Université de Montréal

Transcriptional Interference between Nuclear Receptors and Activator Protein-1

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Thèse présentée à la Faculté des études supérieures
en vue de l'obtention du grade de
Philosophiae Doctor (Ph.D.)
En Biochimie

August 2001

Université de Montréal
Faculté des études supérieures

Cette thèse intitulée

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Résumé

Les récepteurs nucléaires sont des facteurs transcriptionnels impliqués dans la régulation de la croissance cellulaire, en partie dû à leurs effets génomiques. Une voie alternative est l'interférence entre les récepteurs nucléaires et le facteur transcriptionnel AP-1 (activator protein-1), composé de protéines induites par plusieurs facteurs de croissance et molécules de signalisation. Dans ce projet, j'ai analysé les mécanismes d'interférence transcriptionnelle entre les récepteurs des oestrogènes (ER), les récepteurs des glucocorticoïdes (GR) et AP-1.

Premièrement, j'ai étudié la régulation de l'activité de AP-1 par les oestrogènes et les anti-oestrogènes. Les anti-oestrogènes peuvent empêcher la stimulation de la croissance cellulaire par l'estradiol dans les tumeurs mammaires. Cependant, certains anti-oestrogènes comme le tamoxifène (TAM) peuvent induire la croissance cellulaire utérine lorsqu'administrés en absence d'oestrogènes. Il a été proposé que TAM pourrait induire la croissance cellulaire via l'activation d'AP-1. Pour étudier l'activité agoniste histospécifique des anti-oestrogènes comme le tamoxifène, nous avons comparé l'activation transcriptionnelle de vecteurs rapporteurs contenant des éléments de réponse aux oestrogènes (EREs) classiques ou des éléments de réponse à AP-1 dans la lignée cellulaire de carcinome mammaire MCF7 et dans la lignée cellulaire de carcinome de l'endomètre Ishikawa. Nos résultats indiquent que la présence d'EREs dans les promoteurs est suffisante pour médier l'activité agoniste histospécifique des anti-oestrogènes. L'activation d'AP-1 par l'estradiol n'a pas été observée dans des lignées de cellules propageant un gène rapporteur regulé par le TPA. AP-1 peut être

cependant activé par de fortes concentrations (micromolaires) de TAM dans ces cellules. Cependant, ces concentrations d'anti-oestrogènes mènent à une mort cellulaire plutôt qu'à la prolifération cellulaire. Utilisant les mêmes lignées de cellules Ishikawa transfectées de manière stable, nous avons observé que les glucocorticoïdes n'induisent pas de répression de l'activité d'AP-1. Par contre nous observons une répression de l'activité d'AP-1 par les glucocorticoïdes dans les cellules HeLa transfectées de manière stable. Ces résultats suggèrent que AP-1 n'est pas sensible aux hormones stéroïdiennes dans les cellules Ishikawa.

Deuxièmement, j'ai étudié la régulation de l'activité des récepteurs stéroïdiens par les voies de signalisation menant à l'activation d'AP-1. J'ai observé que les activateurs des MAP kinases, tels que TPA et EGF, modulent positivement l'activité transcriptionnelle de GR. Cet effet est précoce, spécifique à GR et peut être aboli par les inhibiteurs de la voie de ERK et de la voie de p38. Pour analyser les mécanismes de cette régulation, nous avons testé quatre hypothèses: 1) régulation positive de l'expression de GR par TPA. 2) régulation positive de la phosphorylation de GR par TPA. 3) régulation positive de la phosphorylation de coactivateurs de GR par TPA. 4) interactions protéine-protéine entre GR et Jun ou Fos, menant à une augmentation de l'activité transcriptionnelle de GR. Nos résultats indiquent que l'expression et la phosphorylation de GR ne sont pas régulées par le TPA *in vivo*, et que la phosphorylation de TIF-2 n'est pas le phénomène limitant dans la stimulation de la transcription dépendante de GR par le TPA. De plus, l'augmentation de l'activité transcriptionnelle de GR par TPA n'est pas médiée via l'induction de Jun ou Fos.

Finalement, j'ai observé que le domaine de liaison à l'ADN de GR est crucial pour médier une stimulation transcriptionnelle de GR par le TPA. Ces résultats suggèrent que le domaine de liaison à l'ADN est la cible de la régulation par les MAP kinases, problement par le recruitment de coactivateurs dont l'activité serait modulée par cette voie de signalisation.

Summary

Nuclear receptors constitute a large superfamily of DNA binding transcriptional regulators that can regulate a diversity of important cellular events, such as development, differentiation, and responses to extracellular stimuli. These effects are mediated mostly by the genomic effects of nuclear receptors which bind selectively to DNA (hormone response element), and regulate target gene transcription. An alternative pathway of transcriptional regulation is the crosstalk between nuclear receptors and other transcription factors, such as NFκB, or activator protein-1 (AP-1). Transcription factor AP-1 is composed of nuclear proteins encoded by the Jun family and Fos family of protooncogenes, whose transcription is rapidly induced by a number of growth factors and other signaling molecules. AP-1 is implicated in diverse aspects of cell growth, differentiation and development. In this project, I have focused on the analysis of the mechanisms of transcriptional interference between nuclear receptors and AP-1.

First I studied regulation of AP-1 activity by steroid hormones, such as estrogen and glucocorticoids. Estrogen has proliferative effects in breast and uterine carcinoma cells. Antiestrogens can block estradiol stimulation of cellular growth in breast cancer cells and in breast tumors (Katzenellenbogen et al., 1995). However, some antiestrogens like tamoxifen (TAM) can, when administered in the absence of estrogen, induce uterine cell growth (van Leeuwen et al.,1994; Fisher et al.,1994). It has been proposed that antiestrogens with tissue-specific agonist activity may induce cell growth through AP-1 activation (Webb et al., 1995). To investigate the tissue-specific agonism of antiestrogens like tamoxifen, we compared transcriptional

activation of reporter constructs containing classical estrogen response elements (EREs) as well as AP-1 response elements in the breast carcinoma cell line MCF7 and in the endometrial carcinoma cell line Ishikawa. Our results indicate that the presence of EREs in promoters is sufficient to mediate cell-specific agonism of antiestrogens. Activation of AP-1 by estradiol was not observed in stable cell lines propagating an AP-1 reporter vector. AP-1 was activated by high concentrations (micromolar) of TAM in stably transfected Ishikawa. However, these high concentrations of antiestrogens induced cell death rather than proliferation.

Using the same stably transfected Ishikawa cell lines that propagate AP-1 responsive reporter vectors, we found that glucocorticoids do not repress AP-1 activity, although we observed AP-1 repression by glucocorticoids in stably transfected Hela cells that propagate the same AP-1 reporter vector. In addition, results from cDNA arrays indicate that AP-1 target genes are not regulated by estrogen in Ishikawa cells. Collectively, these results suggest that AP-1 is not sensitive to steroid hormones in Ishikawa cells.

To study the regulation of steroid receptor activity by mitogenic signaling pathways, we developed a simple and sensitive reporter system to monitor glucocorticoid response element (GRE)-mediated transcription in response to signaling molecules. We found that MAP kinase activators, such as TPA and EGF, positively modulate GR transcriptional activity in epithelial cells. This effect is early, specific to GR, as under the same conditions we did not observe this effect on ER, and can be inhibited by ERK pathway inhibitor PD 98059 and p38 pathway inhibitor SB203580, respectively.

To analyze the mechanisms of this transcription regulation, we tested four hypotheses: 1) upregulation of GR expression by TPA. 2) upregulation of GR phosphorylation by TPA. 3) upregulation of GR coactivator phosphorylation by TPA. 4) Protein-protein interactions between GR and TPA-induced Jun or Fos leading to enhanced GR transcriptional activity. Our results obtained from Northern blot and Western blot indicate that GR mRNA and protein expression are not regulated by TPA. Our data also demonstrate that TPA has no significant effect on GR phosphorylation, and that GR phosphorylation mutant S226E and S226A did not alter GRE-mediated transcriptional activity in COS-7 and Jurkat cells. Our results also demonstrate that overexpression of TIF2 did not further enhance TPA-induced GRE-mediated transcription, suggesting that phosphorylation of TIF-2 is not the limiting phenomena in the potentiation of GR-dependent transcription by TPA. Furthermore our results of transient transfection indicate that overexpression of cJun, JunB, JunD and cFos does not mimic TPA effect, implying that enhancement of GR transcriptional activity by TPA is not mediated through induction of cJun, JunB, JunD and cFos.

In addition, we mapped the GR domain that is targeted by TPA. Using GR and ER chimeric receptor, GR A/B and Gal4 chimeric protein and a series of GR deletion mutants, we found that the GR DNA binding domain is crucial to mediate enhancement by TPA. GR tau1 or AB region was not essential, but the presence of a ligand binding domain was necessary to mediate TPA effects. These results suggest that the effect of TPA on GR-dependent transcription may be mediated by cofactors of GR that interact with the DNA binding domain, or that the DNA binding domain may

allosterically regulate the activity of GR cofactors recruited via the ligand binding domain.

