these cells and they do not interfere with the appearance of high molecular weight DNA fragmentation. Taken together these results suggest that internucleosomal DNA cleavage and higher order DNA fragmentation are triggered by two independent pathways in the apoptotic process induced by camptothecin.

Materials and methods

Chemicals

The radioactive precursor [2-^{14}C]thymidine (59 mCi/mmol; 1 Ci = 37 GBq) was obtained from ICN BioMedicals (Costa Mesa, Calif.). 20-*S*-Camptothecin lactone (CPT) was purchased from Sigma Chemical

[2] E. Schmitt, A. Steyaert, G. Cimoli, and R. Bertrand. Bcl-xL modulates cancer chemotherapeutic-induced apoptosis and prevents the activation of serine protease and caspase activation. Submitted for publication.

[3] E. Schmitt, A. Steyaert, G. Cimoli, and R. Bertrand. Bax promotes cancer chemotherapy-induced apoptosis and accelerates caspase 3-like activation. Submitted for publication.

Co. (St. Louis, Mo.) and dissolved in dimethylsulphoxide (at 10 mmol/L) prior to each experiment. The protease inhibitor TPCK was purchased from Sigma. The peptide derivatives Ac-Asp-Glu-Val-Asp-CHO (Ac-DECD-CHO) and Ac-Tyr-Val-Ala-Asp-CHO (Ac-YVAD-CHO) and the fluorogenic peptide derivatives Ac-Asp-Glu-Val-Asp-amino-4-methylcoumarin (Ac-DEVD-AMC) and 4-(4-dimethylaminophenylazo)benzoyl-Tyr-Val-Ala-Asp-Ala-Pro-Val-5-((2-aminoethyl)amino)-naphthalene-1-sulfonic acid (DABCYL-YVADAPV-EDANS) were purchased from Bachem Bioscience Inc. (King of Prussia, Pa.). All other chemicals were of reagent grade and purchased from Sigma, ICN, or Boehringer-Mannheim, or from local sources.

Cell culture, DNA labeling, and drug treatment

The human Namalwa and U-937 cell lines obtained from the American Type Culture Collection (Rockville, Md.) were grown in suspension culture at 37°C in the presence of 5% CO_2 in RPMI-1640 medium supplemented with 10% heat-inactivated fetal bovine serum, 2 mM glutamine, 100 U/mL penicillin, and 100 μg/mL streptomycin. Cell culture products were purchased from Gibco-BRL Life Technologies (Grand Island, N.Y.). For DNA labeling, cells were grown with [^{14}C]thymidine (0.02 μCi/mL) for 24 h and then chased in isotope-free medium overnight prior to drug treatment.

CrmA transfection and expression

The expression vector pCDNA3 containing the coding sequences of CrmA was generously provided by V.M. Dixit and M. Tewari from the Department of Pathology, University of Michigan Medical School (Ann Arbor, Mich.). Purified vector was transfected in Namalwa cells by electroporation at 0.27 kV (Gene Pulser, BioRad Laboratories, Hercules, Calif.). Transfected cells were grown under neomycin selection at 250 μg/mL to obtain a stable variant line prior to the experiments. CrmA expression was monitored by immunodetection using polyclonal anti-CrmA antibodies provided by J. Yuan, Cardiovascular Research Center, Massachusetts General Hospital-East (Charlestown, Mass.) (data not shown).

DNA filter elution assay

The DNA filter elution assay was designed to monitor and quantitate DNA fragmentation associated with apoptosis (Bertrand et al. 1991, 1993, 1995; Bertrand and Pommier 1995). Each sample (approximately 0.5×10^6 [^{14}C]thymidine-labeled cells) was loaded onto a protein-adsorbing filter (vinyl–acrylic copolymer filters, Metricel membrane, 0.8-μm pore size, 25 mm diameter, Gelman Sciences Inc., Ann Arbor, Mich.) mounted in a Millipore filter holder connected to a 50-mL syringe (see Fig. 1 in Bertrand et al. 1995; Bertrand and Pommier 1995). Cells were then washed with 5 mL of phosphate-buffered saline (PBS). As soon as the washing solution had dripped through, lysis was performed with 5 mL of lysis solution containing 0.2% sodium sarkosyl, 2 M NaCl, and 0.04 M ethylenediaminetetraacetic acid (EDTA), pH 10.0. After the lysis solution had dripped through by gravity, the filter was washed with 5 mL of 0.02 M EDTA (pH 10.0). The filter was then processed as described (Bertrand et al. 1991, 1993, 1995; Bertrand and Pommier 1995). Radioactivity was counted by liquid scintillation spectrometry in each fraction (loading wash, lysis, EDTA wash, filter). DNA fragmentation was determined as the fraction of DNA in the loading wash fraction + lysis fraction + EDTA wash fraction relative to total DNA (loading wash + lysis + EDTA wash + filter). Results are expressed as the percentage of DNA fragmented in treated cells compared with DNA fragmented in control untreated cells (background) using the following formula:

$$((F - F_0)(1 - F_0)) \times 100$$

where F and F_0 represent DNA fragmentation in treated and control cells, respectively.

Analysis of DNA fragmentation by agarose gel electrophoresis

To visualize the oligonucleosome-sized DNA fragments, at specified times after drug treatment, cellular DNA was extracted by a salting-out procedure as described previously (Miller et al. 1988). Electrophoresis was performed in 1.6% agarose gel in Tris-borate buffer (pH 8.0) at 2.5 V/cm for 15 h. Following electrophoresis, DNA was visualized by ethidium bromide staining. High molecular weight DNA fragments were analysed by transverse alternating pulsed field electrophoresis (TAPFE) in a Beckman GeneLine apparatus (Beckman Instruments, Inc., Palo Alto, Calif.). Briefly, agarose blocks containing untreated and CPT-treated cells were prepared at specified times following drug treatment by standard methodology. Plugs were incubated for 24 h at 42°C in a solution containing 1.0 mg/mL proteinase K or 100 μg/mL pronase, 1% *N*-laurylsarcosine, 0.2% sodium deoxycholate, and 100 mM EDTA (pH 8.0). The agarose-embedded DNA was equilibrated in TE buffer, pH 8.0 (20 mM Tris-HCl, 50 mM EDTA), prior to electrophoresis on a 1.2% agarose gel. Gels were subjected to a 30-min run at 170 V with a pulse time of 4 s, followed by a 24-h run at 150 V with a pulse time of 60 s. Electrophoresis was performed at 18°C in TAFE buffer according to the manufacturer's instruction manual. Following electrophoresis, DNA was visualized by ethidium bromide staining.

Cell-free system

Cytosolic extracts and isolated nuclei were prepared by washing control and treated cells twice by centrifugation–resuspension in 10 mL ice-cold PBS (without Ca^{2+} and Mg^{2+}) and incubating cells on ice for 10 min at a density of 1.0×10^7 cells/mL in a lysis buffer containing 10 mM Hepes (pH 7.4), 20 mM NaCl, 80 mM KCl, 5 mM $MgCl_2$, 5 mM EGTA, 1 mM dithiothreitol, 1.0 mM phenylmethylsulfonyl fluoride, 0.15 U/mL aprotinin, 10% glycerol, and 0.1% NP-40. Samples were then centrifuged ($2000 \times g$ for 10 min at 4°C) and supernatants collected as cytoplasmic fractions. Pellets (nuclei) were then washed twice by centrifugation–resuspension in the lysis buffer without detergent. Cytoplasmic extracts from untreated or treated cells were then incubated with isolated nuclei from labeled untreated cells at 30°C for 30 min in the absence or presence of putative inhibitors and DNA fragmentation was then measured by DNA filter elution assays as described above (Bertrand et al. 1991, 1993, 1995; Bertrand and Pommier 1995).

Caspase activity determination

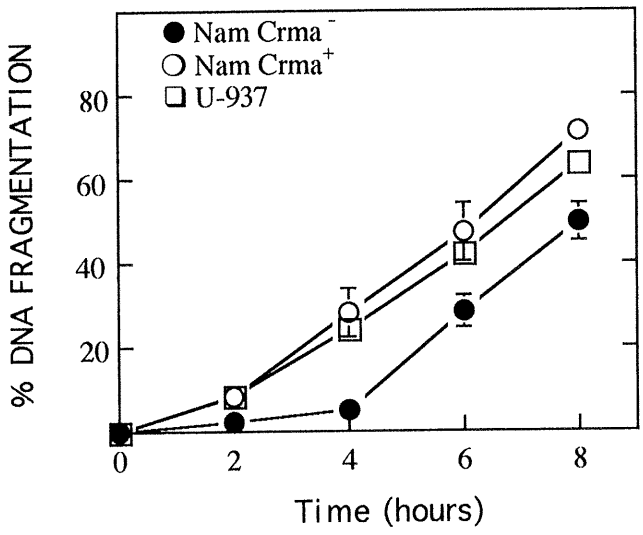
Cytosolic extracts were prepared by washing control and treated cells twice by centrifugation–resuspension in 10 mL ice-cold PBS (without Ca^{2+} and Mg^{2+}) and incubating on ice for 10 min at a density of 1.0×10^7 cells/mL in a lysis buffer containing 100 mM Hepes (pH 7.4), 20% glycerol, 5 mM EGTA, 5 mM dithiothreitol, and 0.1% NP-40. Samples were then centrifuged ($2000 \times g$ for 10 min at 4°C) and supernatants collected as cytosolic extracts. Cascape activities were measured by monitoring fluorescence continuously in a dual luminescence fluorometer (LS 50B Perkin-Elmer) using an excitation wavelength of 360 nm and an emission wavelength of 490 nm for the substrate DABCYL-YVADAPV-EDANS and an excitation wavelength of 380 nm and an emission wavelength of 460 nm for Ac-DEVD-AMC. Reactions were carried out in cuvettes and the temperature was maintained at 37°C using a water-jacketed sample compartment. The assay mixture contained 100 mM Hepes (pH 7.4), 20% (v/v) glycerol, 5 mM dithiothreitol, 5 mM EDTA, and 200 μM fluorogenic peptides. Enzyme activities were determined as initial velocities and expressed as relative intensity per minute for each milligram.

Results

Effect of CrmA on camptothecin-induced oligonucleosome-sized and high molecular weight DNA fragmentation

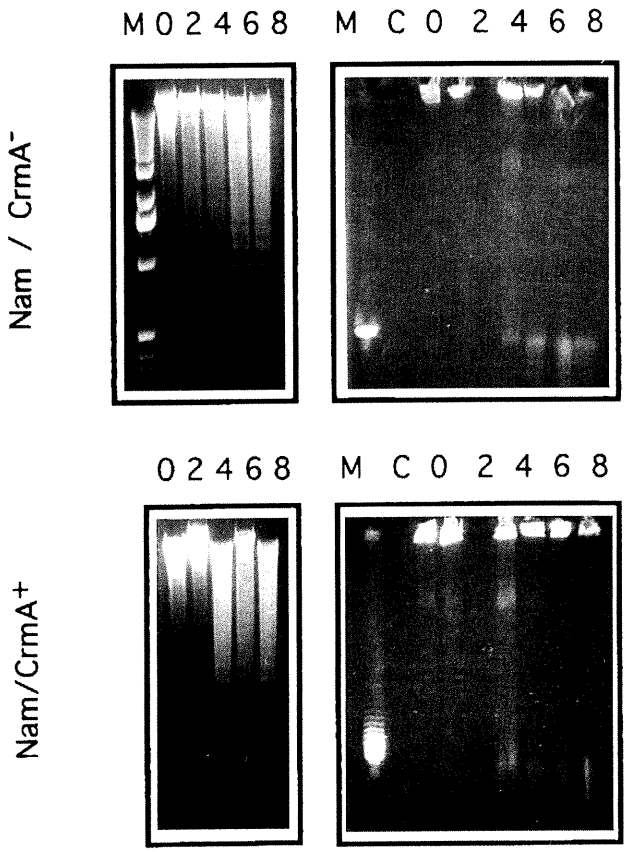
To test whether CrmA expression would modulate camptothecin-induced apoptosis in human cancer cells, DNA fragmentation

Fig. 1. Kinetics of DNA fragmentation induced by CPT in control and CrmA-expressing Namalwa and U-937 cells. [^{14}C]Thymidine-labeled cells were treated for 30 min with 1.0 μM CPT. After drug removal, cells were incubated in drug-free medium and at the indicated times, DNA fragmentation was determined by DNA filter elution assays. Results are expressed as the percent DNA fragmentation of treated cells relative to untreated cells. Values are given as the mean ± SE of four independent experiments performed in duplicate. ●, Nam/CrmA- cells; ○, Nam/CrmA + cells; □, U-937 cells.



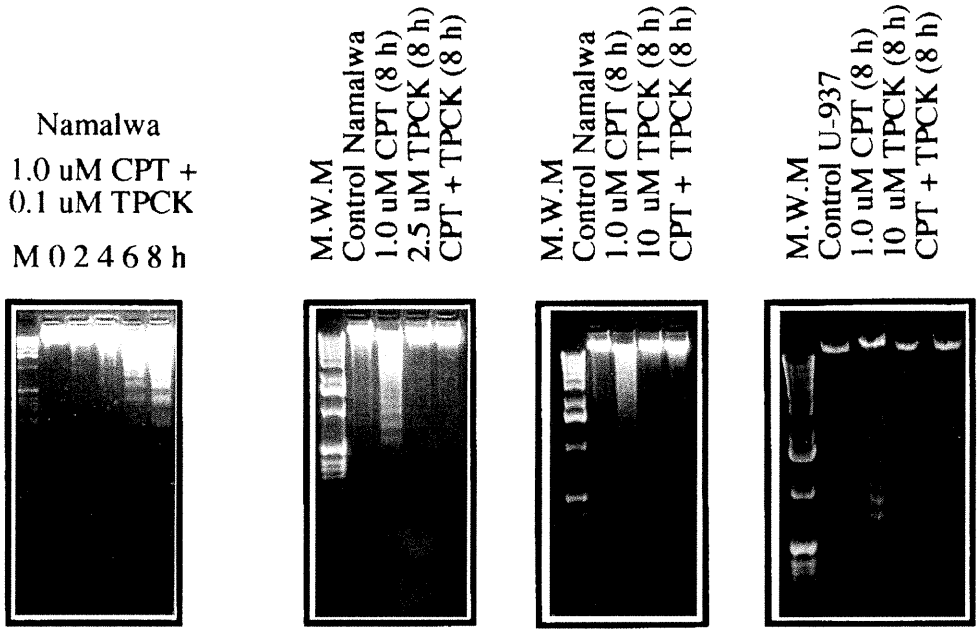
was first measured in control and transfected Namalwa cells expressing CrmA and in U-937 cells using a DNA filter elution assay. To monitor and quantitate the kinetics of DNA fragmentation in cells undergoing apoptosis, we used the DNA filter elution assay that we routinely perform to monitor DNA fragmentation associated with apoptosis (Bertrand et al. 1991, 1993, 1995; Bertrand and Pommier 1995). CrmA expression in Namalwa cells only partially modulated the extent of DNA fragmentation following CPT treatment, a DNA topoisomerase I inhibitor (Fig. 1). Short treatments (30 min) with CPT at 1.0 μM induced apoptosis in Namalwa and U-937 cells with detectable DNA fragmentation as early as 2 h after drug treatment. In contrast, no DNA fragmentation could be detected 2 h after CPT treatment in the transfected Namalwa cells. In these cells, DNA fragmentation began only 4 h after CPT treatment and then increased linearly with time. These results indicate that CrmA expression partially delays the appearance of low molecular weight DNA fragmentation following CPT treatment. To further characterize the effect of CrmA on DNA fragmentation, the oligonucleosome-sized DNA fragments and high molecular weight DNA fragmentation were monitored and visualized by standard agarose gel electrophoresis and TAPFE, respectively (Fig. 2). Control and transfected Namalwa cells were treated with CPT for 30 min (1.0 μM), and at selected times following drug treatment, total DNA was extracted for agarose gel electrophoresis and agarose-embedded DNA was prepared for TAPFE. The oligonucleosome-sized DNA fragmentation was visualized following agarose gel electrophoresis and appeared to be retarded in Namalwa cells expressing CrmA. In contrast, no difference in the kinetics of high molecular weight DNA fragmentation was observed by

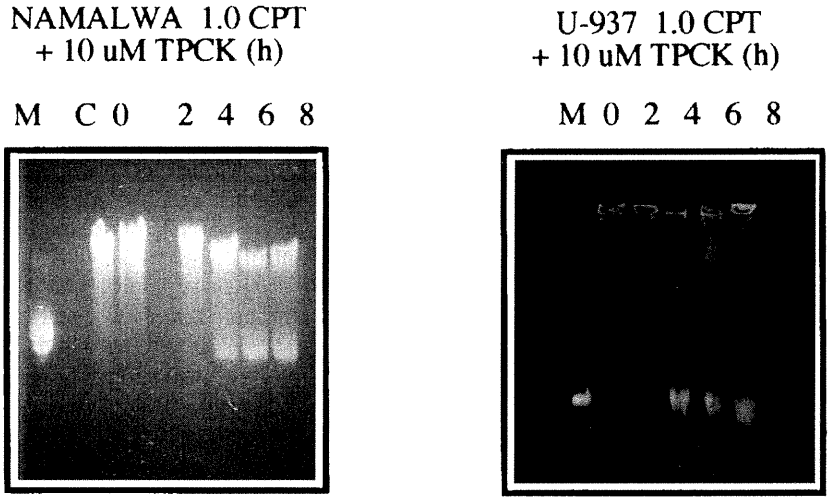
Fig. 2. Oligonucleosome-sized and high molecular weight DNA fragmentation induced by CPT in Namalwa cells. At indicated times (number above each lane) after CPT treatment (1 μM; 30 min), total DNA was extracted from Namalwa (upper panels) and Namalwa–CrmA$^+$ cells (lower panels), DNA fragments were separated by agarose gel electrophoresis (left panels), and high molecular weight DNA fragments were analysed by TAPFE in a Beckman GeneLine apparatus (right panels). Following electrophoresis, DNA was visualized by ethidium bromide staining. Molecular weight markers (M) are 1-kbp DNA markers (Gibco-BRL) and 50-kbp concatemers of lambda DNA (Sigma). C, control untreated cells.