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List of Abbreviations

ACF	ATP-utilizing chromatin assembly and remodeling factor
ACTH	adrenocorticotropic hormone
ACTR	Activator of the Thyroid and Retinoic acid receptor
AF-1	Activation Function-1
AF-2	Activation Function-2
AIB1	Amplified In Breast cancer 1
AP-1	Activator protein-1
AR	Androgen Receptor
ATF	Activating transcription factor
ATP	adenosine triphosphate
BRG-1	brahma-related gene-1
CAF-1	Chromatin assembly factor-1
CaMK	Calmodulin-dependent protein kinase
CAT	Chloramphenicol Acetyl Transferase
CBP	CREB Binding Protein
CDK	Cyclin dependent kinase
CHRAC	Chromatin Accessibility Complex
CRE	cAMP Responsive Element
CREB	CRE Binding protein
CRH	corticotropin releasing hormone
DBD	DNA Binding Domain
E2	17β-estradiol
EGF	Epidermal Growth Factor
ER	Estrogen Receptor
ERK	Extracellular signal Regulated Kinase
FRK	Fos regulating kinase
GR	Glucocorticoid Receptor
GRE	Glucocorticoid Response Element
GRIP-1	Glucocorticoid Receptor Interacting Protein-1
GTFs	General Transcription factors
HAT	Histone Acetyl Transferase
hsp90	heat shock protein 90
hbrm	human brahma
HPA axis	hypothalamic-pituitary-adrenal axis
ISWI	imitation SWI
JAK	Janus kinase
JNK	Jun N-terminal Kinase
LBD	Ligand Binding Domain
MAPK	Mitogen Activated Protein Kinase
MAPKK	Mitogen Activated Protein Kinase Kinase
MAPK KK	Mitogen Activated Protein Kinase Kinase Kinase
MKPs	MAP kinase phosphatases

N-CoR	Nuclear receptor Co-Repressor
NFκB	Nuclear Factor κB
NTP	Ribonucleoside triphosphate
NuA4	Nucleosomal acetyltransferase of histone H4
NURF	Nucleosome remodeling factor
P/CAF	p300/CBP Associated Factor
p/CIP	p300/CBP/co-Integrator-associated Protein
PIC	Preinitiation complex
PKA	Protein kinase A
PKC	Protein kinase C
pol II	RNA polymerase II
PPARs	Peroxisome Proliferator Activated Receptors
PR	Progesterone Receptor
RAC3	Receptor Associated Coactivator 3
RAR	Retinoic Acid Receptor
RSC	remodels the structure of chromatin
RXR	Retinoid X Receptor
SAGA	Spt-Ada-Gcn5-acetyltransferase
Ser	Serine
SIE	Sis-inducible enhancer
SMRT	Silencing Mediator for RXR and TR
SRA	RNA coactivator for steroid receptors
SRB	Suppressor of RNA Polymerase B
SRC-1	Steroid Receptor Coactivator-1
SRE	Serum response element
SRF	Serum response factor
STAT	Signal transducer and activator of transcription
SWI/SNF	(mutants) defective in mating-type switching/sucrose nonfermentable
TBP	TATA-binding protein
TCFs	Ternary complex factors
Thr	Threonine
TIF2	Transcriptional Intermediate Factor 2
TPA	12-O-tetradecanoate-13-acetate
TR	Thyroid hormone Receptor
TRAM-1	Thyroid hormone Receptor Activator Molecule-1
TRE	TPA-responsive element
TSA	Trichostatin A
Tyr	Tyrosine
VDR	Vitamin D_3 Receptor

Acknowledgments

I would like to gratefully acknowledge my research director Dr. Sylvie Mader and my codirector Dr. John White, for their supervision and support, for their patience and encouragement, and for ensuring financial support over the past fiive years.

I would like to express my sincere thanks and appreciation to Dr. B. Franze Lang, Dr. Stephen Michnick, Dr. Normand Brisson and Dr. Daniel Skup for serving on my thesis committee, for taking the time to guide me through my Ph.D.

I am grateful to Dr. Sylvain Meloche for his valuable discussions and suggestions. I thank Kaleish Golpabh for his technical support.

I am also grateful to my colleagues at Dr. Sylvie Mader's lab, for their continued help on my French. Special thanks go to my friends, Geneviève Mêlançon, Denis Nguyen, Etienne Rouault, for providing a constant source of encouragement and support, and for being there for me at all times. I would like to thanks Mathieu Lupien for reading the manuscript.

Finally, I appreciate the studentship I received from FRSQ (Fonds de recherche) and university of Montreal.

To

my parents,

Husband, Yongsen Zhao

daughter, Ming Zhao

and in loving memory of my grandparents

Chapter I

INTRODUCTION

Not too long ago (mid to late 1980s), our view of gene regulation by steroid hormones, growth factors and cytokines was comparatively simple. Steroid hormones lead to gene induction by activation of their corresponding steroid hormone receptors, which are ligand-inducible transcription factors. On the other hand, growth factors, pro-inflammatory cytokines, and phorbol esters initiate signaling at the cell membrane, leading to activation of nuclear transcription factors such as activator protein-1 (AP-1) expression. The two signaling pathways that regulate the activity of these transcription factors were thought to coexist in the nucleus without any exchange or interaction. However, analysis of the human collagenase promoter soon led to disruption of this simple picture. While potently induced by growth factors and cytokines, expression of collagenase is strongly repressed by glucocorticoids. Further analysis demonstrated that a consensus AP-1 binding site such as the one found between positions –73 and –63 in the human collagenase promoter, and synthetic promoters which contain AP-1 binding sites in front of a minimal promoter, are sufficient for mediating repression by glucocorticoids (Jonat et al., 1990; Schüle et al., 1990; Yang-Yen et al., 1990). This form of negative crosstalk (transcriptional interference) is of great physiological significance and clinical importance. Recent experiments have demonstrated that the crosstalk ability of glucocorticoid receptor is essential for mouse development, while the activation of target promoters carrying a glucocorticoid responsse element (GRE), is surprisingly dispensible for suvival under animal house conditions (Reichardt et al., 1998). It is believed that this negative crosstalk and analogous interactions with other transcription factors (e.g. nuclear factor-κB) underlie the anti-inflammatory and immunosuppressive activity of

glucocorticoids (Karin and Chang, 2001). Thus, it could be argued that the most important function of glucocorticoids and glucocorticoid receptor is not necessarily the classical activation of GRE-containing genes, but the repression via transcriptional interference of AP-1 and NF-κB activities. Still the exact molecular mechanisms of the crosstalk remain to be determined.

Crosstalk is not limited to glucocrticoid receptor and AP-1. Rather, many members of the nuclear hormone receptor superfamily, including class II receptors, have been shown to participate in such crosstalk (Göttlicher et al, 1998). This crosstalk between nuclear receptors and AP-1, which is usually inhibitory, can also under certain conditions be stimulatory. It was previously reported that estrogen and the antiestrogen tamoxifen could activate the AP-1 pathway (Webb et al, 1995; Uht et al., 1997). Furthermore, activators of the AP-1 pathway can also modulate nuclear receptor functions. However, there are conflicting reports in the literature regarding the effect of AP-1 activators on glucocorticoid receptor transcriptional activity (Rogatsky et al 1998; Moyer et al., 1993; Maroder et al., 1993; Vacca et al., 1989). In this project, we studied different aspects of the crosstalk between nuclear receptors and AP-1. Current knowledge about signaling by nuclear receptors and by AP-1, and about the crosstalk between these two families of transcription factors are reviewed in this section. Multiple mechanisms of crosstalk are also discussed.

I. SIGNALING BY NUCLEAR RECEPTORS

1. Nuclear receptor classification, structure and function

1.1 Nuclear receptor classification

The nuclear receptor (NR) superfamily comprises over 150 different proteins that have evolved to mediate a complex array of extracellular signals into transcriptional responses. Basically, nuclear receptors are classified as steroid receptors, heterodimeric receptors and orphan receptors. Steroid receptors include estrogen receptor (ER), glucocorticoid receptor (GR), mineralocorticoid receptor (MR), androgen receptor (AR) and progesterone receptor (PR). Retinoid X receptor (RXR) can heterodimerize with thyroid hormone receptor (TR), retinoic acid receptor (RAR), vitamin D_3 receptor (VDR) and peroxisome proliferator-activated receptors (PPARs). Additionally, the superfamily includes numerous "orphan receptors", denoted as such because their endogenous ligands, if they exist, are either unknown, e.g. chicken ovalbumin upstream promoter transcription factor (COUP-TF), or have just been identified e.g. the pregnane receptor (PXR) (Table 1). The evolutionary relationship among the steroid /nuclear receptors has been deduced by the high conservation in their DNA binding domains (DBDs) and in their less conserved ligand binding domains (LBDs) and indicates that this large group of proteins arose from a common ancestral molecule (Laudet 1997).

Steroid receptors are thought to interact with their cognate DNA sequences in regulated genes only in the presence of ligand. These nuclear receptors are also called type I nuclear receptors. Heterodimeric receptors appear to bind their cognate hormone response elements in the presence or absence of ligand. These nuclear receptors are called type II receptors (Klinge, 2000).

Table 1. Nuclear receptors*

Receptors with known ligand(s)		Orphan receptors	
Steroid receptors	Heterodimeric receptors	Orphan receptors With unknown ligand	Orphan receptors with identified ligands
Estrogen ERα	Retinoid X receptor	COUP-TFI/EAR3	BXR (benzoate X
Estrogen ERβ	RXRα	COUP-TFII/ARP-1	receptor)
Glucocorticoid GR	RXRβ	DAX-1	CARβ (constitutive
Mineralcorticoid MR	RXRγ1, γ2	EAR2	androstane receptor)
Androgen AR	Thyroid hormone	hERR1	FXR (farnesoid X
Progesterone PR	receptor	hERR2	receptor)
	TRα1, α2	GCNF	SXR (steroid and
	TRβ1, β2	HNF-3	xenobiotic receptor)
	Retinoic acid receptor	HNF-4	LXR (liver X receptor)
	RARα1, α2	SF-1	PAR (human pregnane
	RARβ1, β2,	nur77/NGF1-B	receptor)
	β3, β4	RTR	PXR (mouse pregnane
	RARγ1, γ2	RZR/ROR	X receptor)
	Vitamin D3 receptor	RZRb	
	Peroxisome proliferator-	SHP	
	activated receptors	TAK1	
	PPARα	TOR	
	PPARβ	TR2-11	
	PPARγ	TR4	

*This table is not exhaustive

1.2 Functional domains of nuclear receptors

All nuclear receptor family members display a similar structural organization in six regions of homology (A to F) (Krust et al., 1986). The amino-terminal region A/B is the most variable region and in some receptors contains transcriptional activation function 1 (AF-1). AF-1 modulates transcription in a ligand independent, cell-type specific and promoter specific manner (Torchia et al, 1998). Region C is highly conserved among nuclear receptors, and contains the DNA-binding domain (DBD) composed of two zinc finger motifs that mediate nuclear receptor interaction with its specific hormone response element. Next to the DNA binding domain is a hinge region (region D) that contains nuclear localization signals. The dimerization and ligand binding domain (LBD, region E) contains transcriptional activation function 2 (AF-2). Some but not all nuclear receptors contain a carboxy-terminal region F of unknown function. The schematic representaion of the functional domains of nuclear receptors is shown in figure 1 (Edwards, 2000).

1.3 DNA recognition by nuclear receptors

Nuclear receptors utilize at least three different modes of DNA recognition. Steroid receptors (type I) form homodimers in the absence of DNA and recognize a core hexanucleotide consensus sequence arranged as an inverted repeat (palindromic stucture) with three unconserved nucleotides seperating the half-site hormone response elements (HREs). The HREs can be divide into two subgroups: the GRE/PRE subgroup (palindromes of PuGAACA) and the ERE subgroup (palindromes of PuGGTCA) (Mader et al.,1989). In the absence of hormone, steroid receptors

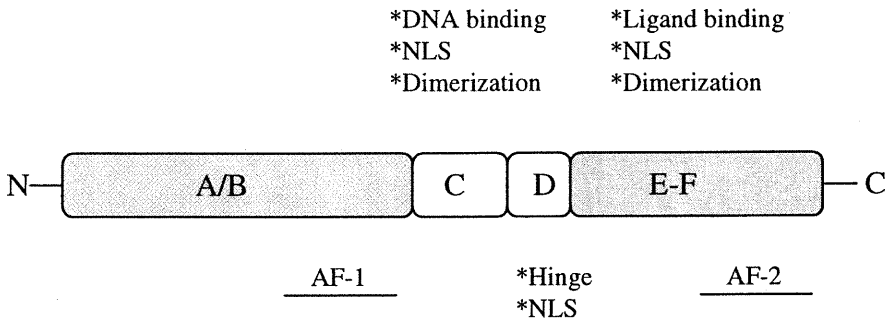

Figure 1. Functional domains of nuclear receptor

Nuclear receptors are modular proteins comprised of less conserved amino-terminal region A/B that contains transcriptional activation function 1 (AF-1), a highly conserved DNA binding domain (DBD) (region C), a hinge region (H) and carboxyterminal ligand binding domain (LBD, region E) that contains transcriptional activation function 2 (AF-2). Some but not all nuclear receptors contain a carboxy-terminal region F of unknown function. Nuclear localization sequences (NLS) and dimerization domains are also shown (adapted from Edwards, 2000).

associate with a chaperone complex composed of heat shock proteins (hsps), immunophilins and other factors. The chaperones help to maintain the receptor in a folded state capable of binding hormone and act as a docking complex to keep the receptor off DNA until activated by hormone. Other classes of nuclear receptors do not appear to form a stable association with hsps and bind to DNA constitutively. Thus, activation of nonsteroid nuclear receptors occurs primarily at a step subsequent to DNA binding. Nuclear receptors for nonsteroid ligands (type II) bind to DNA predominatly as heterodimers with retinoid X receptor (RXR). RXR is a common heterodimer partner required for type II receptors to bind with high affinity to target DNA and to mediate transcription. The type II RXR-heterodimer receptors bind to core hexanucleotide HREs that are arranged as direct repeats (DR). Variable nucletide spacing between the DR half-sites determines the specificity for different RXR-heterodimer receptors (Mangelsdorf and Evans, 1995). Orphan receptors are capable of binding to DRs as homodimers or as heterodimers with RXR. They also recognize extended half site HREs as monomers (Mangelsdorf and Evans, 1995).

1.4 Ligand-dependent nuclear receptor transactivation

Transcriptional activity of most nuclear receptors requires specific ligand binding. Steroids (i.e. estrogens, androgens, progesterones, mineralcorticoids and glucocorticoids), retinoids, thyroid hormones and vitamin D_3 are small lipophilic molecules, and can spontaneously pass through the plasma membranes of their target cells to bind their cognate receptors in the cytosol or nucleus.

The ligand binding domain of nuclear receptors integrates several critical functions: ligand binding, dimerization (homodimerization or heterodimerization),

transactivation, nuclear localization and co-regulator interaction, such as interaction with members of coactivator p160 family and cAMP response element binding protein [CREB] binding protein (CBP)/p300 (see following section on coactivators and corepressors).

To date, the crystal structures of several nuclear receptor ligand binding domains (LBDs) have been solved: the dimeric apo-RXRα, holo (9-cis retinoic acid)-RXRα, monomeric holo-RARγ, monomeric holo-TRα, dimeric holo (estrogen)-and antagonist (raloxifene)-ERα, and apo- and holo (thiazolidinedione)-PPARγ (Bourguet et al. 1995; Egea et al. 2000; Renaud et al. 1995; Shiau et al. 1998; Brzozowski et al. 1997; Uppenberg et al. 1998). These structures revealed that the LBDs are very similar, consisting of 12 highly conserved α helices (numbered H1-H12) and one β turn arranged as an antiparallel α-helical "sandwich" in a three layer structure. Helix H12, which contains the conserved AF-2 core, undergoes an extensive shift upon ligand binding, together with other structural changes (such as bending of helix H3), bring helix H12 into a distinct receptor environment, thus creating the surface(s) which allow binding by coactivators, such as members of the p160 coactivator family and CBP/p300 (Moras and Gronemeyer, 1998).

2. Estrogen receptor, breast cancer and antiestrogens

Lerner and coworkers discovered the first nonsteroidal antiestrogen MER25 in 1958. One year later, Jensen and Jacobson identified the physiological target of estrogen, the estrogen receptor. By the late 1970s, tamoxifen had been developed to treat breast cancer and was approved by the Food and Drug Administration (FDA) in

1985 (Macgregor and Jordan, 1998). Estrogen receptor cDNA was first cloned and sequenced in Dr. Chambon's laboratory in 1986 (Green et al., 1986). All these pivotal results opened the door for the present research on the molecular mechanisms of estrogen and antiestrogen action and have laid the foundation for novel drug discovery for the prevention and treatment of breast cancer, osteoporosis and coronary heart disease.

2.1 Estrogen action

Estrogens exert a wide variety of effects on growth, development, and differentiation, including important regulatory functions within the reproductive system of both females and males, in mammary gland development and differentiation (reviewed in Klinge, 2000). Furthermore, this female steroid hormone is also well known to be deeply involved in many pathophysiological events such as osteoporosis and cancer development in female reproductive organs. In particular, most breast tumors are estrogen dependent for growth (Dickson et al.,1986).

2.2 ERα and ERβ

Estrogens mediate their activities through binding to a specific intracellular receptor protein, the estrogen receptor (ER), encoded by two genes: ERα and ERβ that function both as signal transducers and transcription factors to modulate expression of target genes.

ERα predominates in the uterus and mammary gland, whereas ER beta has significant roles in the central nervous, cardiovascular, and immune systems, urogenital tract, bone, kidney, and lung. The loss of ERα leads to severe gonadal and behavioral phenotypes that result in infertility in both sexes of mice. In contrast,

disruption of the ERβ gene results in subfertility in females whereas male fertility appears unaffected (Lubahn et al., 1993; Weihua et al., 2000; Couse et al., 2000).

2.3 Breast cancer

Breast cancer is the most frequently diagnosed cancer in Canadian women, accounting for about 30% of all new cancer cases each year. It is estimated that in 2001, 19,500 cases will be diagnosed and 5,500 women will die of this disease (National Cancer Institute of Canada: Canadian cancer statistics 2001, refer to http://www.cancer.ca/stats/tables/tab1e.htm.). Statistics also indicate that one out of nine Canadian women is expected to develop breast cancer in her life time and one out of every 25 is expected to die from it (National Institute of Canada: Canadian cancer statistics 1999). Breast cancer is a heterogeneous disease regarding morphology, invasive behavior, metastatic capacity, hormone receptor expression and clinical outcome. Breast cancer is treatable by surgery, radiation therapy, chemotherapy, or hormonal treatment. Early stage cancers are often managed with breast-conserving surgery followed by radiotherapy; however, the exact management, such as the addition of hormone therapy or chemotherapy may be modified by specific patient factors, including hormone receptor status, age, menopausal status and general health (Hortobagyi GN, 1998). Late stage cancers are often managed with chemotherapy or hormone therapy.

2.4 Antiestrogens and breast cancer

Estrogens are well recognized to play a predominant role in breast cancer development and growth and much effort have been devoted to the blockade of estrogen formation and action. The antiestrogen tamoxifen has been most widely used

for the treatment of all stages of breast cancer (Macgregor and Jordan, 2000). In patients with primary breast cancer, tamoxifen delays relapse and prolongs survival in about 20-30%. The frequency of second primary breast cancers in such patients is reduced by about 40% (Powles TJ, 1992). There is tremendous interest in the use of tamoxifen for the prevention of breast cancer because of its demonstrated effect in decreasing the risk of breast cancer recurrence. So, can tamoxifen prevent breast cancer in healthy women? To answer this key question, clinical trials with healthy women have been performed. The largest tamoxifen trial showed approximately 50% reduction in breast cancer incidence in the short term, but two smaller trials did not find any reduction (Fisher et al., 1998; Cuzick et al., 2000). While the antiestrogenic action of tamoxifen is critical to controlling the growth of breast cancer, the increased incidence of endometrial cancers seems to result from a partial agonistic activity of tamoxifen in the uterus. It has been reported that risk of endometrial cancer increased with longer duration of tamoxifen use, with relative risks of 2.0 for 2-5 years and 6.9 for at least 5 years compared with non-users (Fisher et al., 1994; van Leeuwen FE et al., 1994; Bergman L et al., 2000).