TAPFE between the two lines. The high molecular weight DNA fragments were primarily around 45–50 kilo base pairs (kbp) in both cell lines, although higher molecular weight DNA fragments (>600–700 kbp) were seen in early time points following CPT treatment in both cell lines. The transient appearance of DNA bands >600 kbp was reported previously to coincide with DNA–protein complex formation induced by DNA topoisomerase inhibitors and was reversible after drug removal (Beere et al. 1995, 1996; Rusnak et al. 1996). These high molecular weight DNA fragments of >600 kbp were not involved as an initial phase of DNA fragmentation occurring during apoptosis but appeared to be specific damage emerging from DNA topoisomerase inhibitors that would provide signals for the initiation of apoptosis (Beere et al. 1995, 1996; Rusnak et al. 1996). These observations imply that CrmA had no effect on the initial DNA damage induced by DNA topoisomerase in these cells. Furthermore, CrmA did not inhibit the enzymatic pathway leading to the 45- to 50-kbp

Fig. 3. Effect of TPCK on oligonucleosome-sized and high molecular weight DNA fragmentation induced by CPT in Namalwa and U-937 cells. At indicated times after CPT treatment (1 μM; 30 min) in the presence or absence of various concentrations of TPCK (as indicated), total DNA was extracted from Namalwa and U-937 cells and DNA fragments were visualized by ethidium bromide staining following standard agarose gel electrophoresis (upper panels) and TAPFE in a Beckman GeneLine apparatus (lower panels). Molecular weight markers (M.W.M or M) are 1-kbp markers obtained from Gibco-BRL and 50-kbp concatemers of lambda DNA from Sigma. C, control untreated cells.



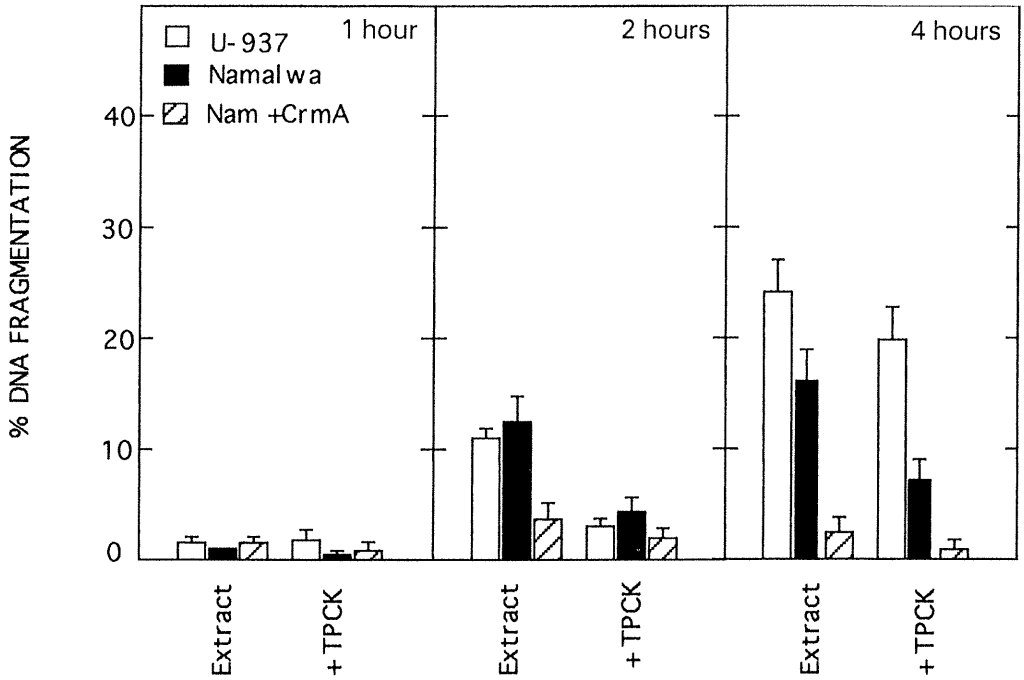


DNA fragments. Instead our results suggest that CrmA partially inhibits unknown activities that are involved in pathways triggering the oligonucleosome-sized DNA fragmentation following CPT treatment.

Effect of TPCK on camptothecin-induced oligonucleosome-sized and high molecular weight DNA fragmentation

Inhibition of oligonucleosome-sized DNA fragmentation using serine protease inhibitors has been reported previously (Bruno et al. 1992; Dubrez et al. 1996; Ghibelli et al. 1995; Hara et al. 1996; Shimizu and Pommier 1996; Solary et al. 1996; Weaver et al. 1993; Yoshida et al. 1996). To analyze and further compare the effect of TPCK in these cells with that of CrmA, Namalwa cells were treated with CPT for 30 min (1.0 μM) in the presence of various concentrations of TPCK, and at selected times following drug treatment total DNA was extracted or agarose-embedded DNA was prepared for electrophoresis (Fig. 3). At low concentrations (0.1 μM), TPCK delayed for 4 h the appearance of oligonucleosome-sized DNA fragmentation induced by camptothecin in Namalwa cells. At higher concentrations (2.5 and 10 μM), TPCK completely

Fig. 4. Detection of TPCK-sensitive activities in a cell-free system. Cytosolic extracts were prepared from U-937 (open bars), Namalwa (solid bars), and CrmA-expressing Namalwa cells (hatched bars) at the indicated times following CPT treatment (1.0 μM for 30 min). [^{14}C]Thymidine-labeled isolated nuclei from untreated cells were then incubated for 30 min with cytosolic extracts alone or in the presence of TPCK (0.1 mM). DNA fragmentation was determined by filter elution assays. Results are expressed as percent DNA fragmentation relative to untreated cells. Error bars represent the SE of four independent experiments performed in duplicate.



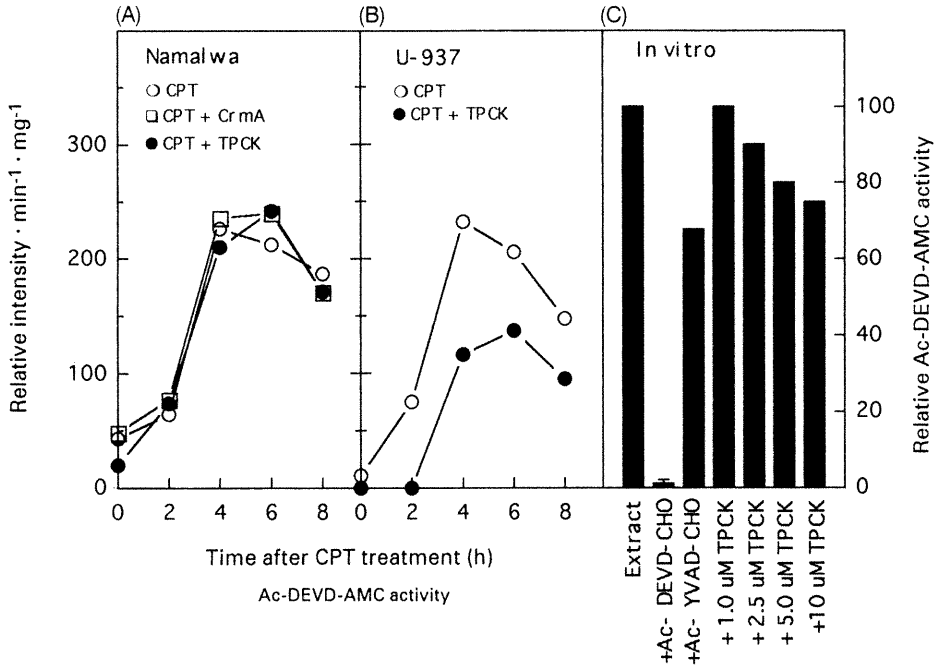
abrogated the oligonucleosome-sized DNA fragmentation for at least 8 h in Namalwa cells. Similarly, 10 μM TPCK completely blocked the oligonucleosome-sized DNA fragmentation induced by camptothecin in U-937 cells. However, even at the highest concentration (10 μM), TPCK had no inhibitory effect on the occurrence of high molecular weight DNA fragmentation in the Namalwa and U-937 cells (Fig. 3; lower panels). The effects of TPCK in these lines were similar to those observed in Namalwa cells expressing CrmA following CPT treatment. Specifically, at low concentrations TPCK did not completely abrogate oligonucleosome-sized DNA fragmentation but significantly delayed its appearance, as did CrmA. These results suggest that CrmA inhibits a TPCK-sensitive pathway involved in oligonucleosome-sized DNA fragmentation. CrmA is a cross-class inhibitor; the potency of CrmA to inhibit putative serine proteases has been described at least with granzyme B (Quan et al. 1995) as well as its inhibitory effect on cysteine proteases such as caspases 1 and 8 (Zhou et al. 1997).

To further investigate the effect of CrmA and TPCK on CPT-induced oligonucleosome-sized DNA fragmentation, we next used a cell-free system that we developed previously. This cell-free system was designed to elucidate the biochemical pathways of apoptosis and has been proven to be convenient for measuring and detecting protease activities in cytosolic extracts prepared from cells undergoing apoptosis. These activated extracts trigger oligonucleosome-sized DNA fragmentation when incubated with isolated nuclei obtained from control untreated cells (Bertrand et al. 1994, 1995; Shimizu and Pommier 1996; Solary et al. 1993). The presence of active TPCK-sensitive proteases involved in DNA fragmentation following DNA topoisomerase inhibitor treatment was recently revealed using this assay (Dubrez et al. 1996; Shimizu and Pommier 1996; Solary et al. 1996).

Cytosolic extracts prepared 1 h after drug treatment showed no activity in the cell-free system, consistent with the lack of detectable DNA fragmentation induced by CPT in cultured cells. However, the cytosolic extracts of CPT-treated Namalwa and U-937 cells prepared 2 and 4 h after drug treatment were able to trigger DNA fragmentation when incubated with isolated control nuclei in the cell-free system (Fig. 4). Activities in those extracts were inhibited predominantly by TPCK. These results indicate that TPCK-sensitive proteases are activated at an early phase of apoptosis following CPT treatment, consistent with other reported observations (Ghibelli et al. 1995; Hara et al. 1996; Shimizu and Pommier 1996; Solary et al. 1996; Yoshida et al. 1996). Early activation of TPCK-sensitive activity in the cell-free system also correlates with the appearance of oligonucleosome-sized DNA fragmentation in cultured cells.

In contrast, cytosolic extracts prepared after 1, 2, and 4 h from CPT-treated Namawa cells expressing CrmA showed a very low level of activity that promotes DNA fragmentation in the cell-free system (Fig. 4). These results are consistent with the protective effect conferred by CrmA on low molecular weight DNA fragmentation in cultured CPT-treated Namalwa–CrmA cells. These results also suggest that CrmA potently inhibits an activity that would otherwise promote DNA fragmentation. In the active 6-h extracts from Namalwa–CrmA cells, activity that was also predominantly inhibited by TPCK was detected (data not shown). Using a similar cell-free system, Lazebnik and colleagues previously showed that

Fig. 5. Caspase-3-like activities in CPT-treated cells. At the indicated times following 30 min treatment with CPT (1.0 μM), cytosolic extracts were prepared from Namalwa (A) and U-937 cells (B) in the absence (○) or presence of 10 μM TPCK (●) and from CrmA-expressing Namalwa cells (□). Caspase activities were monitored continuously at 37°C by detecting fluorescence emission in the presence of Ac-DEVD-AMC at an excitation wavelength of 380 nm and emission wavelength of 460 nm. Enzyme activities were measured as initial velocities and expressed as relative intensity per minute for each milligram. Points represent the means of two independent determinations for Namalwa cells and of a single experiment for U-937 cells. In C, various concentrations of TPCK were added directly to active extracts obtained 4 h after CPT treatment (Namalwa cells) and caspase 3 activities were measured as initial velocities. Results are expressed relative to control activity.



poly(ADP-ribose) polymerase (PARP) and lamin A and B cleavage were not inhibited in the presence of TPCK in their cell-free system extracts (Lazebnik et al. 1995). Our results, together with those of Lazebnik et al. (1995), indicate that the TPCK-sensitive activities detected in cell-free systems are not related to the activities of caspases 3 and 6, which are the caspases involved in PARP and lamin cleavage.

Effect of CrmA and TPCK on caspase activation following CPT treatment

A series of cysteine proteases or caspases are involved in the apoptotic process (Alnemri et al. 1996; Kumar and Harvey 1995; Patel et al. 1996). Their activities are likely associated with the process of cell death itself. In the initial phase of apoptosis triggered by FASR and TNFR1, studies have shown sequential activation of caspases (Enari et al. 1996). To determine the effect of CrmA and TPCK on caspase activities in CPT-treated cells, we monitored caspase-1-like and caspase-3-like activities using the specific fluorogenic peptide substrates DABCYL-YVADAPV-EDANS and Ac-DEVD-AMC, respectively (Fig. 5).

In cytosolic extracts of CPT-treated cells, caspase-3-like activity increased significantly after drug treatment, with peak activity around 4 h after CPT treatment. No significant difference in caspase-3-like activation was observed between Namalwa cells, transfected Namalwa cells with CrmA, and TPCK-treated Namalwa cells following CPT treatment (Fig. 5A). No hydrolysis of DABCYL-YVADAPV-EDANS was detected in these cells following CPT treatment (data not shown). These results indicated that caspase 1 is neither activated nor involved in the apoptotic process induced by CPT and that CrmA and TPCK had no significant effect on the processing and activity of caspase 3 in these cells. Similar results were obtained in U-937 cells (Fig. 5B). To validate these observations, the effect of TPCK on caspase-3-like activity was monitored in vitro. Various concentrations of TPCK were directly added to active extracts and initial velocities of caspase-3-like activities were measured. As shown in Fig. 5C, TPCK only slightly inhibited caspase-3-like activity in vitro whereas the tetrapeptide derivative Ac-DEVD-CHO completely inhibited these activities.

Discussion

The mechanisms by which particular agents trigger the apoptotic response of tumor cells have become a subject of intense study. Although apoptotic cells undergo similar cellular and nuclear changes following physiological and nonphysiological induction, it is clear now that the mechanisms by which these

changes occur vary depending on the stimuli that trigger apoptosis. The antitumor activity and primary mechanism of action of DNA topoisomerase inhibitors have been the focus of a large number of reports, including some reviews (Liu 1989; Pommier and Kohn 1989; Pommier et al. 1994; Pommier and Tanizawa 1993). Antitumor drugs that interfere with mammalian topoisomerase 1 or 2 do so by stabilizing a transient intermediate of the topoisomerase reactions. These drugs trap covalently linked topoisomerase–DNA complexes associated with single- or double-stranded DNA breaks (Liu 1989; Pommier and Kohn 1989; Pommier et al. 1994; Pommier and Tanizawa 1993). Drug-induced stabilization of topoisomerase-linked DNA strand breaks has been recognized as the primary mechanism of action of topoisomerase inhibitors. Formation of cleavable complexes appears to be responsible for the antitumor activity rather than topoisomerase inhibition by itself. Studies using resistant cell lines also supported the concept that formation of cleavable complexes is essential for cell killing (Liu 1989; Pommier and Kohn 1989; Pommier et al. 1994; Pommier and Tanizawa 1993). However, the rapidly reversible nature of these lesions after drug removal strongly suggests that drug trapping of topoisomerase–DNA complexes may be necessary but not sufficient for cell killing and that cell death results from further irreversible DNA lesions and from the induction of intracellular signals or responses leading to cell death. DNA topoisomerase 1 and 2 inhibitors are able in various cell lines to trigger cell death by rapidly inducing the cellular morphological changes, high molecular weight DNA fragmentation, and oligonucleosome-sized secondary DNA fragmentation typical of apoptosis (Barry et al. 1990; Bertrand et al. 1991, 1993; Del Bino and Darzynkiewicz 1991; Jaxel et al. 1988; Kaufmann 1989; Solary et al. 1993; Walker et al. 1991). The mechanisms involved in low and high molecular DNA fragmentation are unclear, but several observations including ours indicate that the high molecular weight and extensive oligonucleosome-sized DNA fragmentation are triggered by two independent mechanisms in cells treated with camptothecin, a DNA topoisomerase 1 inhibitor.

Several biochemical events that contribute to apoptotic cell death have recently been elucidated in mammalian cells. The triggering of apoptosis by various stimuli involves cytosolic proteolytic cascades. An emerging family of caspases or Ced-3–Ice-like cysteine proteases has been identified and were proposed to be a pivotal trigger of apoptosis; several studies have revealed the importance of the caspases in executing the process of cell death itself (Alnemri et al. 1996; Kumar and Harvey 1995; Patel et al. 1996).

Active serine proteases were proposed also to play an essential role in apoptosis induced by DNA topoisomerase inhibitors (Bruno et al. 1992; Ghibelli et al. 1995; Hara et al. 1996; Shimizu et al. 1996; Solary et al. 1996; Weaver et al. 1993; Yoshida et al. 1996). Moreover, a recent report indicated that in the hepatocellular carcinoma Hep 3B cells, apoptosis is induced by CPT without activation of the proteolytic caspase cascade involved in PARP and lamin B cleavage and that a trypsin-like protease activity appears to activate apoptosis in these cells (Adjei et al. 1996). Another serine protease, the well-known granzyme B, is able to activate apoptosis via a proteolytic cascade in the instance of apoptosis mediated by cytotoxic T lymphocytes in targeted cells (Berke 1995; Darmon et al. 1995, 1996; Fernandez-Alnemri et al. 1996; Greenberg 1996).

The finding that CrmA and TPCK prevent oligonucleosome-sized DNA fragmentation in human cancer cell lines following CPT treatment without interfering with caspase activation and the occurrence of high molecular weight DNA fragmentation indicate that CrmA- and TPCK-sensitive pathways are involved in CPT-induced oligonucleosome-sized DNA fragmentation. Our data also suggest that proteolytic caspase pathways may be associated with pathways triggering high molecular weight fragmentation whereas other pathways may be associated with the occurrence of oligonucleosome-sized DNA fragmentation specifically after CPT treatment. Although it is well known that CrmA is a potent inhibitor of caspase 1 and 8, it has been shown recently that CrmA is unlikely to inhibit caspases 3 and 6 in vivo (Zhou et al. 1997), which are the main caspases involved with apoptosis induced by DNA topoisomerase inhibitors (Dubrez et al. 1996; Martins et al. 1997; Sané et al.[4]; Schmitt et al., see footnotes 2 and 3). Although the importance of the role of serine proteases in apoptosis has been supported by several observations, only a few studies have identified putative enzymes. A 24-kDa serine protease has been identified and purified from apoptotic U937 cells (Wright et al. 1994) and others have partially purified from Jurkat T cells a serine protease activity in FAS-induced apoptosis (Schlegel et al. 1995). More recently, Liu et al. (1997) identified from HeLa cells a caspase-activated DNA fragmentation factor that induces DNA fragmentation when coincubated with nuclei. This novel heterodimeric protein functions downstream of caspase 3 to trigger DNA fragmentation and shows no DNase activity on naked DNA. Therefore, it is likely that DNA fragmentation factor plays a role in a more complex pathway that triggers DNA fragmentation between caspase 3 activation and putative endonucleases. However, the mechanisms by which DNA fragmentation factor acts to trigger nuclear degradation and whether CrmA and TPCK interfere with DNA fragmentation factor activity are still unknown.

In summary, our results, with those of others, suggest that CrmA and TPCK may partially inhibit uncharacterized but sensitive activities that are involved in pathways triggering the oligonucleosome-sized DNA fragmentation following CPT treatment.

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[4] A.T. Sané, A. Steyaert, D. Meyer, and R. Bertrand. Differential effect of CrmA upon CD95/Fas-, TNF-, and DNA topoisomerase inhibitor-induced apoptosis. Submitted for publication.

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ANNEXE II

The Bcl-xL and Bax-α control points: modulation of apoptosis induced by cancer chemotherapy and relation to TPCK-sensitive protease

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(Biochem Cell Biol. 75, 301-314, 1997)

REVIEW / SYNTHÈSE

The Bcl-xL and Bax-α control points: modulation of apoptosis induced by cancer chemotherapy and relation to TPCK-sensitive protease and caspase activation

E. Schmitt, A.T. Sané, A. Steyaert, G. Cimoli, and R. Bertrand

Abstract: Defective control of apoptosis appears to play a central role in the pathogenesis of human diseases including neoplasic, autoimmune, and neurodegenerative diseases. Conversely, cancer chemotherapy and ionizing radiation can induce cancer cell death by apoptosis, and deregulated apoptosis following cancer chemotherapy could define a new category of drug resistance mechanism. By understanding the role that some major regulators of apoptosis play either at the commitment or execution phases of cell death in a given tissue and pathology, we will be in a better position to design and explore new therapeutic modalities. The Ced-9 – Bcl-like and Ced-3 – Ice-like gene family products are intrinsic proteins regulating the decision of a cell to survive or die and executing part of the cell death process itself, respectively. Among the various Bcl-like proteins, the effects and functions of the Bcl-x and Bax proteins in controlling apoptosis induced by cancer chemotherapy have been studied recently. In human cancer variant cell lines showing differential expression of the Bcl-xL protein, a preventive effect of Bcl-xL on cell death induced by various cytotoxic drugs is observed, with greater effects in cells containing the highest level of Bcl-xL expression. Similarly, overexpression of Bax-α in cancer cell lines sensitizes these cells to some cancer chemotherapy compounds. Modulation of apoptosis either negatively by Bcl-xL or positively by Bax-α resides downstream of the primary mechanism of action of anticancer drugs, suggesting that they act primarily as intrinsic control points following cytotoxic drug injuries. An emerging family of Ced-3 – Ice like cysteine proteases (caspases) has been also identified and several studies have revealed their importance in executing the process of cell death. More recently, activation of a *N*-tosyl-L-phenylalanylchloromethyl ketone (TPCK)-sensitive pathway was also suggested to play an important role in apoptosis induction following cancer chemotherapy. Evidence obtained using a combination of assays including cell-free systems and enzyme activity assays now suggests that Bcl-xL and Bax-α control points function upstream of TPCK-sensitive protease and caspase activation. Bcl-xL delays and prevents activation of apoptotic protease cascades whereas Bax-α shows the opposite effect, accelerating their activation.

Key words: Bcl-xL, Bax-α, serine protease, caspase, DNA topoisomerase inhibitors, apoptosis.

Résumé : Une régulation défectueuse de l'apoptose semble jouer un rôle central dans le développement de cancers et de maladies auto-immunes et neurodégénératives. Les agents anticancéreux utilisés en chimiothérapie ainsi que les radiations ionisantes induisent la mort des cellules tumorales par apoptose et une dérégulation des mécanismes d'activation de l'apoptose serait une nouvelle forme de résistance aux drogues. Une meilleure compréhension du rôle joué par certains régulateurs majeurs intervenant dans la phase d'engagement ou la phase d'exécution de l'apoptose pourrait permettre le développement de nouvelles stratégies thérapeutiques. La famille de gènes semblables à Ced-9 – Bcl et Ced-3 – Ice codent des protéines intracellulaires intervenant dans la phase d'engagement de l'apoptose ou dans l'exécution du processus de mort cellulaire lui-même, respectivement. Des travaux récents ont été réalisés afin de comprendre les effets et les fonctions des

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Abbreviations: CPT, camptothecin; TPCK, *N*-tosyl-L-phenylalanylchloromethyl ketone.

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protéines Bcl-x et Bax dans la régulation de l'apoptose induite par la chimiothérapie. Dans les cellules tumorales humaines exprimant différents taux de protéines Bcl-xL, l'effet protecteur de Bcl-xL contre l'apoptose induite par certains agents cytotoxiques est d'autant plus important que son taux d'expression est élevé. De la même manière, une surexpression de Bax-α sensibilise les cellules cancéreuses à certaines drogues utilisées en chimiothérapie. La modulation négative de l'apoptose par Bcl-xL ou la modulation positive par Bax-α est indépendante du mécanisme d'action primaire des agents anticancéreux suggérant que ces protéines jouent un rôle au niveau des signaux intracellulaires qui émergent d'une lésion cytotoxique. Des cystéine-protéases (caspases) de la famille des protéases semblables à Ced-3 – Ice ont aussi été identifiées et plusieurs études ont montré leur implication dans l'exécution du processus de mort cellulaire. Il semblerait également, selon des données plus récentes, que l'activation de sérine-protéases joue un rôle important dans l'induction de l'apoptose par des agents anticancéreux. Nos résultats obtenus en utilisant un système acellulaire et en mesurant les activités enzymatiques suggèrent que Bcl-xL et Bax-α interviennent à des points de contrôle en amont de l'activation des protéines effectrices de l'apoptose. Bcl-xL retarde l'activation en cascade des protéases alors que Bax-α a l'effet contraire en accélérant leur activation.

Mots clés : Bcl-xL, Bax-α, sérine-protéase, caspase, inhibiteurs de topo-isomérase, apoptose.

Introduction

Programmed cell death (apoptosis) is a fundamental process essential for normal regulation of cell homeostasis (Ellis et al. 1991; Raff et al. 1994; Thompson 1995; Wyllie 1987, 1992). Apoptosis is well defined by characteristic cellular morphological changes associated with internucleosomal DNA fragmentation, which results from endonuclease activation (Kerr et al. 1972, 1995; Wyllie et al. 1984). Genetic studies in the nematode *Caenorhabditis elegans* have provided major evidence that specific genes can inhibit or activate the apoptotic process. One gene, Ced-9, has been found to repress apoptosis whereas two others, Ced-3 and Ced-4, are essential for apoptosis in *C. elegans*. (Hengartner et al. 1992; Hengartner and Horvitz 1994*a*, 1994*b*; Horvitz et al. 1994; Yuan 1993). Recent studies in human cell lines have uncovered many Ced-9 – Bcl-like and Ced-3 – Ice-like gene products that regulate either negatively or positively the apoptotic threshold of a human cell. The human Ced-9 – Bcl-like family includes a series of related gene products such as Bcl-2 (Hockenbery et al. 1990; Korsmeyer 1992; Reed 1994; Vaux et al. 1988), Bax (Oltvai et al. 1993), Bcl-x (Boise et al. 1993), Mcl-1 (Kozopas et al. 1993), A1 (Lin et al. 1993), Bak (Chittenden et al. 1995; Farrow et al. 1995; Kiefer et al. 1995), Bag (Takayama et al. 1996, 1995), Bfl-1 (Choi et al. 1995), Bik (Boyd et al. 1995), Bcl-w (Gibson et al. 1996), Brag-1 (Das et al. 1996), and Bid (Wang et al. 1996*a*). Bcl-2 has been found translocated (t(14/18)) in human B cell lymphoma and is thought to contribute to the development of B-cell neoplasia (Clarke et al. 1986; Tsujiimoto et al. 1984), while Bcl-xL is often highly expressed in B lymphoma tumor cells (Xerri et al. 1996). Extensive work has been done to understand the function and role of Bcl-2. Overexpression of Bcl-2 is often associated with cell death protection induced by anticancer compounds and ionizing radiation (Hockenbery 1995; Korsmeyer 1995; Reed 1994, 1995*a*, 1995*b*). Although the mechanisms and functions of all of the others have been less studied, recent observations suggested that Bcl-xL (Datta et al. 1995; Dole et al. 1995; Fang et al. 1995; Ibrado et al. 1996) has protective effects against anticancer drugs, whereas Bax-α (Chresta et al. 1996; Krajewski et al. 1995; Sakakura et al. 1996; Thomas et al. 1996; Wagener et al. 1996; Zhan et al. 1994) and Bcl-xS (Ealovega et al. 1996; Sumantran et al. 1995) accelerate cell death induced by anticancer drugs.

An emerging family of Ced-3 – Ice-like cysteine proteases (caspases) has been also identified and cloned on the basis of a conserved peptapeptide sequence (QACXG) that contains the active cysteine residue. The caspases are separated into three subfamilies on the basis of their degrees of homology and by phylogenetic analysis; the ICE-related family includes Ice – caspase-1 itself with its spliced isoforms (Alnemri et al. 1995; Yuan et al. 1993), Tx – Ich-2 – Ice rel-II – caspase-4 (Faucheu et al. 1995; Kamens et al. 1995; Munday et al. 1995), Ty – Ice rel-III – caspase-5 (Faucheu et al. 1996; Munday et al. 1995), and Ich-3 – caspase-11 (Wang et al. 1996*b*); the CPP32-related family includes CPP32 – Yama – Apopain – caspase-3 (Fernandes-Alnemri et al. 1994; Nicholson et al. 1995; Tewari et al. 1995), Mch-2 – caspase-6 (Fernandes-Alnemri et al. 1995*a*), Mch-3 – Ice-Lap3 – Cmh-1 – caspase-7 (Duan et al. 1996*a*; Fernandes-Alnemri et al. 1995*b*; Lippke et al. 1996), Mch-4 – caspase-10 (Fernandes-Alnemri et al. 1996), and Mch-5 – Flice – Mach – caspase-8 (Boldin et al. 1996; Fernandes-Alnemri et al. 1996; Muzio et al. 1996); and the third group includes Ich-1(l and s) – caspase-2 (Wang et al. 1994) and Ice-Lap6 – Mch6 – caspase-9 (Duan et al. 1996*b*). Recent studies have also revealed that serine proteases play an important role in the apoptotic process. Granzyme B, a granule serine protease injected into target cells by cytotoxic lymphocytes, triggers apoptosis in association with perforin proteins (Greenberg and Litchfield 1995; Heusel et al. 1994). AP24, an elastaselike serine protease induced by tumor necrosis factor and ultraviolet light, has been reported to activate apoptosis in U-937 cells (Wright et al. 1994) and two other serine proteases have been partially isolated from cells undergoing apoptosis (Marthinus et al. 1995; Schlegel et al. 1995). Other studies using either cultured cells or cell-free systems have also shown the importance of as-yet-unknown serine proteases in the apoptotic process (Bruno et al. 1992; Ghibelli et al. 1995; Hara et al. 1996; Shimizu and Pommier 1996; Solary et al. 1996; Weaver et al. 1993; Yoshida et al. 1996). More recently, it has been also reported that a dichloro-isocoumarin-sensitive pathway may lead to low molecular weight DNA fragmentation in cells following VP16 (Dubrez et al. 1996) whereas trypsinlike protease activity appeared to activate apoptosis following camptothecin (CPT) treatment in a hepatoma cell line (Adjei et al. 1996).

Chemotherapeutic drugs such as DNA topoisomerase I (top 1) and II (top 2) inhibitors (CPT and etoposide (VP16),

respectively) trigger apoptosis in various cell lines (Barry et al. 1990; Bertrand et al. 1991, 1993; Del Bino and Darzynkiewicz 1991; Jaxel et al. 1988; Kaufmann 1989; Solary et al. 1993; Walker et al. 1991). These drugs are known to stabilize a transient intermediate of topoisomerase reactions where enzymes are linked to the 3′ (top 1) or 5′ (top 2) terminus of a DNA duplex producing DNA single- or double-stranded breaks (Liu 1989; Pommier and Kohn 1989; Pommier et al. 1994; Pommier and Tanizawa 1993). These protein-linked DNA single- or double-stranded breaks are prelethal DNA lesions that inhibit DNA metabolism, such as DNA synthesis (Bertrand et al. 1992; D'Arpa et al. 1990; Holm et al. 1989; Hsiang et al. 1989). Top 2 inhibitors are currently among the most active anticancer drugs and top 1 inhibitors are in clinical trials with very encouraging results. The mechanisms of action of these drugs as well as some of the factors that determine their antitumor activities have been extensively reviewed recently (Gupta et al. 1995; Pommier 1997).