2.5 Overview of Antiestrogens

Antiestrogens, which antagonize the actions of estrogens, can be classified into two major groups: antiestrogens which have mixed estrogenic/antiestrogenic actions in laboratory assays (type I) and pure antiestrogens (type II, such as ICI 164,384) that have no estrogen-like properties in laboratory assays. The structures of some of these antiestrogens are shown in figure 2, along with the structures of the naturally

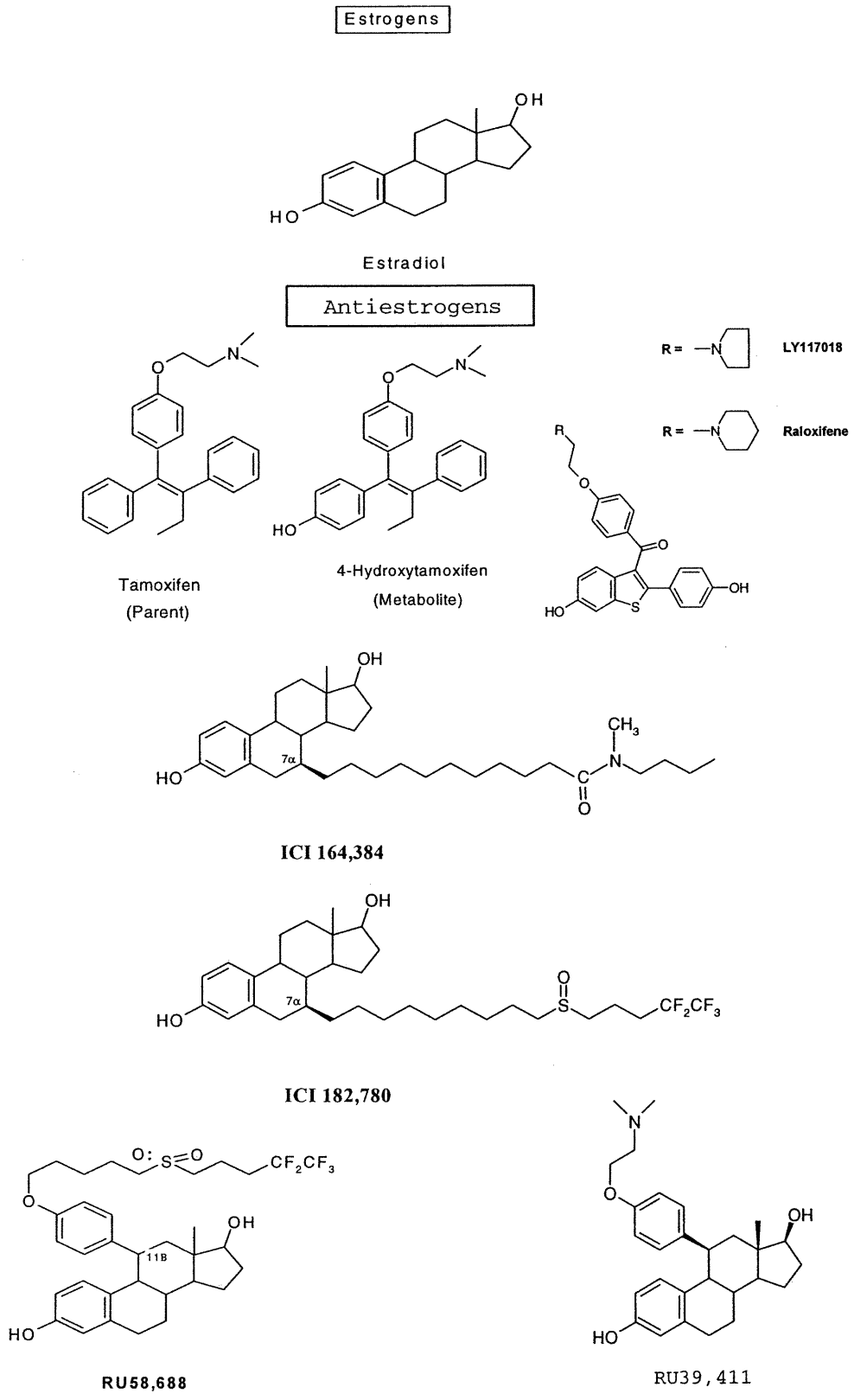

Figure 2. Structures of estrogen and antiestrogens

occurring estrogen 17β-estradiol. Partial antiestrogens can be either steroidal (RU39,411) or non-steroidal, such as the triphenylethylene tamoxifen. Tamoxifen is hydroxylated at the 4-position to produce 4-hydroxytamoxifen (OHT), a metabolic derivative of tamoxifen with increased binding affinity for the ER (Jordan et al., 1977).

The compounds LY 117, 018 and raloxifene (also referred to LY 139,481-Hcl, LY 156,758 and keoxifene) are benzothiophene based antiestrogens (figure 2). They have high binding affinity for the ER, and can block the uterotropic effects of estradiol. It has been suggested that compared to tamoxifen, LY 117, 018 and raloxifen might act at separate sites or by different molecular mechanisms (Black et al., 1980, 1981,1983; Macgregor and Jordan, 1998). Additionally, raloxifene exhibits beneficial effects on bone (preservation of bone density) and the cardiovascular system (decrease in serum cholesterol) (Jordan et al., 1987; Black et al., 1994). Raloxifene has been approved for the prevention and treatment of postmenopausal osteoporosis, and shown to reduce breast cancer incidence in a three year clinical trial. The evaluation of its effect on cardiovascular disease is underway (Cauley et al., 2001).

Pure antiestrogens were discovered by Wakeling and colleagues (Wakeling and Bowler, 1987). The lead compound, ICI 164,384, is a 7α-alkyl amide analogue of oestradiol (figure 2) that is entirely free of oestrogen partial-agonist activity (Wakeling and Bowler, 1987; Wakeling, 1993). The compound ICI 182,780 has significantly increased antiestrogenic potency and retains pure estrogen antagonist activity. Both ICI 164,384 and ICI 182,780 are poorly soluble and have low oral activity (Wakeling

et al., 1991). Nevertheless, ICI 182,780 has entered clinical trial as an effective antitumor antiestrogen by depot injection. Preclinical models and clinical studies demonstrate that ICI 182,780 is devoid of agonist activity, and inhibits growth of breast and endometrium epithelia. ICI 182,780 down-regulates the estrogen receptor and is active in tamoxifen-resistant breast carcinoma (Howell et al., 2000).

The compound RU58,668 is substituted in the 11β-position with a long hydrophobic side chain (figure 2). Studies *in vitro* and *in vivo* have demonstrated that RU58,668 has the properties of a pure antiestrogen (Van de Velde et al., 1994).

The compound EM-800 is an orally active pure antiestrogen, developed by Labrie's group (Gauthier et al., 1997). EM-652 is the de-esterified version of EM-800, and has higher affinity for the estrogen receptor than ICI 182,780, hydroxytamoxifen and raloxifene. EM-652 has a very potent inhibitory activity on both ER alpha and ER beta, and on growth of human breast cancer cells in vitro (Labrie et al., 1999). Laboratory studies demonstrate that EM-800 prevents bone loss in ovariectomized female rats, and lowers serum cholesterol. EM-800 and raloxifene achieve the same degree of effectiveness on bone and serum cholesterol at higher doses, but EM-800 is at least three to ten times more potent than raloxifene at lower concentrations and has no stimulatory effect on the uterine epithelium (Martel et al., 2000). Clinical trials with EM-800 are under way (Hermenegildo and Cano, 2000).

2.6 Mechanisms of antiestrogen action

As described previously, antiestrogens can be divided into two major catogories based on their mechanisms of action. Both type I and type II antiestrogens

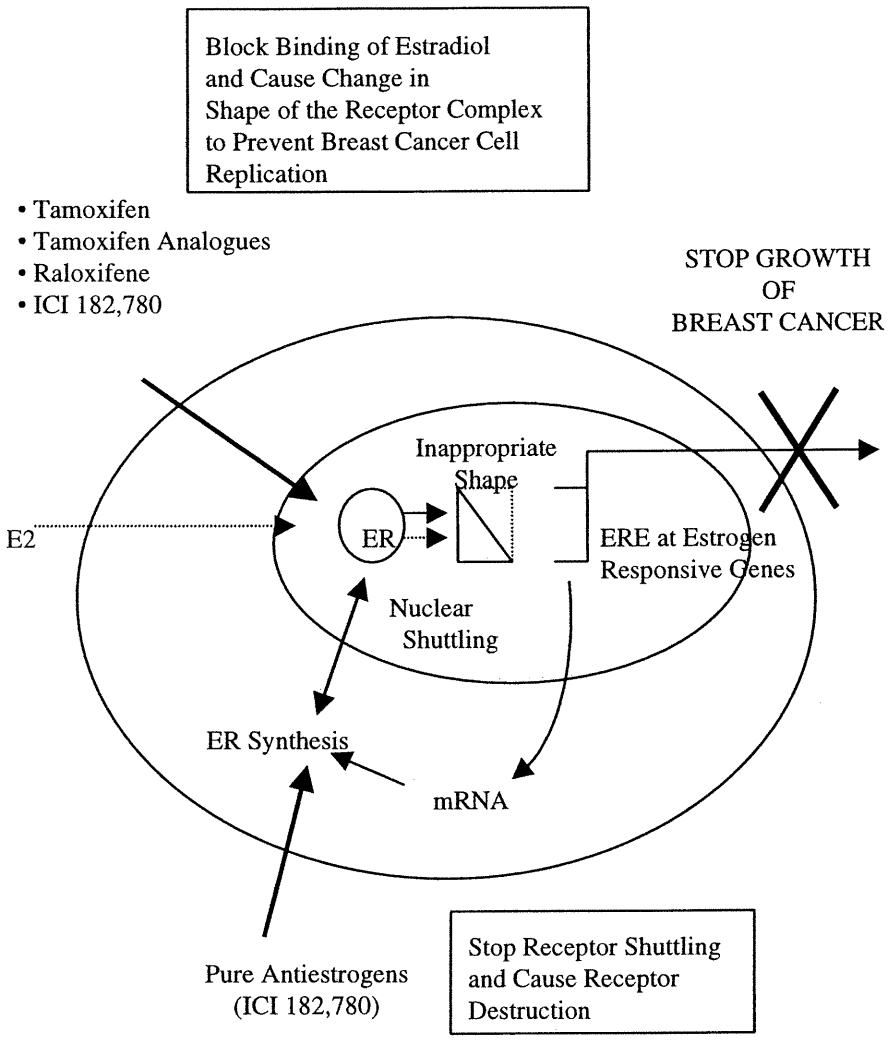

Figure 3. The current molecular model of antiestrogen action.

Different antiestrogens act at several points of weakness in the ER signal-transduction pathway (from Macgregor and Jordan, 1998).

are competitive inhibitors of the binding of E2 to the ER but there the similarity ends (figure 3). Type I antestrogens seem to form a receptor complex that is converted incompletely to the fully activated form. As a result of the different changes in the tertiary structure of the protein, the complex is only partially active in initiating the programmed series of events necessary to orchestrate gene activation (Macgregor and Jordan, 1998).

The mechanism by which the pure anti-oestrogens (type II) produce their effects remains in question, but all of them are competitive antagonists of the estrogen receptors. Moreover, pure antiestrogens have been proposed to provoke ER degradation (Dauvois et al., 1992) and block the shuttling of estrogen receptors into the cell nucleus (Dauvois et al., 1993; Hermenegildo and Cano, 2000).

3. Glucocorticoid receptor

3.1 Biological effects of glucocorticoids

Glucocorticoids have two principal physiological effects: metabolic and anti-inflammatory. The metabolic effects include the regulation of protein, carbohydrate, lipid and nucleic acid metabolism. The anti-inflammatory effects are multifaceted and involve many different aspects of the inflammatory response. Glucocorticoids have been widely used as anti-inflammatory drugs in chronic inflammatory diseases such as asthma, rheumatoid arthritis, inflammatory bowel disease and autoimmune diseases, all of which are associated with increased expression of inflammatory genes. Glucocorticoids have also been used as immunosuppressive and antineoplastic agents.

The clinical efficacy of synthetic glucocorticoids such as prenisolone or dexamethasone stems from their ability to mimic natural glucocorticosteroids (Newton, 2000). Glucocorticoid therapy has so far been associated with severe metabolic side-effects, e.g. thinning of the skin, decalcification of the skeleton, and HPA insufficiency (Newton 2000). Separation of the anti-inflammatory effects from the metabolic effects of glucocorticoids would increase the clinical potential for treatment of inflammatory diseases such as asthma.

3.2 Secretion of glucocorticoids is under control of HPA axis

Stress, including inflammation, pain, infection or even mental stress, leads to activation of the hypothalamic-pituitary-adrenal (HPA) axis (figure 4). These stimuli cause excitation of the hypothalamus, which responds by releasing corticotropin releasing hormone (CRH), CRH then acting on the anterior pituitary to induce synthesis and release of adrenocorticotropic hormone (ACTH). ACTH in turn stimulates the adrenal cortex to release glucocorticoids such as cortisol. Once within the blood, cortisol is transported to target organs where it elicits numerous metabolic effects including increased blood glucose levels, stimulation of gluconeogenesis in the liver, and mobilisation of both amino and fatty acids, as shown in figure 4. However, in addition to these metabolic effects, glucocorticoids are also potent endogenous immunological supressors (Newton, 2000).

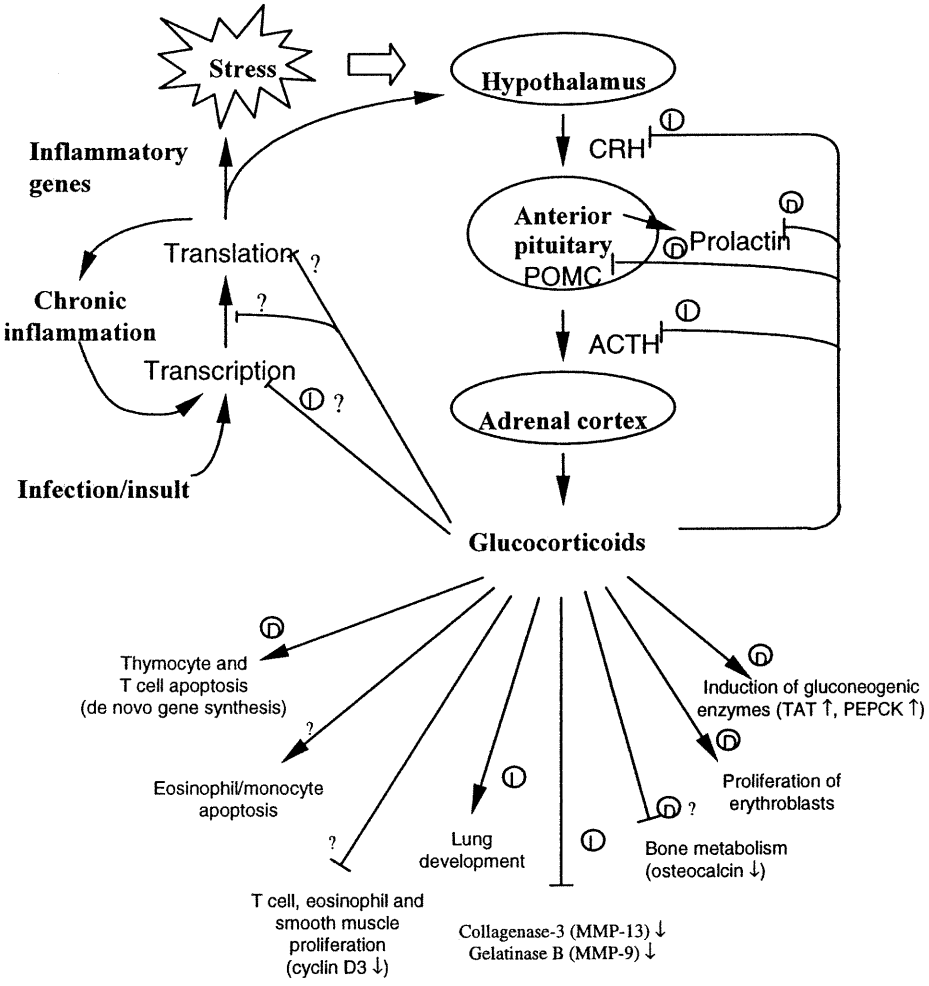

Figure 4. Effects of glucocorticoids on the hypothalamic-pituitary-adrenal (HPA) axis and biological effects of glucocorticoids.

This scheme shows the sites of synthesis and action of the main HPA hormones and the targets of glucocorticoid action. Based on analysis of dimerization defective mice many of the effects of glucocorticoids are labelled as either dependent on (D) or independent of (I) GR DNA binding. Question marks indicate uncertainty as to the mechanism of action (from Newton, 2000).

3.3 DNA-binding dependent glucocorticoid signaling

It is generally believed that most, if not all, the effects of glucocorticoids on cells are mediated via an intracellular receptor protein, glucocorticoid receptor (GR). Glucocorticoid receptor was the first nuclear receptor to be cloned. Rat liver glucocorticoid receptor complementary DNA (cDNA) was cloned by Yamamoto's group in 1984 (Miesfeld et al., 1984). Human GR (777 amino acid) was cloned in 1985. In common with other nuclear receptors, GR has a modular structure with principal functions (transactivation, DNA binding, and ligand binding) localized to specific domains. The glucocorticoid-binding domain is at the C-terminal end of the molecule (residues 501-777) and the DNA binding domain (residue 390-500) is localized in the central portion of the molecule. An N-terminal domain (τ_1, residues 77-262) is involved in transcriptional activation of genes once binding to DNA has occurred and this region may also be involved in binding to cofactors. Deletion analysis has demonstrated a 41-amino-acid core at the C-terminal end of the τ_1 domain that is critical for transactivation. In human GR there is another transactivating domain (τ_2, residues 526-556) localized just C-terminally of the DNA-binding domain (DBD) and this region is also important for the nuclear translocation of the receptor (Wright et al., 1993).

In the absence of hormone, GR is predominantly maintained in the cytoplasm as an inactive multi-protein complex. This consists of two hsp90 (heat shock protein) molecules plus a number of other proteins including the immunophilins p59 and calreticulin. Entry of glucocorticoids into the cell and subsequent binding to the ligand-binding domain of GR leads to a conformational change in the receptor. This

causes dissociation of the multi-protein complex and allows nuclear translocation of GR by virtue of the nuclear localisation sequence within the DNA binding domain (DBD). Once within the nucleus, GR homodimer binds to specific glucocorticoid response elements (GREs) to induce target gene transcription (Wright et al., 1993; Newton, 2000).

This classical mode of action of GR involves binding of the receptor to GREs in the promoter and enhancer regions of responsive genes. Subsequently, transcription is activated by interaction of the receptor with the basal transcription machinery (Beato et al., 1995). As described previously, the consensus sequence for GRE is the palindromic 15-bp sequence GGTACAnnnTGTTCT (where n is any nucleotide) (Barnes, 1998). The gluconeogenic enzymes tyrosine aminotransferase and phosphoenolpyruvate carboxykinase are regulated by glucocorticoids via this classical mode of GR action (Reichardt and Schütz, 1998). Another mode of GR action is mediated through so called negative GREs (nGREs) which mediate transcription repression. In contrast to GREs, nGREs posess a much less conserved consensus sequence. However, such elements have been identified in a few genes, such as proopiomelanocortin (POMC) (Reichardt and Schütz, 1998). The number of genes per cell directly regulated by glucocorticoids is estimated to be between 10 and 100 (Barnes, 1998).