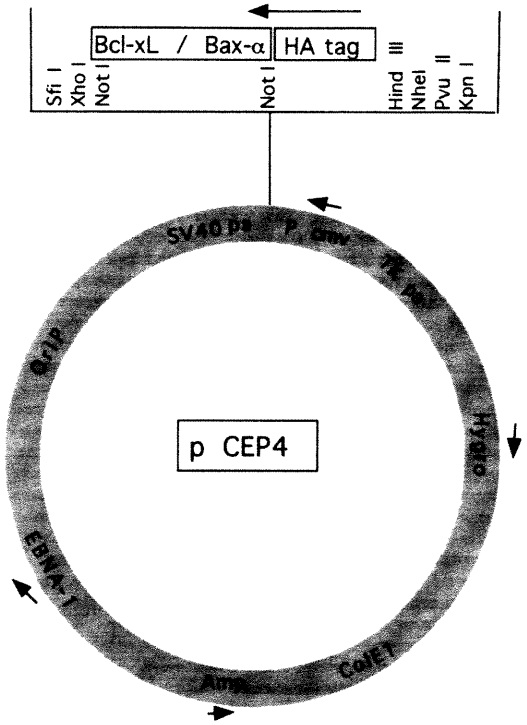
Similarly, all of the anticancer chemotherapeutic drugs used clinically can induce apoptosis in various cell lines (Hickman 1996; Hickman et al. 1994). However, the mechanisms by which these drugs activate programmed cell death are just beginning to be examined. Signals emerging from DNA-damaging agents induce complex cellular responses involving DNA repair and transcription regulation and are influenced by a series of cellular checkpoints and related to specific gene expression and activation. The decision for a given cell population to undergo cell growth or cell death is influenced by a series of extrinsic and intrinsic signals. The Ced-9 – Bcl-like and Ced-3 – Ice-like gene products are intrinsic proteins regulating the decision of a cell to survive or die and executing the process of death itself, respectively. Thus, expression and activation of these genes might determine drug sensitivity and resistance (Korsmeyer 1995; Kumar and Harvey 1995; Patel et al. 1996; Reed 1994, 1995*a*, 1995*b*; Thompson 1995; Wyllie 1992, 1995).

In this review, we will discuss the mechanisms of apoptosis induced by anticancer drugs with emphasis on the effects of Bcl-xL and Bax-α in human cancer cells in relation to TPCK-sensitive protease and caspase activation. Although we will focus on our own work, we will also compare our studies with those of others.

Bcl-xL and Bax-α expression

The human Bcl-x and Bax genes belong to the Ced-9 – Bcl-like family that regulates cell survival and cell death. Alternative splicing of the Bcl-x gene results in at least two distinct Bcl-x mRNAs, Bcl-xL and Bcl-xS (Boise et al. 1993). A third form of spliced mRNAs, named Bcl-xES, has been detected in a series of haematopoietic cell lines; its role and function are under investigation (Schmitt et al. 1995). Several Bax mRNA isoforms have also been isolated and they probably are derived from alternative splicing of a single Bax gene mapped to chromosome 19q13.3-q13.4 in humans (Apte et al. 1995; Oltvai et al. 1993). The first studies with Bcl-x and Bax gene products indicated that Bcl-xL inhibits cell death upon growth factor withdrawal whereas Bax-α and Bcl-xS have opposite effects: they inhibit the protective effect conferred by Bcl-2 (Boise et al. 1993; Oltvai et al. 1993). Immunohistochemical analysis of Bcl-x expression showed that Bcl-x proteins are expressed in a wide variety of cell types, predominantly in a variety of

Fig. 1. Schematic representation of pCEP4-HA-BCL-xL and pCEP4-HA-Bax-α expression vectors. The pCEP4 vector was first modified by adding Kosak and HA epitope tag sequences at the *Hin*dIII –*Not*I restriction sites; Bcl-xL and Bax-α ORF cDNA were then inserted in frame with the HA epitope tag sequences at the unique *Not*I restriction site. Vectors were sequenced prior to transfection.



neuronal, haematopoietic, reproductive, and epithelial cells (Gonzales-Garcia et al. 1995 ; Krajewski et al. 1994*a*). Similar analysis of Bax expression showed that Bax proteins are expressed in a wide variety of cell types, predominantly in a variety of epithelial cells in the thymic medulla, in the germinal center lymphocytes of lymph nodes, and in several populations of reproductive and neuronal cells (Krajewski et al. 1994*b*). The central role of Bcl-x in regulating the apoptotic threshold of haematopoietic cells and central nervous system tissues has been revealed by gene knock-out studies in mice (Motoyama et al. 1995). Bax-deficient mice displayed thymocyte and B cell hyperplasia, atypical ovarian granulosa cells, and immature male germ cells (Knudson et al. 1995). In human tumors, high levels of expression of Bcl-xL were found in several B lymphomas, Reed-Sternberg cells of Hodgkin's disease, and neuroblastoma cells (Dole et al. 1995; Schlaifer et al. 1995; Xerri et al. 1996). Reduced expression of Bax-α was associated more recently with poor response rates to chemotherapy in women with metastatic breast adenocarcinoma (Bargou et al. 1995; Krajewski et al. 1995).

To further define the role and effect of Bcl-xL and Bax-α in cancer chemotherapy, we initiated a series of investigations involving gene transfer. The human Bcl-xL and Bax-α cDNAs were cloned by RT-PCR from polyA(+) RNA using specific

Table 1. DNA fragmentation induced by vinblastine paclitaxel and *cis*-platinum in control and transfected U-937 and Namalwa cells.

	U-937	U-937 bcl-xL$^{1.50}$	Namalwa	Nam-bax$^{1.50}$
Vinblastine				
1.0 μM	22.6±1.7	10.0±2.5	5.5±1.3	25.3±1.3
10 μM	36.5±2.6	17.6±0.8	8.9±1.1	46.9±1.8
25 μM	62.3±2.0	23.2±1.3	15.8±1.6	62.3±2.8
100 μM	84.9±0.8	37.8±6.7	37.3±5.2	72.8±0.8
Paclitaxel				
1.0 μM	12.4±1.8	6.7±1.2	7.0±1.0	10.2±1.4
10 μM	19.1±3.6	11.1±1.0	8.8±1.4	10.3±0.8
25 μM	24.5±1.6	10.9±0.7	15.2±1.3	15.4±2.3
100 μM	35.5±0.8	13.5±3.6	13.6±1.8	21.1±2.8
cis-Platinum				
1.0 μM	9.6±4.5	5.4±0.9	4.2±15.8	8.8±15.8
10 μM	15.6±1.0	4.7±0.8	9.4±10.4	18.9±15.8
25 μM	29.1±1.9	9.5±0.6	15.3±9.2	22.9±15.8
100 μM	61.5±1.8	38.4±2.1	20.5±9.2	20.3±15.8

Note: Cells labeled with [^{14}C]thymidine were treated with various concentrations of vinblastine, paclitaxel, and *cis*-platinum for 8 h and DNA fragmentation was measured by DNA filter elution assays. Values are expressed as the percentage of DNA fragmentation in treated cells relative to control untreated cells and are given as the mean ± SE ($n = 6$). U-937 bcl-xL$^{1.50}$ and Nam-bax$^{1.50}$, U-937 cells transfected with Bcl-xL and Namalwa cells transfected with Bax-α selected at 1.5 mg/mL hygromycin, respectively.

adapter primers and the amplified fragments were subcloned in the eucaryotic expression vector pCEP4 (InVitrogen) that had been modified to include hemagglutinin epitote Tag sequences (HA-tag) and Kosak consensus sequences (Fig. 1). Purified pCEP4-HA-Bcl-xL and pCEP4-HA-Bax-α were transfected in human leukemic monocytelike U-937 and B lymphoma Namalwa cell lines, respectively. Transfected variant cell lines were grown under hygromycin selection at 0.5 and 1.5 mg/mL for 2–3 months to obtain stable variant lines. Western blot analysis indicated that exogenous Bcl-xL and Bax-α proteins were expressed at different levels with respect to the hygromycin concentration used for the selection. The highest level of expression was found in transfected variant cells selected at 1.5 mg/mL hygromycin (Fig. 2*a*). Cytofluorometric assessment of the exogenous proteins using anti-HA rhodamine-conjugated monoclonal antibodies suggested that all cells expressed the exogenous proteins with median fluorescence intensity displaced from 2.55 to 5.05 and from 2.64 to 4.53 in the transfected Bcl-xL and Bax-α lines, respectively (Fig. 2*b*). Exogenous proteins were also detected and visualized by immunohistochemical staining with a monoclonal antibody raised against the HA-epitope tag followed by a biotin – streptavidin – alcaline phosphatase complex. Figure 2*c* shows that all cells expressed the transfected proteins.

Effect of Bcl-xL and Bax-α upon apoptosis induced by DNA topoisomerase I and II inhibitors

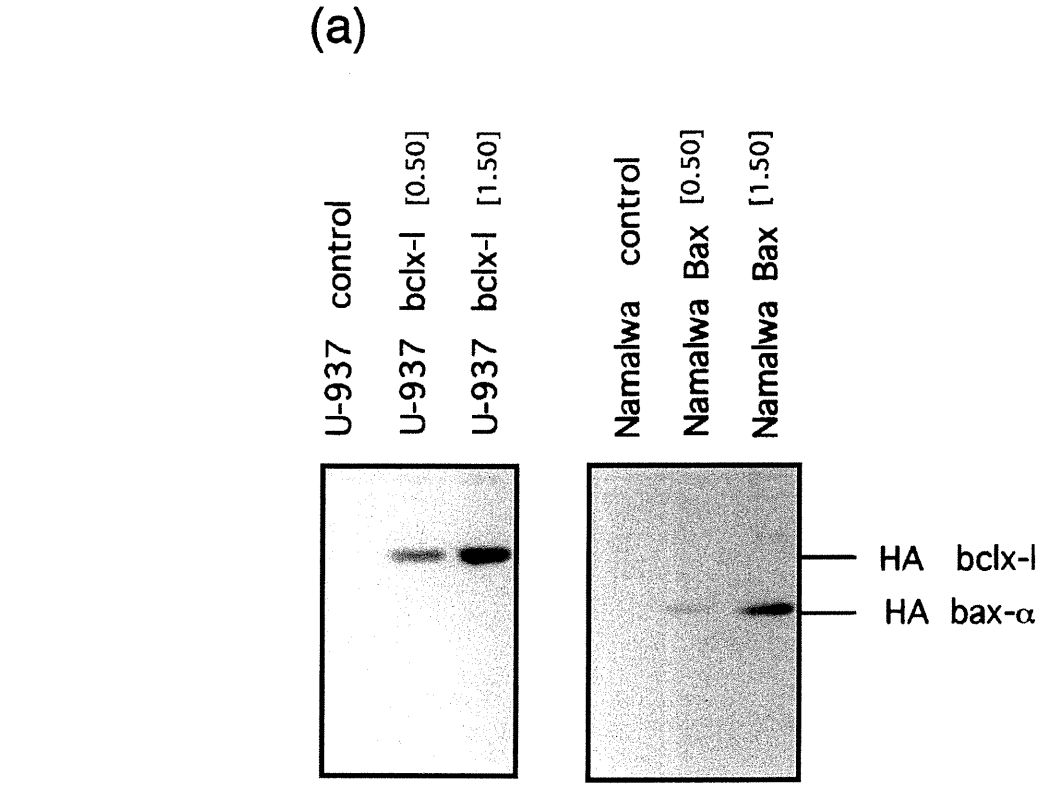
Several studies have shown that CPT, a DNA topoisomerase I inhibitor, and etoposide (VP16), a DNA topoisomerase II inhibitor, activate the morphological changes and internucleosomal DNA fragmentation associated with apoptosis in several cell lines (Barry et al. 1990; Bertrand et al. 1991, 1993; Del Bino and Darzynkiewicz 1991; Kaufmann 1989; Solary et al. 1993; Walker et al. 1991). We routinely used a DNA filter assay to monitor and quantitate kinetics of DNA fragmentation in cells undergoing apoptosis (Bertrand et al. 1991, 1993, 1995; Bertrand and Pommier 1995).

Short treatments (30 min) with CPT at various concentrations induced apoptosis in U-937 cells with more extensive DNA fragmentation in cells treated at the highest CPT concentrations (Fig. 3). The variant transfected U-937 cells selected at 500 μg/mL and 1.5 mg/mL were almost completely protected from CPT-induced DNA fragmentation 8 h after drug treatment (Fig. 3). Twenty-four hours after drug treatment, the degree of protection was nearly complete in the Bcl-xL transfected line selected at 1.5 mg/mL hygromycin, and more than 50% of the cells in the Bcl-xL transfected line selected at 0.5 mg/mL hygromycin were protected compared with those of control treated cells (data not shown (Schmitt et al.[3])). These results indicate a direct relation between the amount of Bcl-xL protein and the degree of protection against CPT-induced DNA fragmentation and apoptosis. In the B lymphoma Namalwa cells, short treatment with CPT (30 min) at moderate concentrations (0.1–0.5 μM) induced apoptosis in control Namalwa cells whereas low concentrations of CPT (such as 0.01 μM) did not trigger apoptosis significantly (Fig. 3). However, in the Bax-α transfected Namalwa cells, both the kinetics and the extent of DNA fragmentation were greater after treatment with a low concentration of CPT (Fig. 3). Moreover, cell-death promoting activity of Bax-α after such treatment was greater in cells expressing the highest level of Bax-α. At 0.01 and 0.05 μM CPT, the cell-death promoting effect of Bax-α in the transfected lines was greater than the effect of CPT in control Namalwa cells. At higher CPT concentrations (0.1–0.5 μM) the difference between the CPT-treated control and Bax-α transfected cell lines seemed to be less significant (Fig. 3). These results indicate a direct relation between the amount of Bax-α and the degree of apoptosis induced by a low concentration of CPT. Thus, higher levels of Bax-α protein sensitize these cells to low drug concentrations.

Similarly, continuous treatments of these cells with VP16, a DNA top II inhibitor, activated apoptosis and DNA fragmentation; the U-937 cells were more sensitive than Namalwa cells (Fig. 4). The extent of protection conferred by Bcl-xL in U-937 cells (Fig. 4) was significant at or below 25 μM. At 100 μM, the protective effect was lost. The cell-death promoting activity of Bax in Namalwa cells increased linearly as a function of drug concentration. While control Namalwa cells did not show high levels of DNA fragmentation when treated at or below 10 μM VP16, the Bax expressing Namalwa cells revealed approximately 60% DNA fragmentation at 10 μM VP16 (Fig. 4). As with CPT, these results suggest that higher levels of Bax-α sensitize these cells to VP16 (Schmitt et al. 1997).

[3] E. Schmitt, A. Steyaert, G. Cimoli, and R. Bertrand. Bcl-xL modulates cancer chemotherapeutic-induced apoptosis and prevents the activation of serine protease and caspase activation. Submitted for publication.

Fig. 2. Expression of Bcl-xL and Bax-α in control and transfected lines. (*a*) Whole-cell extracts were prepared from control and transfected cells selected at 0.50 and 1.5 mg/mL hygromycin. Following SDS-polyacrylamide gel electrophoresis and electrophoretic transfer, HA-Bcl-xL and HA-Bax-α proteins were detected using anti-HA peptide and peroxidase-conjugated monoclonal antibodies. Immuno-complexes were revealed by enhanced chemiluminescence (Amersham) and autoradiography. (*b*) Fluorescence intensity distribution of permeable cells was analyzed in the absence or presence of anti-HA rhodamine-conjugated monoclonal antibodies (10 μg/mL) using Becton Dickinson Immunocytometry Systems. Left panel: control U-937 (1, unstained; 3, stained) and U-937 Bcl-xL cells (2, unstained; 4, stained). Right panel: control Namalwa (1, unstained; 3, stained) and Namalwa Bax-α cells (2, unstained; 4, stained).