3.4 DNA-binding independent glucocorticoid signaling

Glucocorticoids can exert their biological effects via two pathways: (1) DNA-binding dependent direct modulation of target gene transcription. (2) Crosstalk with other transcription factors, such as NF-κB and AP-1. The most striking effect of glucocorticoids is to inhibit the expression of multiple inflammatory genes (cytokines, enzymes, receptors and adhesion molecules). This is not due to a direct interaction between glucocorticoid receptors and glucocrticoid response elements (GREs), as no GRE was found in the promoter regions of most inflammatory genes. However, it is direct inhibitory interaction between activated glucocorticoid receptors and activated transcription factors, such as nuclear factor-κB and AP-1, which regulate the inflammatory gene expression (Barnes, 1998).

To separate DNA binding-dependent and -independent glucocorticoid signaling, a mouse strain carrying a missense mutation in the second zinc finger of the GR was generated (Reichardt et al., 1998). This A458T substitution in the D-loop which is located in the DBD of the receptor, interferes with binding to a GRE and thereby abolishes transactivation of linked genes. In contrast, repression via crosstalk with other transcription factors such as AP-1, remains largely intact. Mice homozygous for this mutation are viable, indicating that DNA binding is not essential for survival (Reichardt et al., 1998). This is in sharp contrast to the observed lethality of mice with a disrupted GR gene, which die immediately after birth due to atelectasis of the lungs (Cole, 1995). Obviously, crosstalk of GR with other transcription factors is sufficient for proper development and inflation of the lung at birth.

4. Nuclear receptor coactivators and corepressors

4.1 Updated list of nuclear receptor coactivators and corepressors

Transcriptional regulation of nuclear hormone receptors involves the participation of basal transcription factors and other cofactors, known as nuclear transcriptional coactivators or corepressors, which enhance or repress transactivation by nuclear receptors. Over 30 potential coactivators have been identified by their ability to bind various receptor domains and to alter the transcriptional activity of nuclear receptors in overexpression studies. Mostly studied coactivators identified in recent years (as shown in table 2) include CBP/p300, steroid receptor coactivator-1 (SRC-1)/nuclear receptor coactivator-1 (NCoA-1), transcriptional intermediate factor 2 (TIF-2)/glucocorticoid receptor interacting protein-1 (GRIP-1)/nuclear receptor coactivator-2 (NCoA-2), p300/CBP/co-integrator-associated protein (p/CIP)/activator of the thyroid and retinoic acid receptor (ACTR)/amplified in breast cancer 1(AIB1)/receptor-associated co-activator-3 (RAC3)/thyroid hormone receptor activator molecule (TRAM-1), and p300/CBP associated factor (P/CAF). The nuclear receptor targets and functions of these coactivators are listed in table 2. Other identifed coactivators include: androgen receptor activator (ARA70), PPARγ coactivator-1 (PGC-1), p68, steroid receptor RNA activator (SRA) (lanz et al., 1999), the DRIP/TRAP complex (Freedman, 1999) and components of the BRG-1 (SWI/SNF) complex (Fryer and Archer, 1998; Collingwood et al., 1999; Wallberg et al., 2000). In addition, two coactivators with protein methyltransferase activities have been recently characterized: coactivator-associated arginine methyltransferase 1(CARM1), and protein arginine methyltransferase 1(PRMT1) (Koh et al., 2001).

Table 2. Nuclear receptor cofactors

Proteins	Nuclear receptor target in vitro	Function
Coactivators		
SRC-1, NCoA-1	ER, GR, PR, TR, RXR	Acetyltransferase Interacts with CBP
TIF2, GRIP1, NCoA2	ER, AR, GR, PR	Interacts with CBP
P/CIP, ACTR, RAC3, or AIB1, TRAM1	ER, PR, TR, RAR, RXR, VDR	Acetyltransferase Interacts with CBP, PCAF
PCAF	RAR, RXR	Acetyltransferase Interacts with p300/CBP
P300/CBP	ER, TR, RAR, RXR	Acetyltransferase Interacts with SRC-1, PCAF
Corepressors		
NCoR, SMRT	TR, RAR, RXR ER+hormone antagonist PR+hormone antagonist	Interacts with SIN3 to recruit histone deacetylase
TIF1α, TIF1β	RAR	Interacts with HP1 and Histone deacetylase

Transcriptional corepressors identified so far include nuclear receptor co-repressor (N-CoR) (Hörlein et al., 1995), silencing mediator for RXR and TR (SMRT) (Chen and Evans, 1995) and transcription intermediary factor-1 (TIF1), their nuclear receptor targets and functions are also shown in table 2. A few newly identified corepressors are : polypyrimidine tract-binding protein-associated splicing factor (PSF) (Mathur et al., 2001), and SMRT/HDAC1 Associated Repressor Protein (SHARP) (Shi et al., 2001).

Very recently, a bifunctional nuclear receptor interacting protein that can act as either a coactivator or a corepressor depending on the receptor type was identified: FKHR (forkhead homologue in rhabdomyosarcoma, a member of the hepatocyte nuclear factor3/forkhead homeotic gene family). Transient transfection of FKHR into mammalian cells dramatically represses transcription mediated by ER, GR and PR. In contrast, FKHR stimulates rather than represses RAR- and TR-mediated transactivation (Zhao et al., 2001).

4.2 p160 family coactivators

Among the nuclear receptor co-activators identified and cloned, p160 family members have drawn particular attention. These proteins were initially identified biochemically as 160 kDa proteins (p160) which interacted directly with nuclear receptors in an agonist and AF-2 dependent manner. To date, three distinct but related p160 family members have been identified, with each family member having a number of splice variants. These include SRC-1/NCoA-1, SRC-2/TIF2/GRIP1/NCoA2 and SRC3/p/CIP/ACTR/AIB1/RAC3/TRAM-1. It has been demonstrated that p160 family coactivators can enhance the transactivation function of

several nuclear receptors (such as TR, RAR, RXR, VDR, GR, ER) in mammalian cells (Torchia et al., 1997; Chen et al., 1997; Anzick et al., 1997; Li et al., 1997; Takeshita et al, 1997).

Members of the p160 coactivator family share 40% overall sequence homology. They are modular proteins with regions of conserved sequence that serve as distinct functional domains. The most conserved region is the N-terminus that contains homology to the basic helix-loop-helix (bHLH) motif and a PAS (period protein self-association domain) domain (figure 4). The bHLH is a DNA binding motif present in several families of eukaryotic transcription factors and PAS function as a dimerization domain in Period (Per), single minded (SIM) and the arylhydrocarbon receptor (AHR). Whether the bHLH motif in p160 coactivators is capable of DNA binding or whether the PAS domain is a site for p160 interaction with other proteins, or for the formation of homo- or heterodimer complexes between SRC family members, is unknown. The N-terminal bHLH/PAS domain is dispensable for p160 binding to nuclear receptors and for coactivation function suggesting that this region has a role that is not yet appreciated (Edwards, 2000).

A nuclear receptor interaction domain (NID) is found in the central portion of all three members of p160 family coactivators. The NID contains three highly conserved LXXLL motif (L stands for leucine, X stands for any amino acid) termed nuclear receptor boxes that are responsible for recognition of AF-2. Analysis of these interaction regions by the self-optimized prediction method strongly suggested that they represent helical domains, often with amphipathic characteristics. It has been

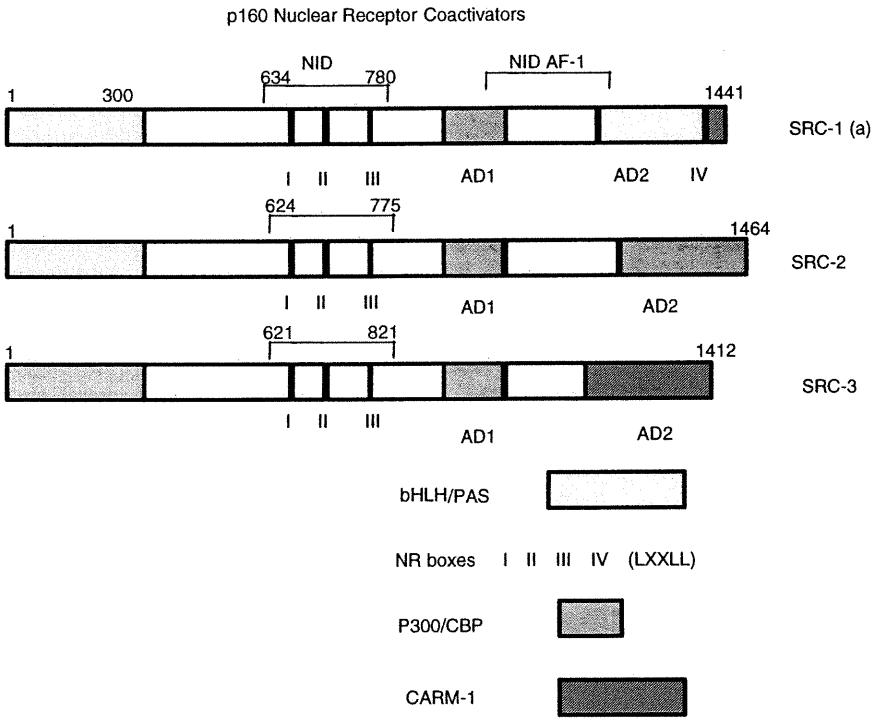

Figure 5. Schematic representation of the functional domains of the p160 family of nuclear hormone receptor coactivators.