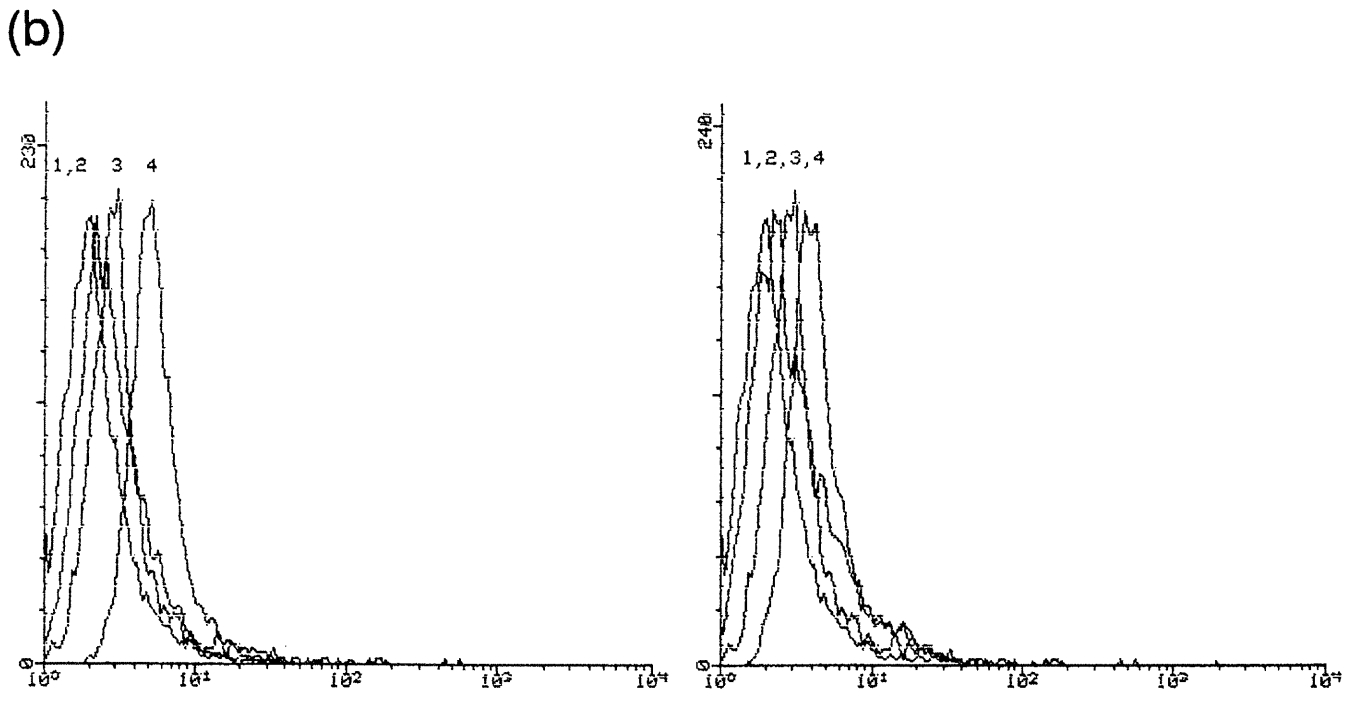
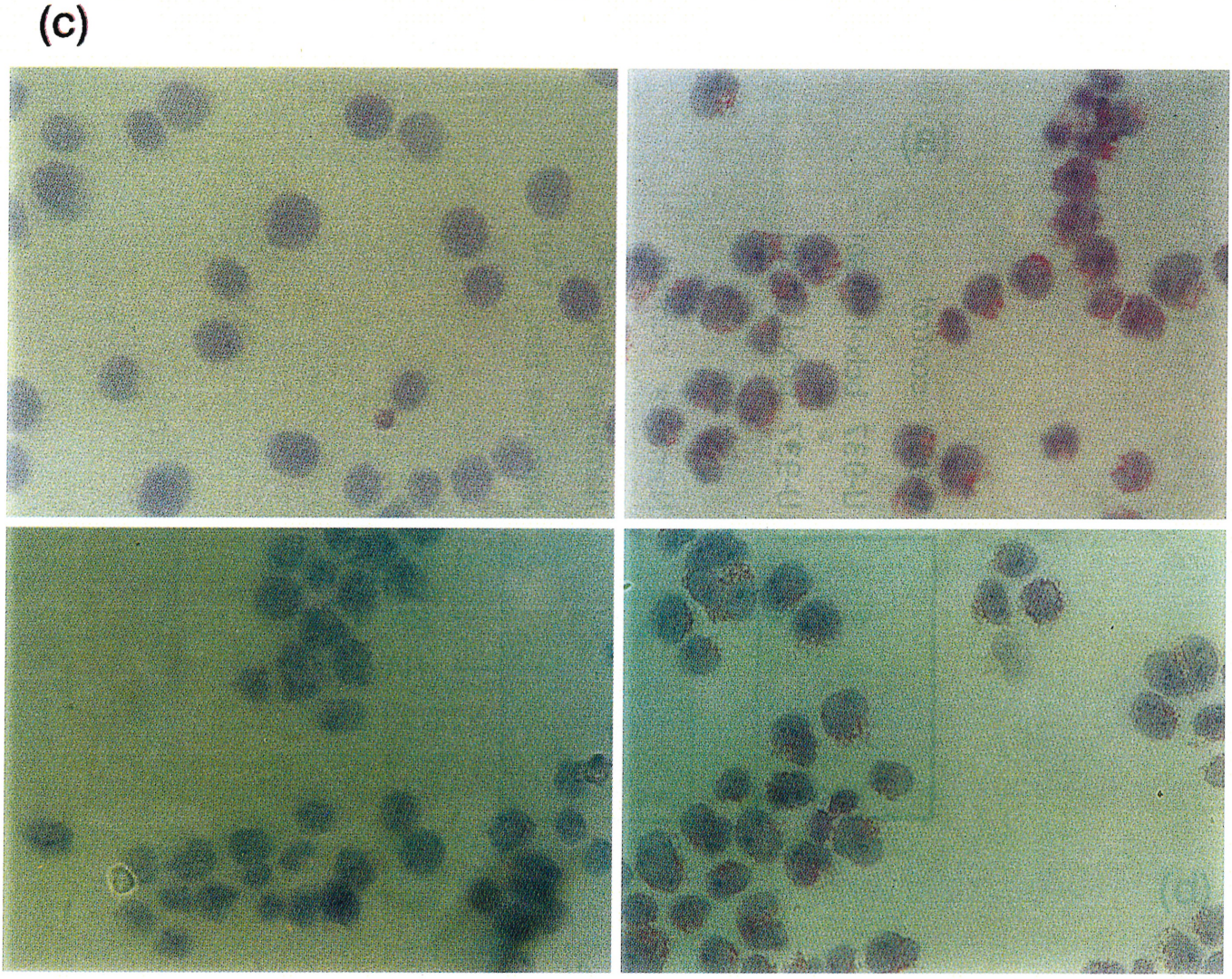


Fig. 2. (*c*) Expression of HA-Bcl-xL and HA-Bax-α proteins by immunohistochemistry using anti-HA mouse monoclonal antibodies as primary antibody (10 μg/mL) and a biotinylated goat anti-mouse as secondary antibody followed by a preformed streptavidin – alcaline phosphatase complex and colorimetric reaction (FastRed; Sigma Chemical Co., St. Louis, Mo.). Note the absence of staining in control U-937 (upper left) and Namalwa cells (lower left) and the intense positivity of U-937 Bcl-xL (upper right) and Namalwa Bax-α cells (lower right).



Modulation of cell death induced by vinblastine, paclitaxel, and *cis*-platinum

Modulation of cell death by Bcl-xL in U-937 cells was also observed in cells treated continuously for 8 h with a series of anticancer drugs that act primarily by different mechanisms of action (vinblastine, a tubulin polymerization inhibitor; paclitaxel, a microtubule depolymerization inhibitor; *cis*-platinum(II) diamine dichloride, a DNA adducts inducing agent) (Table 1). The protective effect of Bcl-xL was observed at various drug concentrations for all drugs tested. The cell-death promoting activity of Bax was significant in vinblastine-treated cells; however, Bax-α overexpression did not significantly sensitize Namalwa cells to paclitaxel or *cis*-platinum either 8 h (Table 2) or 24 h (data not shown (Schmitt et al. 1997)) after drug treatment. Taken together, these results show that Bcl-xL acts primarily downstream of primary mechanisms of action of these drugs, conferring a multidrug resistance modulation, whereas Bax-α has a more selective effect among these drugs in Namalwa cells.

Other gene transfer studies in human cell lines have also indicated that high expression of Bcl-xL protects B lymphocytes exposed to γ-irradiation and reactive oxygen species from apoptosis (Fang et al. 1995). Multidrug-resistance variant cell lines have also been isolated on the basis of Bcl-xL expression rather than P-glycoprotein or Bcl-2 expression (Datta et al. 1995; Han et al. 1996). Other studies have reported that Bcl-xL provides resistance to ionizing radiation and various cytotoxic drugs including DNA topoisomerase inhibitors, taxol, *cis*-platinum, and vincristine in leukemia and neuroblastoma cells (Datta et al. 1995; Dole et al. 1995; Ibrado et al. 1996). Similarly, overexpression of the Bcl-xS protein was reported

Fig. 3. CPT-induced DNA fragmentation in control, Bcl-xL, and Bax-α transfected lines. [^{14}C]Thymidine-labeled cells were treated for 30 min at various CPT concentrations (*x* axis; micromolar). After drug removal, cells were incubated in drug-free medium for 8 h and DNA fragmentation was determined by DNA filter elution assays. Results are expressed as the percentage of DNA fragmentation in treated cells relative to untreated cells (*y* axis). Points and bars represent the means ± SD of four independent experiments performed in duplicate. Experiments were conducted with U-937 cells (●), Bcl-xL-transfected U-937 cells selected at 0.50 mg/mL (■) and 1.5 mg/mL hygromicin (▲), Namalwa cells (○), and Bax-α transfected Namalwa cells selected at 0.50 mg/mL (□) and 1.5 mg/mL hygromicin (△).

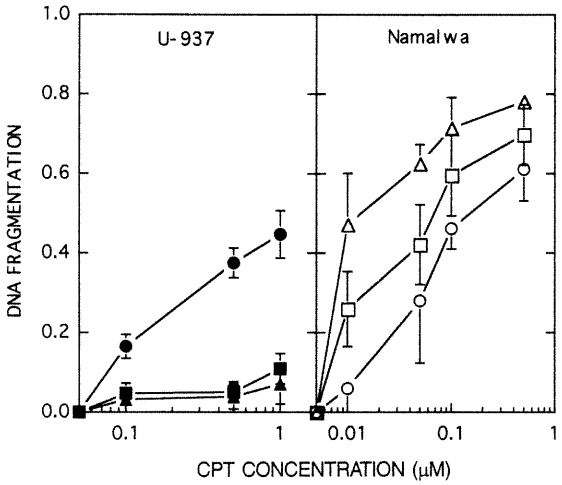
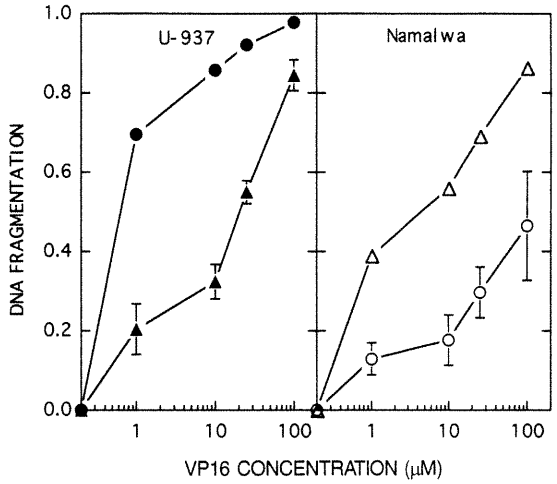


Fig. 4. VP16-induced DNA fragmentation in control, Bcl-xL, and Bax-α transfected lines. [^{14}C]Thymidine-labeled cells were treated continuously at various VP16 concentrations for 8 h (*x* axis; micromolar). DNA fragmentation was determined by DNA filter elution assays. Results are expressed as the percentage of DNA fragmentation of treated cells relative to that of untreated cells (*y* axis). Points and bars represent the means ± range of two independent experiments performed in triplicate. Experiments were conducted with U-937 cells (●), Bcl-xL-transfected U-937 cells selected at 1.50 mg/mL (▲), Namalwa cells (○), and Bax-α-transfected Namalwa cells selected at 1.5 mg/mL hygromicin (△).



to sensitize human breast carcinoma and neuroblastoma cells to chemotherapeutic drugs by trapping the Bcl-xL protein (Dole et al. 1996; Ealovega et al. 1996; Sumantran et al. 1995). Gene transfer studies with Bax-α in human breast cell lines showed that Bax-α sensitizes these cells to radiation- and drug-induced apoptosis (Sakakura et al. 1996; Wagener et al. 1996). Other studies have indicated that the Bax:Bcl-2 ratio in human testicular tumours and in B-cell chronic lymphocytic leukemia is related to cell susceptibility to apoptosis (Chresta et al. 1996; McConkey et al. 1996; Thomas et al. 1996). Others have reported that Bax-α can antagonize the protective effects of Bcl-xL during VP16-induced apoptosis in FL5.12 cells and in a lesser degree during *cis*-platinum-induced apoptosis (Simonian et al. 1996). The mechanism underlying the reduced effect of Bax-α in promoting *cis*-platinum-induced apoptosis is not yet known. Although our observations suggest that Bax-α does not promote paclitaxel-induced apoptosis in B lymphoma Namalwa cells, others have reported that a mean 10-fold increase in the level of murine Bax expression in human SW626 ovarian cancer cells promotes cell death induced by paclitaxel (Strobel et al. 1996). Ordered balance within the ratio of effector and repressor proteins regulating the decision of a cell to survive or die has already been suggested to form an important checkpoint for cell death (Oltvai and Korsmeyer 1994). Taken together, our results and those of others suggest that even a slight difference in expression among these proteins influences apoptosis induced by cancer chemotherapy.

Inhibition of DNA synthesis by camptothecin in Bcl-xL and Bax-α transfected cells

Inhibitors of DNA top I and II stabilize a reaction involving a transient intermediate of topoisomerases in which enzymes are linked to the 3′ (top I) or 5′ (top II) terminus of a DNA duplex producing DNA single- or double-stranded breaks (Pommier and Bertrand 1993; Pommier et al. 1994; Pommier and Tanizawa 1993). These enzyme-linked DNA adducts on active replication forks inhibit DNA synthesis. DNA replication inhibition is one of the most widely explored processes to be implicated in the cytotoxic mechanism of action of DNA top I inhibitors (Bertrand et al. 1992; D'Arpa et al. 1990; Holm et al. 1989; Hsiang et al. 1989; Pommier et al. 1994). To determine whether Bcl-xL and Bax-α proteins act primarily upstream or downstream of CPT translated effects on DNA synthesis, rates of DNA synthesis were measured by thymidine pulse incorporation immediately after CPT treatment. Table 2 shows that in U-937 control cells, immediately following CPT exposure at 0.1, 0.5, and 1.0 μM, DNA synthesis was inhibited to approximately 19, 30, and 45% of that of control untreated cells, respectively. The extent of DNA synthesis inhibition was similar in all transfected U-937 cells treated with 0.1, 0.5, and 1.0 μM CPT, with rates of approximately 27, 36, and 47% of that of the transfected untreated cells, respectively. These results suggest that Bcl-xL does not interfere with the primary

Table 2. DNA synthesis inhibition induced by camptothecin in control and transfected U-937 and Namalwa cells.

CPT	U-937	U-937bcl-xL$^{0.50}$	U-937bcl-xL$^{1.50}$	Namalwa	Nam-bax$^{0.50}$	Nam-bax$^{1.50}$
0.01 μM				15.0±9.9	19.5±9.8	19.3±3.8
0.05 μM				25.3±5.8	26.3±5.2	28.8±10.9
0.1 μM	19.1±9.2	28.8±7.8	26.2±15.8	34.5±6.4	35.3±2.9	36.5±8.2
0.5 μM	30.2±9.1	35.5±6.4	36.3±10.4	40.8±7.5	43.8±4.8	42.5±4.8
1.0 μM	45.0±5.0	45.8±6.2	48.3±9.2			

Note: Cells were treated with various concentrations of CPT for 30 min. Incorporation of [^{3}H]thymidine was determined by 10-min pulse experiments. Values are expressed as the percentage of DNA synthesis inhibition in treated cells relative to control untreated cells and given as the mean ± SE (n = 6). U-937 bcl-xL$^{0.50}$ and U-937 bcl-xL$^{1.50}$, U-937 cells transfected with Bcl-xL and selected at 0.50 and 1.5 mg/mL hygromycin, respectively. Nam-bax 0.50 and Nam-bax$^{1.50}$, Namalwa cells transfected with Bax-α and selected at 0.50 and 1.5 mg/mL hygromycin, respectively.

Table 3. The effect of caspase-3-like, serine protease inhibitors and r Bcl-xL protein in a cell-free system.

	CPT	+Ac-DEVD- CHO	+TPCK	+r Bcl-xL
2-h extracts				
U-937	11.0±0.9	08.6±0.6	03.0±0.6	11.0±0.3
U-937 bcl-xL	00.5±0.2	00.6±0.2	00.4±0.1	00.6±0.2
4-h extracts				
U-937	24.2±2.8	10.5±2.3	19.2±2.5	23.6±2.6
U-937 bcl-xL	00.3±0.2	00.5±0.3	00.5±0.5	00.6±0.2
8-h extracts				
U-937	77.4±3.7	72.8±6.0	75.2±3.0	68.0±1.5
U-937 Bcl-xL	13.4±1.4	07.7±1.6	01.3±0.8	13.2±1.8

Note: Cytosolic extracts were prepared from U-937 cells and Bcl-xL-tranfected U-937 cells at the indicated times following CPT treatment (1.0 μM for 30 min). Isolated nuclei labeled with [^{14}C]thymidine from untreated cells were then incubated for 30 min with cytosolic extracts alone or in the presence of TPCK, Ac-DEVD-CHO, or purified recombinant Bcl-xL protein. DNA fragmentation was determined by DNA filter elution assays. Results are expressed as the percentage DNA fragmentation relative to untreated cells. Values are given as the mean ± SE (n = 9). The Bcl-xL transfected U-937 line used in these experiments was the one selected at 1.5 mg/mL hygromycin.

mechanism of action of CPT and does not prevent CPT-induced DNA synthesis inhibition. Similarly, in Namalwa control cells immediately following CPT exposure at 0.01, 0.05, 0.1, and 0.5 μM, DNA synthesis was reduced to approximately 15, 25, 35, and 41% of that of control untreated cells, respectively. The extent of DNA synthesis inhibition was similar in the two transfected Namalwa cells treated at the same drug concentrations with rates of approximately 19, 27, 36, and 43%, respectively. These results suggest that Bax-α does not interfere with the primary mechanism of action of CPT and neither modulates nor amplifies CPT-induced DNA synthesis inhibition.

Although signals emerging from unfinished replication and damaged DNA in these cells are not yet completely known, our studies indicate that Bcl-xL and Bax-α are control points of these signals and influence the decision of a cell to survive or die. Others have reported that Bcl-xL significantly inhibits taxol-induced apoptosis in human HL60 cells without affecting the tubulin polymerization effect and mitotic arrest caused by taxol (Ibrado et al. 1996). A recent study has suggested that a sufficient level of Bax may bypass the need for upstream molecules such as p53 in triggering chemotherapy-induced apoptosis (Strobel et al. 1996). It has been proposed that Bax-α is a wild-type p53 primary response gene in the p53-regulated pathway for apoptosis activation (Miyashita et al. 1994; Miyashita and Reed 1995). Namalwa cells are p53 double mutant cells (O'Connor et al. 1993) and it is unlikely that mutated p53 plays a role in the induction of apoptosis in these lines. Nevertheless, elevation of the pro-apoptotic Bax-α protein by gene transfer sensitizes these cells to low drug concentrations in a p53-independent manner.

Differential effect of Bcl-xL and Bax-α on the activation of TPCK-sensitive proteases and caspases

A series of serine proteases and caspases are involved in the apoptotic process (Kumar and Harvey 1995; Patel et al. 1996). Their activities are likely associated with the process of cell death itself. To determine whether Bcl-xL protein functions upstream of the proteases to prevent their activation, we used a cell-free system that we developed previously. This cell-free system is designed to elucidate the biochemical pathways of apoptosis and has proven to be convenient to measure and detect protease activities in cytosolic extracts prepared from cells undergoing apoptosis (Bertrand et al. 1994, 1995; Bertrand and Pommier 1995; Shimizu and Pommier 1996; Solary et al. 1993). These activated extracts trigger DNA fragmentation when incubated with isolated nuclei obtained from control untreated cells, and activities could be modulated in vitro (Bertrand et al. 1995, 1994; Shimizu and Pommier 1996; Solary et al. 1993).

Cytosolic extracts of CPT-treated U-937 cells prepared 2 and 4 h after drug treatment are able to trigger DNA fragmentation when incubated with isolated control nuclei in the cell-free system (Table 3). Cytosolic extracts prepared 1 h after drug treatment showed no activity in the cell-free system, consistent with a lack of detectable DNA fragmentation induced by CPT in cultured cells. Activities in the 2-h extracts were inhibited almost completely by *N*-tosyl-L-phenylalanylchloromethyl ketone (TPCK), a serine protease inhibitor, and slightly inhibited by the tetrapeptide derivative Ac-Asp-Glu-Val-aspartic acid aldehyde (Ac-DEVD-CHO), a caspase-3-like inhibitor (Table 3). Activities in the 4-h extract were significantly inhibited by Ac-DEVD-CHO and to a lesser extent by TPCK. The tetrapeptide Ac-Tyr-Val-Ala-aspartic acid aldehyde (Ac-YVAD-CHO), a caspase 1-like inhibitor, had no effect on either the 2- or 4-h extracts (data not shown (Schmitt et al., see footnote 3)). These results suggest a cascade of protease activation in U-937 cells undergoing apoptosis with activation of TPCK-sensitive proteases as early as 2 h after drug

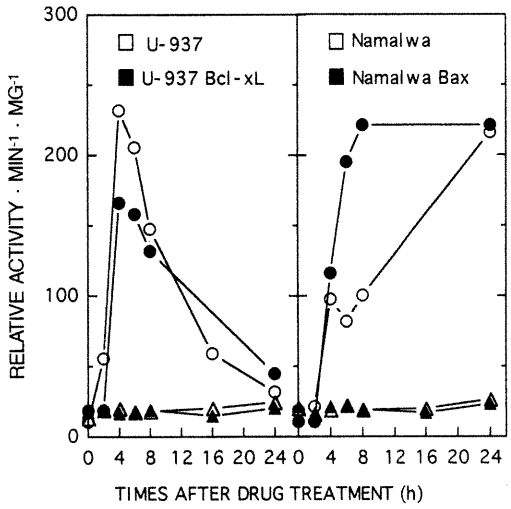
treatment followed by caspase-3-like activation. Neither TPCK nor Ac-DEVD-CHO could inhibit DNA fragmentation in the 8-h extracts, suggesting the presence of other factors that trigger DNA fragmentation in the cell-free system.

In contrast, in cytosolic extracts of CPT-treated Bcl-xL-transfected U-937 cells, neither protease activities nor DNA fragmentation was detected 2 and 4 h after drug treatment in the cell-free system, consistent with the protective effect conferred by Bcl-xL in CPT-treated cultured cells. However, 8 h after drug treatment, activities were found in the cell-free system, consistent with the beginning of DNA fragmentation observed in cultured cells. Furthermore, activities in the 8-h extracts were predominantly inhibited by TPCK and slightly inhibited by Ac-DEVD-CHO (Table 3). Taken together these results show that Bcl-xL protein prevents and delays the activation of TPCK-sensitive activity and caspase-3-like activities. Furthermore, when the protective effect conferred by Bcl-xL protein was lost in cultured cells, a similar ordered cascade of activation was detected in the cell-free system. These results suggest that following CPT treatment, TPCK-sensitive activity and caspase-3-like activity are activated. Similar activation of a TPCK-sensitive activity and caspase-3-like cysteine proteases was detected in Namalwa cells treated with CPT (Sané et al. 1997). Interestingly, using a similar cell-free system, Lazebnik and colleagues previously showed that neither PARP, nor lamin A and B cleavage were inhibited in the presence of TPCK in their cell-free system extracts (Lazebnik et al. 1995). Together these results indicate that the TPCK-sensitive activity found in cell-free systems is not related to the caspases involved in PARP and lamin A and B cleavage.

Sequential activation of caspase-1-like and caspase-3-like activity during CD95–Fas-induced apoptosis has been reported recently (Enari et al. 1996). Other studies have revealed the role played by serine proteases in the apoptotic process. Early studies showed that a series of serine protease inhibitors can block apoptosis in cultured cells or in cell-free systems following DNA damaging agent treatment (Bruno et al. 1992; Ghibelli et al. 1995; Hara et al. 1996; Shimizu et al. 1996; Solary et al. 1996; Weaver et al. 1993; Yoshida et al. 1996). The well-known granzyme B serine protease is able to activate caspase 3 both in vivo and in vitro in some instances of apoptosis mediated by cytotoxic T lymphocytes in targeted cells (Darmon et al. 1996, 1995; Fernandes-Alnemri et al. 1996). More recently, AP24, a serine protease purified from U-937 cells, was reported to be rapidly activated in U-937 cells undergoing apoptosis induced by TNF and ultraviolet light (Wright et al. 1994). Moreover, consistent with our observations, a recent report showed that in the hepatocellular carcinoma Hep 3B cells, apoptosis is induced by CPT without activation of the proteolytic caspase cascade involved in PARP and lamin B cleavage and that a trypsinlike protease activity appeared to activate apoptosis in these cells (Adjei et al. 1996). The TPCK-sensitive activities proteases that are activated and involved in CPT-induced apoptosis are presently being characterized.

To test whether Bcl-xL was actually preventing the activation of serine and cysteine proteases or directly interfering with already activated proteases, a large amount of purified recombinant Bcl-xL protein was added to extracts in the cell-free system. Addition of recombinant Bcl-xL protein to extracts containing TPCK-sensitive and caspase-3-like activities

Fig. 5. Caspase-1-like and caspase-3-like activities in control, Bcl-xL, and Bax-α transfected lines. Cytosolic extracts were prepared from U-937 and Bcl-xL-transfected U-937 cells at the indicated times following CPT treatment (0.5 μM; left panel) and from Namalwa and Bax-α tranfected Namalwa cells at the indicated times following VP16 treatment (10 μM; right panel). Enzyme activities were measured as initial velocities and expressed as relative intensity per minute for each milligram. Points represent the average of two independent determinations (range within 10%). Open symbols are for control cells and solid symbols are for transfected cells; triangles represent caspase-1-like activity and circles represent caspase-3-like activity.



did not inhibit these activities or DNA fragmentation in the cell-free system whereas Ac-DEVD-CHO and TPCK showed potent inhibition (Table 3). These results suggest that Bcl-xL has no inhibitory effect on these activated proteases. Taken together, these results indicate that Bcl-xL acts primarily upstream of serine proteases and caspase-3-like activity in the apoptotic process, preventing their activation. Thus, Bcl-xL is an important control point that can block or delay signals that would otherwise activate these proteases.

The kinetics of caspase activation were also monitored in control and Bcl-xL U-937 cells treated with CPT and in control and Bax-α transfected Namalwa cells treated with VP16. Caspase activities were measured by monitoring fluorescence continuously in a dual luminescence fluorometer using the specific fluorogenic peptide derivatives DABCYL-YVADAPV-EDANS and Ac-DEVD-AMC, substrates of caspase-1-like and caspase-3-like activity, respectively. In cytosolic extracts obtained from Bcl-xL transfected U-937 cells, a slower increase in caspase-3-like activity was detected compared with that of control U-937 cells after CPT treatment (Fig. 5). No increase in DABCYL-YVADAPV-EDANS hydrolysis was detected following CPT treatment in both lines, suggesting that caspase-1-like activity is neither activated nor involved in the apoptotic process induced by CPT in U-937 cells. Similar results were obtained in Namalwa cells treated with VP16 (Fig. 5). The kinetics of caspase-3-like activity correlate with the slow activation of DNA fragmentation following VP16

treatment in control Namalwa cells; in contrast, a more significant increase in caspase-3-like activity was detected in Bax-α transfected Namalwa cells (Fig. 5). No increase in DABCYL-YVADAPV-EDANS hydrolysis was detected following VP16 treatment, suggesting that caspase-1-like activity is neither activated nor involved in the apoptotic process induced by VP16 in these cells. Taken together, these results indicate that caspase-3-like activity correlates with the occurrence of DNA fragmentation and that Bcl-xL delays whereas Bax-α accelerates its activation. Interestingly, the effect of Bax-α on caspase activation was opposite to that of Bcl-xL. Other studies have reported that Bcl-2 and Bcl-xL prevent the activation of cysteine proteases in cultured cells (Monney et al. 1996; Shimizu et al. 1996) or in similar cell-free systems using activated cytosolic extracts from *Xenopus laevis* oocytes (Newmeyer et al. 1994). Cosulich et al. (1996) reported that Bcl-2 prevents apoptotic chromatin condensation and DNA cleavage only when added prior to the activation of the caspases. Our recent results with Bcl-xL and Bax-α in human cultured cells and using a cell-free system are in agreement with these studies and suggest that Bcl-xL, as Bcl-2, prevents whereas Bax-α accelerates protease activation.

In summary, accumulating evidence indicates that Bcl-xL and Bax-α are primary control checkpoints that can block or promote transmission of cell death signals emerging from cell injuries induced by anticancer drugs. Although the exact mechanisms by which these proteins transduce cell survival or cell death signals have yet to be identified, Bcl-xL blocks and Bax-α promotes the activation of key proteases involved in the execution of apoptosis. Recent progress suggests that the Ced-9 – Bcl-like family functions both as an adaptor or docking protein and as an ion channel (Reed 1997). The localization of these proteins, namely intracellular membranes such as the outer mitochondrial membrane, endoplasmic reticulum, and nuclear envelope, suggests that they may influence more specifically the opening of mitochondrial megachannels (Bernardi et al. 1994; Zoratti and Szabo 1996) and the release of apoptogenic protease activators from mitochondria such as the apoptosis-inducing factor (Zamzami et al. 1995) and cytochrome *c* (Yang et al. 1997; Kluck et al. 1997). Other data suggest that Ced-9 – Bcl-like binding proteins such as Ced-4 or its equivalent in mammalian cells APAF-1 (Zou et al. 1997) may be important factors controlling caspase activation by a trappinglike mechanism that involves a multimolecular complex between Ced-9 – Bcl-like, Ced-4 like, and Ced-3 – Ice-like proteins (Spector et al. 1997; Chinnaiyan et al. 1997; Wu et al. 1997). Signals emerging from the major regulators of cell survival and cell death represent complex cellular checkpoints that influence cellular sensitivity and resistance to cancer chemotherapy.

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ANNEXE III

Activation and role of caspases in chemotherapy-induced apoptosis

Estelle Schmitt, Alain Théophile Sané, Richard Bertrand

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Activation and role of caspases in chemotherapy-induced apoptosis

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Abstract The importance of caspase activation during apoptosis has become eminently apparent in the last few years. The caspases participate in a proteolytic cascade activated in response to various stimuli, including anticancer drugs, that results in the systematic and orderly eradication of the cell. The core machinery of caspase activation is now emerging and involves multiple molecular complexes. We describe the two best-studied models of caspase activation, the mitochondrial pathway and the cell death receptor pathway, and discuss their involvement in caspase activation induced by various anticancer drugs used in chemotherapy. Defective apoptosis contributes to tumor growth and drug resistance. Understanding the activation and role of caspases in apoptosis may help develop new therapeutic strategies to circumvent drug resistance.

INTRODUCTION

Apoptosis or programmed cell death is a natural process that plays a crucial role in development, regulation of cell homeostasis and elimination of abnormal and harmful cells.[1] Excessive or insufficient cell death contributes to human diseases including neurodegenerative disorders, autoimmune and neoplastic diseases.[2] Apoptosis contributes to tumor development since the rate of cell death as well as the rate of cellular proliferation are determining factors of tumor growth. In cancer therapy, the interest in apoptosis arises because of observations that ionizing radiation, antitumor drugs, cytokines and growth suppressor factors often trigger this mode of cell death. Moreover, the efficacy of several antitumor drugs correlates with their ability to induce apoptosis in human cancer cells and defective control of apoptosis in treated tumors constitutes a form of drug resistance mechanism.[3] Programmed cell death is characterized by an ordered sequence of morphological changes such as cytoplasmic shrinkage, membrane blebbing, chromatin condensation, nuclear breakdown and distinctive DNA fragmentation. Core components of the cell death machinery were defined recently by genetic studies of the nematode *Caenorhabditis elegans* where four key cell death genes, *ced-9*, *ced-4*, *ced-3* and *egl-1* have been identified.[4-7] Both *ced-4* and *ced-3* are essential for cell death to occur whereas *ced-9* antagonizes the function of *ced-4* and *ced-3* by protecting cells from death. *Egl-1* promotes cell death and negatively regulates the function of *ced-9*. Mammalian homologs of these genes have recently been identified. Ced-9 protein presents significant homologies to the mammalian Bcl-like family that contains combination of Bcl-2 homology domains (BH1, BH2, BH3 or BH4 domain) and includes antiapoptotic Bcl-2, Bcl-xL, Mcl-1, A-1/Bfl-1/Grs, Bcl-w, Brag-1 and proapoptotic Bax, Bak, Bcl-xS, Mtd/Bok/Bod members. Egl-1 contains only BH3 domain, similar to the mammalian cell death activators that includes Bik/Nbk (and its close murine homolog mBlk), Bid, Hrk, Bad, Bim and BNip3.[8,9] Ced-3 is related to the cysteine protease interleukin-1β converting enzyme (ICE), a member of a growing family of ICE-like proteases now called caspases, for *c*ysteine *a*spartic *a*cid-specific prote*ases*.[10-12]

Recently, Apaf-1, the first human homolog of ced-4 was identified (13). Functional analysis of ced-9, ced-3 and ced-4 proteins and their mammalian counterparts has given rise to a molecular framework for cell death in which the caspases play a central role.[14-17] After cell death initiation by diverse stimuli and insults like anticancer drugs, initiator caspases are activated and process other downstream members of the death protease family, resulting in sequential proteolytic events that converge to amplify the initial apoptotic signal. The activation of this proteolytic cascade ends with the cleavage of a variety of cytosolic, cytoskeletal, nuclear or other cellular proteins resulting in the systematic and orderly morphological changes of the cell undergoing apoptosis. In this review we report and discuss recent progress in the understanding of the molecular frameworks that regulate caspase activation in cells that inititate the apoptotic program. We describe the two best-studied models of caspase activation, the mitochondrial pathway and the CD95/Fas/APO-1 death receptor pathway, and discuss their involvement in caspase activation induced by various anticancer drugs used in chemotherapy.