The three members of p160 family coactivators, termed steroid receptor coactivators (SRC) include: SRC-1 (SRC-1a, SRC-1e), SRC-2 (GRIP1, TIF-2) and SRC-3 (p/CIP, ACTR, RAC3, TRAM-1 and AIB1). They all contain conserved sequence regions that serve as distinct functional domains. The amino terminal 300 residues contains basic helix-loop-helix (bHLH) and PAS dimerization domains. A centrally located nuclear receptor interaction domain (NID) is found in all three members of SRCs. The NID contains three LXXLL motifs, or nuclear receptor boxes (NR), that recognize AF-2 through a hydrophobic site on the surface of the LBD. A splice variant SRC-1(a) contains a fourth NR box at the C-terminus. Two autonomous transcriptional activation domains (AD1 and AD2) are found in all members of SRCs. AD1 overlaps the binding site for the general coactivators CBP/p300 and AD2 interacts with a novel protein methylase termed coactivator associated arginine methyltransferase (CARM1). CBP/p300 and CARM1 are functionally cooperative, and they function independently through different activating domains AD1 and AD2 respectively. A second nuclear receptor interaction domain (NIDAF-1) that interacts with AF-1 in the N-terminus of steroid receptor is located between AD-1 and AD-2. Histone acetyltransferase activity (HAT) is located in a C-terminal segment of SRC-1a and SRC-3 (adapted from Edwards, 2000).

demonstrated that a LXXLL sequence motif is necessary and sufficient for the binding of several coactivators to nuclear receptors (Torchia et al., 1997; Herry et al., 1997). Similar motifs have been identified in virtually all of the many factors whose genes have been cloned, based on their ability to interact with liganded nuclear receptors, including CBP, TIF1 and RIP140. It was demonstrated that residues immediately adjacent to the motif modulate the affinity of the interaction and its specificity towards different nuclear receptors (Darimont et al.,1998; McInerney et al., 1998).

Structure–function analyses have revealed that co-activators are composed of distinct domains responsible for NR binding (NR interaction domain[NID]) and transactivation (transactivation domain AD1 and AD2). It has been demonstrated that all three related p160 family members contain two major transactivation domains: a weaker transactivation domain located in the far carboxyl terminus (AD2), and a stronger transactivation domain (AD1) which directly overlaps with a conserved region that mediates interactions with the CBP/p300 (Torchia et al., 1998). Recently, AD2 was found to interact with a novel protein methylase termed coactivator associated arginine methyltransferase (CARM1). CBP/p300 and CARM1 are functionally cooperative (Chen et al., 2000).

In vivo Biological function of p160 coactivators: Initial evidence for a biological role of p160s in nuclear receptor function *in vivo* has been provided by disruption of the SRC-1 gene by O'Malley's group. Mice bearing a homozygous deletion of the SRC-1 gene exhibit partial resistance to multiple hormones including estrogen, progesterone, androgen, and thyroid hormone. SRC-1 null mutant female mice exhibit reduced growth and development of the mammary gland and uterus in

response to estrogen and progesterone, and males show reduced growth and development of the prostate and testis in response to androgen. SRC-$1^{-/-}$ mice, however, are viable and fertile and were reported to overexpress TIF-2 suggesting that different members of the p160 coactivator family have overlapping redundant functions (Xu et al.,1998; Weiss et al.,1999).

Not only SRC-1 null mice, but also SRC-3 null mice were generated by O'Malley's group. They constructed a targeting vector which allowed disruption of the SRC-3 gene and expression of Lac Z under the control of the endogenous SRC-3 promoter simultaneously. Compared with SRC-1, mouse SRC-3 is expressed in a tissue and cell type-specific manner, distributed mainly in the oocytes, vaginal epithelium, mammary glands, smooth muscle, hepatocytes, and specific regions of the brain. Genetic disruption of SRC-3 in mice results in a pleiotropic phenotype showing dwarfism, delayed puberty, reduced female reproductive function, and blunted mammary gland development, but did not exhibit the generalized resistance to steroid hormones in most target tissues that characterized the phenotype of SRC-1 null mice (Xu et al.,1998; Xu et al.,2000). The different expression patterns of SRC-1 and SRC-3 and the distinct physical and functional phenotypes of the SRC-1 and SRC-3 knockout mice clearly indicate that members of the p160 coactivator family play differential roles in development and disease (Xu et al., 2000).

4.3 Cointegrators CBP and p300

CBP and p300 are considered to be 'cointegrators', because they form intranuclear complexes with TBP and bind to a large number of transcription factors, including CREB, nuclear receptors, c-Jun, NFκB, Myb, C/EBPβ, p53, and STATs.

CBP and p300 also participate in the assembly of multiprotein complexes that contain p/CIP, P/CAF. In addition to serving as molecular scaffolds, CBP and p300 each possess intrinsic histone acetyltransferase activities that are required for their function as coactivators (Glass et al., 1997; Torchia et al., 1998; Klinge, 2000)

4.4 Amino terminal AF-1 interacting coactivators

The vitamin D receptor-interacting protein 150 (DRIP150) was identified as a protein that interacts specifically with a functional GR AF-1 surface, utilizing a modified yeast two-hybrid approach. In yeast and mammalian cells, DRIP 150 enhances GR AF-1-mediated transactivation. DRIP 205, another member of the DRIP complex (see immediately below), interacts with the GR ligand-binding domain in a hormone-dependent manner and facilitates GR transactivation in concert with DRIP 150. These results suggest that DRIP150 and DRIP205 functionally link GR AF-1 and AF-2, and represent important mediators of GR transcriptional enhancement (Hittelman et al.,1999).

p68, an RNA helicase, was isolated from MCF-7 nuclear extract by a GST pull-down assay with the ER AB region. Previous studies indicate that p68 is important in RNA processing, transcription, translation, cell growth and division. Moreover, p68 binds calmodulin in a Ca^{2+}-dependent manner and is phosphorylated by PKC. p68 RNA helicase binds *in vitro* and *in vivo* with AF-1 of ERα, but not the LBD. p68 was specific for ERα as it had no effect on either AF-1 or AF-2 of ERβ, AR, MR, or RARα. Overexpression of p68 enhanced AF-1 activity of ERα, but had no effect on AF-2 activity. Phosphorylation of ERα Ser118 by mitogen-activated protein kinase potentiated the interaction with p68. The RNA helicase activity of p68

is dispensable for its ERα coactivation function suggesting p68 is a dual function protein (Endoh et al., 1999).

Using AF-1 of the progesterone receptor A form as the bait in a yeast two-hybrid screen, a novel coactivator, termed steroid receptor RNA activator (SRA) was identified and characterized. SRA is selective for steroid hormone receptors and mediates transactivation via their N-terminal AF-1. In transiently transfected Hela cells, SRA enhanced CAT activity from reporters for PR, GR, AR, and ER, but not TR, RAR, RXR, and PPAR. SRA is unique in that it does not exhibit characteristics of a protein, but appears to act as an RNA transcript *in vivo*. Biochemical fractionation revealed that SRA co-purified with SRC-1 in a large protein complex corresponding to a size of 2 Mda. In contrast, SRA was not detected in fractions containing p300/CBP. These results indicate that SRA is present with SRC-1 in a distinct steroid receptor coactivator complex (Lanz et al., 1999).

4.5 Nuclear receptor–coactivator complexes

The two nuclear receptor–coactivator complexes that are recruited by most nuclear receptors are: 1) the complex comprised of p160s, CBP/p300 and p/CAF (p300/CBP associated factor), recruited to nuclear receptors through ligand-dependent AF-2 interactions. The net effect of these interactions would be to provide HAT activity, resulting in chromatin acetylation. 2) A distinct coactivator complex comprised of DRIP/TRAP (composed of 14-16 proteins) and Mediators/SRB (Mediator complex together with SRB proteins associates with RNA polymerase II) subunits, recruited to nuclear receptors in a ligand dependent manner. Several DRIP/TRAP subunits are homologous to proteins described as components of the Mediator. It is a key question

regarding nuclear receptor coactivation to know how the DRIP/TRAP complex interfaces with the p160/CBP/pCAF system, independently or in a step-wise fashion (Freedman, 1999).

4.6 Mechanisms of coactivator effect on transcription

Initially, a two-step mechanism has been proposed for how p160 proteins mediate nuclear hormone receptor transcriptional activation. The first is to promote the local remodeling of chromatin structure via the HAT activity of the recruited p160 coactivator complex and enable access of DNA to general transcription factors. The second is recruitment/stabilization of the basal transcription machinery by direct or indirect protein-protein interaction with general transcription factors associated with the RNA polymerase II holoenzyme (figure 6).

Recently, based on *in vitro* analyses, it has been suggested that SRCs mediate transcriptional activation through multiple mechanisms including: (1) direct interaction with ligand-bound nuclear receptors; (2) direct contact with certain general transcription factors such as TFIIB and TBP; (3) interaction with common transcriptional coactivators such as CBP, p300, and p/CAF; (4) interaction with other coactivators such as coactivator–associated arginin methyltransferase 1 (CARM-1), cancer-amplified transcription coactivator ASC-2, PPARγ coactivator-1 (PGC-1), and steroid receptor RNA coactivator (SRA); (5) participation in chromatin remodeling through their intrinsic histone acetyltransferase activity; (6) enzymatic modification of other constituents of the coactivator comoplex (Xu et al.,2000).