CASPASE ACTIVATION IN APOPTOSIS; GENERAL FEATURES

To date, more than 12 human caspases are identified. They are separated in three subfamilies based on their degree of homology and phylogenetic analysis: the ICE-related family includes ICE/caspase-1, TX/ICH-2/ICE_{rel}-II/caspase-4, Ty/ICE_{rel}-III/caspase-5, ERICE/caspase-13; the CED3/CPP32-related family includes CPP32/Yama/Apopain/caspase-3, Mch-2/caspase-6, Mch-3/ICE-LAP3/CMH-1/caspas7, Mch5/Flice/Mach/caspase-8, Mch-4/Flice-2/caspase-10; and the third group includes ICH-1/caspase-2, ICE-LAP6/Mch6/caspase-9 and Viz/caspase-14. Two more murine caspases, mICH-3/caspase-11 and mICH-4/caspase-12, have been described with no known human counterparts identified yet.[10-12] Ectopic overexpression of many caspases results in apoptosis, and deficient mice for caspase -2, -3, -8 and -9, generated by homologous recombination, exhibit severe cell death abnormalities.[18-22] However, mice deficient for caspase -1 and -11, a close homolog of caspase-4, do not show altered cell death phenotype.[23,24] These data showed that some caspases, including caspase -1, -11 (and presumably -4 and -5), are unlikely to be involved directly in apoptosis and are believed to play a role in pro-cytokine activation. These observations also reveal that individual members of the caspase superfamily play non-redundant roles during development and perhaps in pathways of induced apoptosis. Mice deficient in various members of the Bcl-like family, such as

Bcl-2, Bcl-x, Bax and Bcl-w, and in Apaf-1 show similar non-redundant role for those proteins in regulating apoptosis.[25-27]

Caspases have a conserved peptapeptide sequence (QACXG) that contains the active cysteine residue. They are synthesized as inactive zymogens that require at least two sequential proteolytic cleavages at aspartic acid residue to generate the mature catalytically active heterodimers. Caspases have an autocatalytic activity when they are produced at very high concentration (by ectopic expression using eucaryotic or procaryotic expression vector systems), but their activation during apoptosis is sequential; some caspases process and activate others.[10-12] Two types of distinct conserved protein-protein interaction motifs, named death effector domain (DED) and caspase recruitment domain (CARD), are found at the amino-terminal prodomains of several caspases. These domains play a central role in the formation of caspase activating apparatus in vivo. DED are present on caspase -8 and -10 and on the death adapter protein FADD/MORT-1. CARD are present on caspase -1, -2, -4, -5, -9 and -13 and on the death adapter proteins RAIDD/CRADD and Apaf-1. In the mitochondrial pathway of caspase activation, Apaf-1 eases caspase-9 autoactivation by oligomerizing its precursor molecules via CARD domain interactions in presence of cytochrome c and dATP.[15] Similar models are proposed for caspase -2, -8 and -10 activation. Caspase -8 and -10 precursors present a very low level of intrinsic caspase activity but after being recruited to ligand-activated cell death receptors by FADD/MORT-1 via DED domain interactions, they reach a sufficient local concentration and presumely a configuration to be activated and initiate apoptosis triggered by activated CD95/APO-1/FasR, TNF-R1 and Dr3/TRAMP/WSL-1/LARD death receptors. Caspase-2 is targeted to activated TNF-R1 receptor via the death adapter protein RAIDD/CRADD at the RIP-TRADD-TNF-R1 complex through CARD-CARD interactions. Caspases such as caspase -2, -8, -9 and -10 that have long amino-terminus prodomains containing DED or CARD modules, act as initiator caspases while others such as caspase-3, -6 and -7 that contain short prodomains and no DED or CARD prodomains, act as effector caspases in the apoptotic process.[11,12]

Activation of caspases after death stimuli result in morphological cellular changes that culminate in disassembly of the dying cell and engulfment by neighboring cells. Although multiple protein substrates of caspases are identified, the functional significance of their proteolysis in cell death is often poorly understood. Cleavage site specificity of the caspases was outlined using a positional scanning peptide substrate combinatorial approach. Based on their individual specificity, caspases are divided into three groups; caspase -2, -3 and -7 have preference for DEXD motif, caspase -6, -8 and -9 present preference for (I/L/V)EXD, while caspase -1, -4 and -5 prefer (W/L)EHD sequence.[28] Caspase cleavage leads to the activation of proteins that contribute directly to the apoptotic program like Bid, other pro-caspases and activate several kinases like PKC δ and θ, MEKK-1, PAK2, MST/Krs, PKN, although their exact role in apoptosis remains elusive. Caspase cleavage will lead to the inactivation of proteins that could interfere with cell death directly such as Bcl-2, Bcl-xL and presumably p28Bap31 or indirectly such as AKT, PARP, DNA-PK, MDM2, U1-70K, Rb, p21, p27. The cleavage of cytoskeletal proteins or proteins involved in cytoskeleton and cell-cell adhesion regulation (fodrin, actin, gelsolin, Gas2, keratins, γ and β-catenins, αII and αIII spectrin, focal adhesion kinase) and proteins involved in maintaining the integrity of the nuclear structure (NuMA, lamins, SAF-1) contribute to cellular structural disassembly. Finally, caspase cleavage of nuclease inhibitors such as DFF/mICAD initiate the endonucleotic DNA cleavage pathway by activating nucleases like CPAN/mCAD.[29-31] However, other DNA fragmentation promoting activity and endonucleases are involved for complete oligonucleosome-sized DNA digestion during apoptosis.[32-34]

Many studies have shown that the Ced-9/Bcl-like proteins are key regulators of caspase activation and apoptosis triggered by anticancer drugs like camptothecin, etoposide, vinblastine, taxol, Ara-C, cis-platinum.[35-39] Clues about the molecular mechanisms by which these proteins regulate caspase activation are emerging. Though a clear understanding of how caspases are activated after death stimuli remains elusive, considerable understanding has been obtained by intensive studies on the mitochondrial pathway and CD95/APO1/Fas cell death receptor pathway of caspase activation.

MITOCHONDRIAL REGULATION OF CASPASE ACTIVATION

Mitochondria are considered as major players in apoptosis of mammalian cells and undergo functional and structural changes early during the death process.[40] Mitochondrial permeability transition (MPT), and subsequent mitochondrial transmembrane potential ($\Delta\psi m$) disruption, is a general feature of cells undergoing apoptosis. MPT involves the opening of permeability transition pores or mitochondria megachannels at the contact regions of the inner and outer membranes of mitochondria. These events have been detected in many different cell types after diverse apoptosis-inducing stimuli including anticancer drugs.[41] Permeability transition pore opening leads to the release of the mitochondrial apoptogenic factor AIF and cytochrome c.[40] MPT pore opening appears to culminate in large amplitude swelling of the mitochondria matrix, and both cytochrome c and AIF seem to be released from a mechanical damage of the outer mitochondrial membrane.[42] AIF is a protease activator that can activates caspase-3 and perhaps other caspase-3 like proteases and induces DNA fragmentation in isolated nuclei. Once liberated, AIF contributes to further induction of MPT and thus, amplifies the mitochondrial initiating signal.[40]

Considerable clarifications for the role of mitochondria in apoptosis have been obtained by three recent works published by Wang's group (Fig. 1). This group has established an in vitro system based on cytosols of growing HeLa cells in which apoptosis is initiated by the addition of dATP. Fractionation of these activated cytosol extracts and reconstitution of caspase-3 activation, a major caspase activity in apoptotic cells, leads to the purification of three apoptotic protease-activating factors termed Apaf-1, -2 and -3. Apaf-2 was identified as mitochondrial cytochrome c, Apaf-1 as the human homolog of Ced-4, and Apaf-3 as caspase-9.[13,15,43] All three factors are required for caspase-3 activation and DNA

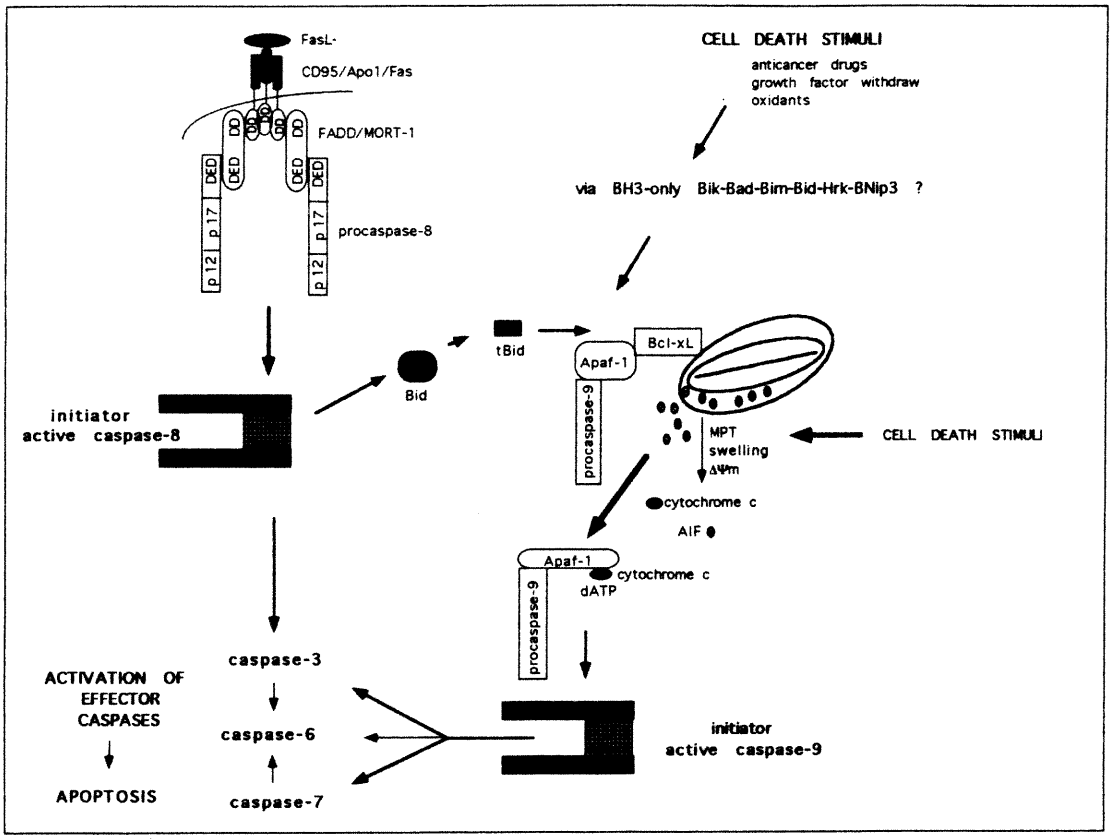

Fig. 1 **Schematic diagram of caspase activation via the mitochondrial pathway and CD95/APO-1/FAS cell death receptor pathway.**

fragmentation of exogenous added nuclei. Wang and coworkers have demonstrated that Apaf-1 binds to caspase-9 in the presence of dATP and cytochrome c and this interaction is mediated by the CARD domains found on the two proteins. The complex formation between Apaf-1 and caspase-9 zymogen leads to the cleavage and activation of caspase-9, probably through an autoproteolytic processing and caspase-9 in turn activates directly the effector caspase -3 and -7 (Fig. 1).[15,41] In this system, cytochrome c and dATP are required to generate an active Apaf-1/caspase-9 complex and a model is proposed in which cytochrome c and dATP induce conformational changes in Apaf-1 necessary to Apaf-1/caspase-9 activation. Apaf-1 and caspase-9 deficient mice have been generated and these mice exhibit many apoptosis defects with a global phenotype similar, although not identical to caspase-3 deficiency.[21,22,26,27] Apaf-1 and caspase-9 deficiency in vivo impaired the activation of caspase-3 but mitochondrial cytochrome c translocation appears not affected in deficient cells after DNA damage.

These results suggest that Apaf-1 and caspase-9 act downstream of cytochrome c release and upstream of caspase-3 activation and are consistent with their functional interactions shown in vitro. Wang's group has shown that caspase-9 is the protease that lies immediately downstream of Apaf-1. It has been shown also that Apaf-1 could interact in vivo with other caspases containing long prodomain like caspase -8 and -4.[17] Similarly, its homolog ced-4 interacts with caspase-8.[14] However, others have revealed that none of the other CARD-and DED-containing caspases, such as caspase -1, -2, -4, -8, -10 and -13, were activated in the presence of Apaf-1, cytochrome c and dATP indicating the high specificity of the Apaf-1/caspase 9 activating apparatus.[15]

Cytochrome c release from mitochondria is an early event in the initiation of the apoptotic program. It has been shown that Bcl-2 and Bcl-xL overexpression prevents caspase-3 like protease activation and apoptosis by blocking cytochrome c translocation from mitochondria to the cytosol,[46-48] whereas overexpression of the proapoptotic protein Bax-α induces release of cytochrome c.[49] Many data correlate an MPT-mediated $\Delta\psi m$ disruption with induction of apoptosis and suggest that the antiapoptotic Ced-9/Bcl-like proteins suppress apoptosis through inhibition of MPT,[9,40] whereas the proapoptotic Bax protein that binds to the MPT pore complex can induce loss of mitochondrial membrane potential and promotes cytochrome c release and caspase activation.[50,51] Mitochondrial cytochrome c release, however, can

occur independently of MPT and dissipation of the voltage gradient $\Delta\psi m$.[40,52] Although the mechanisms by which cytochrome c is translocated from mitochondria remain elusive, mitochondrial volume dysregulation with matrix swelling in response to death stimuli leads to outer-mitochondrial membrane rupture that results in the translocation of intermembrane proteins to the cytosol and these events can occur while $\Delta\psi m$ is maintained.[42] Bcl-xL and perhaps Bcl-2 function to prevent the loss of outer membrane integrity possibly via their ion channel activity and so impede mitochondrial cytochrome c release.[9] Since it was shown that caspases can directly induce permeability transition and that caspase inhibitors block MPT under certain circumstances, mitochondrial $\Delta\psi m$ disruption resulting from caspase activation may amplify the initial signal triggered by cytochrome c/Apaf1/caspase-9 complex.[52,53]

Direct interaction between Bcl-xL and cytochrome c has been shown.[48] Bcl-xL interacts also with Apaf-1/caspase 9 and inhibits the oligomerization and activation of caspase-9.[16,17] Moreover, Apaf-1-dependent activation of caspase-9 is inhibited by recombinant Bcl-xL in a dose dependent manner.[17] Ced-4-induced apoptosis by ectopic expression in mammalian cells is abrogated by Ced-9 and Bcl-xL. Ced-4 interacts with ced-9 and Bcl-xL and the proapoptotic proteins Bax, Bik and Bak disrupt the Ced-4/Bcl-xL interaction.[54] Like Bcl-xL and Bcl-2, ced-9 protein localizes primarily to intracellular membranes. Coexpression of ced-4 and ced-9 into cells results in a shift in the cellular distribution of ced-4 protein from the cytoplasmic compartment to a membrane fraction enriched in mitochondria.[54] These results suggest an alternate mechanism of regulation of caspase activation by ced-9/bcl-like proteins at mitochondrial level; ced-9/bcl-like proteins may interact with and sequester the essential molecules (Apaf-1, cytochrome c) implicated in the initiation phase of caspase activation (Fig. 1). Such a mechanism could explain the protection from apoptosis induced by microinjected cytochrome c in cells overexpressing Bcl-2.[55]

CELL DEATH RECEPTOR-MEDIATED CASPASE ACTIVATION

Activation of some cell surface death receptors can lead to a near-to-immediate activation of initiator caspases and subsequent cell death. This growing novel family of cell death receptors includes TNF-R1, CD95/APO-1/Fas, DR-3/WSL-1/TRAMP/LARD/APO-3, DR-4/TRAIL-R1, DR-5/TRAIL-R2 and DR-6.[56,57] These receptors are characterized by the presence of a death domain (DD) in their cytoplasmic region and the ability to transduce proliferative and/or apoptotic signals by recruiting cytoplasmic proteins that contain a similar DD motif. The death-signaling pathways emerging from aggregation of the cell surface receptor CD95/APO-1/Fas and TNF-R1 are the best characterized. For Fas, stimulation by its natural ligand or by agonist antibodies leads to the formation of a death-inducing signaling complex (DISC). The DISC contains FADD/MORT-1, a *Fas-a*ssociated *d*eath *d*omain adapter protein that binds to the aggregated receptor through its C-terminal DD. FADD/MORT-1 also contains a N-terminal death effector domain and recruits caspase-8 or -10 proenzyme through a DED-DED interaction (Fig. 1). Once caspase-8 zymogen is recruited to the DISC, it is proteolytically processed to the active protease containing p17 and p12 subunits and released from the DISC.[56] Recent data suggest that caspase-8 activation at the DISC occurs by autoprocessing after oligomerization of the zymogen.[58] Active caspase-8 in turn can process other downstream effector caspases like caspase-3.[59]

Similarly, oligomerization of TNF-R1 by TNF-α binding induces a DISC with recruitment of the adapter molecule TRADD (*TNFR-a*ssociated *d*eath *d*omain) that recruits FADD that binds to caspase-8. Alternatively, TRADD can recruit caspase-2 via the TRADD/RIP/CRADD/RAIDD complex. Caspase-8 activation after CD95/Fas engagement is the initiator step of a protease cascade amplification that leads to an irreversible point resulting in cellular disassembly. Whether mature caspase-8 cleaves and activates directly downstream effector caspases or whether the amplifying step involves the mitochondrial apoptotic pathway remain unresolved questions. It has been shown in some cell types that Fas-induced apoptosis is not inhibitable by Bcl-2 and/or Bcl-xL.[60] These results suggest that in some cells, caspase-8 activates directly downstream effector caspases in a mitochondria-independent manner and that Fas can trigger an apoptotic pathway that bypasses the CED-9/Bcl-like mitochondrial checkpoint. However, other studies show that CED-9/Bcl-like family members can inhibit CD95/Fas-mediated apoptosis.[61,62] In MCF7-Fas cells overexpressing Bcl-xL, caspase-8 is processed normaly after anti-Fas or TNF-α stimulation but cells are protected from apoptosis. Moreover, apoptosis induced by microinjection of recombinant active caspase-8 is inhibited by Bcl-xL suggesting that Bcl-xL blocks apoptosis downstream of caspase-8 activation.[61]

Susin and coworkers have proposed a pathway for Fas-induced apoptosis that involves a Bcl-2-non-inhibitable mitochondrial effector phase.[63] In this scenario, Fas-crosslinking and subsequent activation of caspase-8 lead to activation of downstream caspases that induce MPT and $\Delta\psi m$ disruption. These events cause the release of mitochondrial AIF that acts as an effector protease and induces DNA fragmentation, or contributes to further induction of MPT and activation of downstream effector caspases like caspase-3. In mitochondria overexpressing Bcl-2, caspase-induced MPT and AIF release occurs to a similar extent as in control mitochondria, providing an explanation for the failure of Bcl-2 to inhibit Fas-mediated apoptosis in some cells. In a cell-free system based on *Xenopus* egg extracts in which apoptosis requires mitochondria, it has been shown that high concentration of active caspase-8 triggers apoptosis through a mitochondria-dependent and -independent pathway. In this system, a small amount of active caspase-8 triggers the apoptotic process, predominantly, if not exclusively, through the mitochondria-dependent pathway that is inhibitable by Bcl-2. In this mitochondria-dependent context, the inhibitory effect of Bcl-2 on cytochrome c translocation can be lost if sufficient amount of caspase-8 is added to the extract.

These results support a model in which the mitochondrial release of cytochrome c amplifies signals initiated by caspase-8.[64] The inhibitory effect of CED-9/Bcl-like on this amplification loop is bypassed if sufficient amount of

caspase-8 is activated at the initiation step that leads to direct activation of the effector caspase-3.[59] Scaffidi and al. have recently defined two cell types based on the dependence of caspase activation and apoptosis on mitochondrial function in CD95-mediated apoptosis. In type I cells, caspase-8 is activated at the DISC level and promotes activation of caspase-3 in a mitochondria-independent manner. In type II cells, strong activation of caspase-8 and caspase-3 occurs downstream of mitochondria activation and requires mitochondrial function. In these type II cell lines, CD95-mediated apoptosis is inhibited by Bcl-2 and Bcl-xL.[65] According to the model, inhibition of CD95-triggered apoptosis by Bcl-xL downstream of a strong activation of caspase-8 in MCF7-Fas cells is explained by a deficiency in caspase-3 expression.[61] The lack of sufficient amount of caspase-3 would impede a direct activation of downstream caspases by caspase-8 and thus engages the CED-9/Bcl-like inhibitable mitochondrial pathway.

More direct information about the role of caspase-8 in apoptosis triggered by CD95/Fas/APO-1, TNF-R1 and DR-3/WSL-1/TRAMP/LARD/APO-3 is provided by caspase-8 deficient mice.[20] In this recent work, Varfolomeev and coworkers show that fibroblasts derived from the caspase-8$^{-/-}$ embryos are resistant to apoptosis induced by TNF-R1, CD95/Fas/APO-1 and DR-3/WSL-1/TRAMP/LARD/APO-3 receptors. This study suggests a central role of caspase-8 for these cell death receptor signaling pathways, and supports a model in which caspase-8 activation at the DISC level, is the initiation step. Recently, two independent studies established a molecular link between the activated caspase-8 at the CD95 receptor and the mitochondrial death machinery.[66,67] In these studies, it has been shown that the BH3-containing death agonist Bid protein is a specific proximal substrate of caspase-8 during Fas-induced apoptosis. Cleavage of Bid by caspase-8 yields a C-terminal, BH3-containing fragment (tBID) that activates downstream mitochondrial apoptotic events. After cleavage by caspase-8, tBID translocates onto mitochondria where it colocalizes with Bcl-xL, leading to cyochrome c release, $\Delta\psi$m disruption, and ultimately cell shrinkage and nuclear condensation (Fig. 1). tBID-induced mitochondrial damage is antagonized by Bcl-xL or Bcl-2 overexpression. These results provide new evidence for the involvement of the mitochondrial death pathway in apoptosis triggered by CD95/Fas/APO-1 receptor. Apaf-1/caspase-9 complex has been described as the upstream activator of caspase-3 and -7 in cytochrome c mediated apoptosis.[15] However, Apaf-1 deficient thymocytes or activated T lymphocytes and caspase-9 deficient thymocytes remained sensitive to CD95-mediated apoptosis.[21,22,26,27] These results do not necessarily rule out the mitochondrial implication in CD95-induced apoptosis but suggest that other caspases and/or Apaf-1 homologs might be implicated in this pathway.

CASPASE ACTIVATION IN CHEMOTHERAPY-INDUCED APOPTOSIS

Several studies have revealed the activation of various caspases in cells treated with anticancer drugs and generally the effector caspase-3, -7 and -6 but also caspase -9, -2 and in some cases -8, are found activated.[27,55,58,59,68,74] Although, the precise molecular mechanisms triggered by apoptosis-inducing agents used in cancer therapy remain unknown, many studies strongly suggest now that the mitochondrial pathway of caspase activation plays the central role in cell death induced by genotoxic drugs (Fig. 2).

1. Chemotherapeutic agents, including the DNA topoisomerase I and II inhibitors camptothecin and etoposide, the antimetabolites 1-β-D-arabinofuranosylcytosine(cytarabine) and 2-chloroadenosine, the anthracycline antibiotic doxorubicin and the microtuble-disrupting agent paclitaxel, induce mitochondrial $\Delta\psi$m disruption that is inhibitable by Bcl-2 and Bcl-xL.[41]
2. The ced-9/Bcl-like protein members are key regulators of caspase activation and downstream nuclear events in apoptosis triggered by a variety of drugs like camptothecin, etoposide, vinblastine, paclitaxel, cisplatin and cytarabine.[35-39]
3. Mitochondrial cytochrome c release before or concurrent with caspase activation is observed in different cell types after drug treatments.[46,47]
4. Overexpression of Bcl-xL or Bcl-2 antagonizes cytochrome c release from mitochondria and subsequent caspase-3 activation triggered by anticancer drugs.[46,47]
5. Apaf-1 overexpression sensitizes cancer cells to paclitaxel or etoposide and this is accompanied with increased caspase-9 and -3 activities.[75]
6. Cells deficient in Apaf-1 or caspase-9 are protected from apoptosis induced by anticancer drugs such as cisplatin and etoposide[21,27] whereas cells deficient in caspase-8 and -2 show no protecting effect against anticancer drugs such as etoposide or doxorubicin.[18,20] Cells deficient for caspase-3 show only reduced chemosensitivity to doxorubicin suggesting that other effector caspases can compensate for caspase-3[19] (perhaps caspase-7).

In some cells, a cross-resistance between anticancer drugs and CD95-mediated apoptosis is observed. This observation reflects alterations in common downstream components of the apoptotic pathways triggered by CD95 and DNA damaging agents or suggests that drug-induced apoptosis could be mediated in some cases by the death receptor Fas signaling pathway. The implication of the CD95 death pathway in chemotherapy-mediated apoptosis remains an unresolved question documented by controversial data. Some data suggest that the CD95-receptor system plays a central role in apoptosis induced by anticancer drugs with different mechanisms of action. For example, it has been shown in neuroblastoma cells that cytotoxic compounds such as etoposide, doxorubicin and cisplatin lead to induction of CD95-L and upregulation of CD95 receptor by de novo gene expression. In addition, treatment of these cells with $F(ab')_2$ anti-CD95 antibody fragments that interfere with CD95 receptor/ligand interaction, strongly reduces apoptosis induced by cytotoxic drugs, suggesting that the cell-death mechanism activated requires the CD95 death-signaling pathway.[76] Involvement of the CD95 death pathway in drug-induced-apoptosis is suggested also by studies in leukemia and hepatoma cells.[77]

The molecular mechanism that leads to the activation of the CD95 cell-surface death receptor after genotoxic stresses

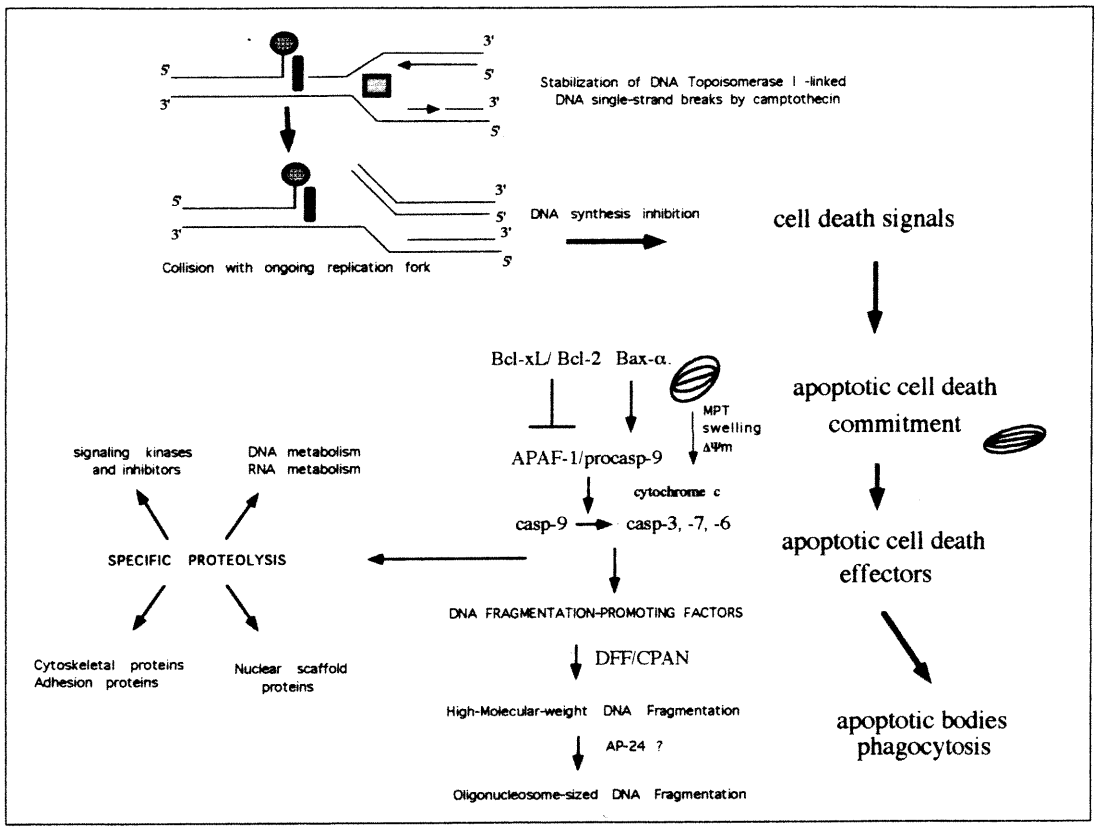

Fig. 2 **Schematic representation of activation and role of caspases in apoptosis mediated by the DNA topoisomerase I inhibitor camptothecin.**

remains obscure. Herr and coworkers showed that ceramides strongly induce apoptosis, CD95 expression and caspase activation. CD95 signaling is required for ceramide-induced apoptosis because inactivation of the CD95 pathway by dominant negative FADD or antisense strategy leads to a strong reduction of cell death mediated by ceramides. In fibroblasts from Niemann-Pick patients, that present a deficiency of the acid sphingomyelinase gene, ceramide production and apoptosis after doxorubicin treatment is strongly reduced and these cells fail to upregulate CD95-L. In these cells, stimulation with exogenous C2-ceramide induces CD95-L expression and apoptosis; moreover, a direct activation of the CD95 receptor with an agonist antibody induces apoptosis.These data indicate that the activation of the CD95 receptor/ligand system in anticancer drug-induced apoptosis is mediated by the sphingomyelin pathway.[78] However, several finding show that chemotherapeutic agents trigger apoptosis independently of Fas signaling. For example, etoposide, doxorubicin, methotrexate, topotecan and cisplatin triggered apoptosis in Fas-resistant Jurkat cells and apoptosis activation was comparable to that of parental cells.[79]

It has been shown that fludarabine-, doxorubicin- and cisplatin-induced apoptosis in human CEM cells is accompanied by upregulation of Fas-L expression. However, in these cells, inhibition of CD95 signaling pathway by CrmA expression or application of neutralizing anti-Fas/FasL antibodies did not inhibit apoptosis induced by these agents.[80] The Fas-independent mechanism of chemotherapy-induced caspase activation is also highlighted by studies using caspase-8 or FADD/MORT-1 deficient cells[20,81] and in cells overexpressing CrmA, a potent inhibitor of the initiator caspase-8.[82,83] From all these studies, it appears that the implication of the Fas signaling system after chemotherapy remains questionable. Some studies suggest that it could participate in some cases but most of the studies indicate that it is not essential for the activation of caspases and apoptosis in cells treated with anticancer agents. However, gene activation, processing and secretion of FasL or other death ligands by treated cells during drug treatment may have significant effects clinically on neighboring and even distant cells in humans.

FUTURE PERSPECTIVES

Although we understand better how caspases are activated following anticancer drug treatment, the exact mechanisms

by which different drugs that mediate different types of damage, converge on the mitochondrial pathway of caspase activation remain elusive. The search to identify signals that link chemotherapy to alterations in mitochondria is just beginning. Strategies to overcome drug resistance to cancer treatment could involve the induction of apoptosis through caspase activation. Several strategies can be envisioned to promote caspase activation in tumor cells. First, new compounds that block the activity of the antiapoptotic members of the Ced-9/Bcl family such as Bcl-2 and Bcl-xL may be designed.[5] Such compound could be targeted to the BH4 domain to prevent the interaction of antiapoptotic proteins with adapter molecules like Apaf-1 or putative close homologs. An alternate approach could be to design drugs that will impede the pore-forming domain of Bcl-xL or Bcl-2 that lies between the two central hydrophobic α-helices, α5 and α6. Third, new compounds that act on the mitochondria megachannels are expected to promote the dysfunction of mitochondria and thus provoke AIF or cytochrome c release that accelerate caspase-9 activation. Several proteins that form the MPT pores may be targeted such as the adenine nucleotide translocator protein (ANT), porins and cyclophilin D.[41] An other alternative approach is to design agents that will activate the death receptor complexes associated with some initiator caspases.[56] The persistence of growth factor dependency in malignant cells transformed by oncogenes is a central feature of cancer cell growth. Inhibition of these growth-factor signaling pathways leads to caspase activation and apoptosis in cancer cells.[84] Therefore, antigrowth factor cancer therapies may be an additional strategy to promote tumor cell death. The main challenge for all these strategies is selectivity. Understanding how apoptosis is regulated in specific cancer cells may lead to the development of new strategies to circumvent drug resistance in cancer specific tumors.

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