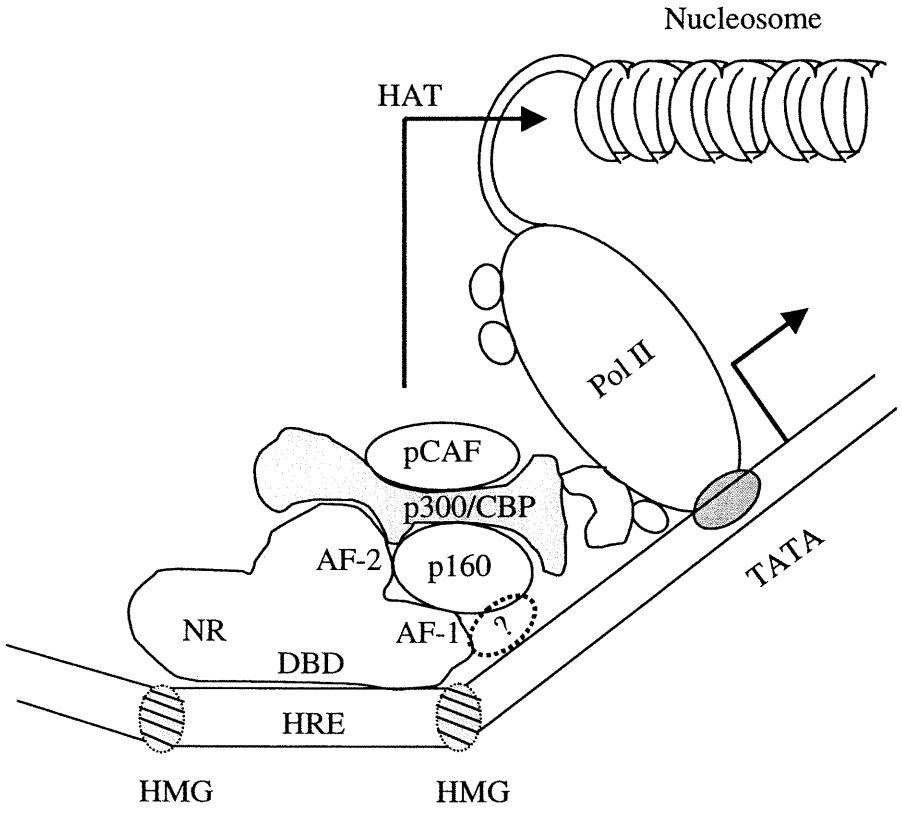

Figure 6. Mechanism of action of p160 nuclear receptor coactivators.

A two-step mechanism has been proposed for how p160 proteins mediate nuclear receptor transcriptional activation. As an initial step, HAT activity of the recruited p160 coactivator complex modulates local chromatin structure resulting in general transcription factors gaining access to DNA at the promoter. This step is followed by recruitment or stabilization of the RNA polymerase II holoenzyme (pol II) through direct or indirect binding of coactivators with general transcription factors associated with pol II. The high mobility group protein HMG-1/2 enhances transcription by facilitating steroid receptor binding to specific hormone response elements (HREs) and stabilizing the receptor-DNA complex. DBD, DNA binding domain; HAT, histone acetyltransferase activity (from Edwards, 2000).

4.7 Nuclear receptor corepressors

Type II nuclear receptors function as transcriptional repressors in the absence of hormone. The repressor activity resides in a distinct region of the ligand-binding domain of RAR and TR and is functionally separable from the C-terminal AF-2 domain. Two-distinct, but highly related proteins, identified as Silencing mediator for retinoid and thyroid receptor (SMRT) (Chen and Evans, 1995) and nuclear receptor co-repressor (NcoR) (Hörlein et al., 1995), serve as co-repressors that mediate transcriptional repression by TR and RAR. Ligand binding triggers dissociation of these corepressors and recruitment of coactivators.

Co-repressors mediate transcriptional silencing by mechanisms that include direct inhibition of the basal transcription machinery and recruitment of chromatin-modifying enzymes: histone deacetylases. Histone deacetylation is thought to lead to a compact chromatin structure to which the accessibility of transcriptional activators is impaired (Burke and Baniahmad, 2000).

II. EUKARYOTIC TRANSCRIPTION

In the last five years, our knowledge and understanding of chromatin structure and of its function in gene regulation has dramatically expanded (Hark and Triezenberg, 2001). Now it is evident that eukaryotic transcription is achieved by an interlaced network of transcription factors and chromatin-modifying complexes. It now seems impossible to study transcriptional regulation in a manner that does not involve chromatin (Kadonaga, 1998). Schematic diagrams of potential linkage

between chromatin and transcription are shown in figure 7. Yeast and Mammalian protein complexes involved in transcription regulation are listed in table 3. Nuclear receptors provide the best examples of transcriptional control through the targeted recruitment of large protein complexes that modify chromosomal components and reversibly stabilize or destabilize chromatin. In this section, first we will talk about current models of the RNA polymerase II transcription initiation machinery, then we will explain the chromatin modifying machines, including the chromatin remodeling complexes and the histone acetyltransferases (HATs) and deacetylases (HDACs). Finally we will discuss some links between glucocorticoid receptor transcription and chromatin modification.

1. RNA Polymerase II transcription initiation machinery

1.1 Ordered assembly of transcriptional preinitiation complexes

Transcriptional activation in eukaryotes requires concerted action of multiple protein factors: RNA polymerase II holoenzyme, general transcription factors (GTFs), sequence-specific DNA-binding activators, and coactivators. The GTFs include TATA-binding protein (TBP), TFIIB, TFIIE, TFIIF, and TFIIH (These factors are designated as TFIIB, TFIIE, etc., for transcription factor for RNA polymerase II plus an identifying letter) and were identified biochemically as factors required for accurate, basal level transcription initiation in vitro. These proteins can assemble as a preinitiation complex (PIC) in an ordered fashion onto promoter DNA *in vitro*. Clearly, order-of-addition experiments (foot-printing and gel retardation assays) demonstrated that PIC assembly is nucleated *in vitro* by TBP binding to the TATA

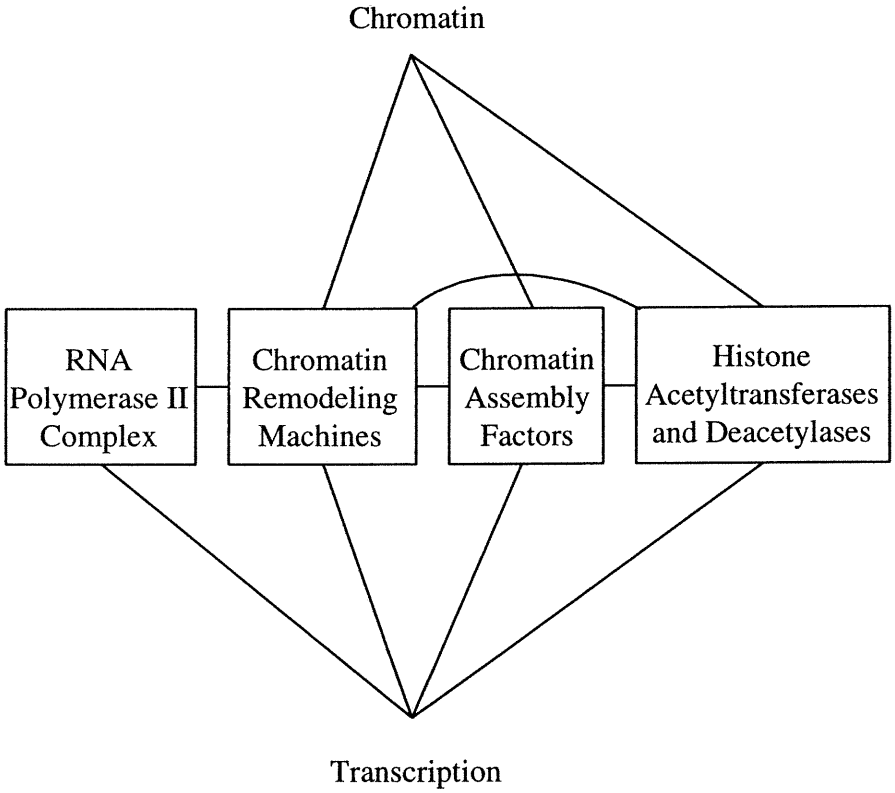

Figure 7. chematic diagram of potential linkage between chromatin and transcription.

This figure depicts some linkages that have been suggested by experiments in the literature. Although there is not universal agreement with regard to some of these postulated connections.Additionally this diagram is not meant to be comprehensive. For example, it does not include the relation between chromatin/DNA modification and gene regulation by DNA methylation (from Kadonaga, 1998).

Table 3. Yeast and Mammalian protein complexes involved in transcription regulation

	Yeast	Mammalian
RNA Polymerase II Mediator Complex	RNA polymerase II Basal transcription factors SRB mediator	
Chromatin Remodeling Machines	SWI/SNF complex RSC complex	BRG-1 or hbrm-associated complexes
Chromatin Assembly Factors	yeast CAF-1	mammalian CAF-1
Histone Acetyltransferases	Gcn5-dependent SAGA complex Gcn5-dependent Ada complex NuA4, NuA3	SRC-1, SRC-3, CBP/P300, P/CAF
Histone Deacetylases	Rpd3p, Hda1p, Hos1p, Hos2p, Hos3p, Sir2p	Class-I HDACs: HDAC1,-2, and-3 Class-II HDACs: HDAC4,-5,-6 and-7

element followed by binding of TFIIB, RNA pol II-TFIIF, TFIIE, and TFIIH as shown in figure 8 (reviewed by Hampsey, 1998). Some general transcription factors can interact with RNA polymerase II in the absence of DNA however, suggesting that RNA polymerase II may also assemble into a multi-component complex containing a subset of initiation factors before binding to promoter DNA. This suggests that ordered assembly might not occur *in vivo* (Koleske and Young, 1994; Hampsey, 1998).

1.2 RNA polymerase II complex (holoenzyme)

Based on genetic and biochemical studies, recently an RNA polymerase II multi-subunit complex has been discovered, commonly termed the "RNA polymerase II holoenzyme". It contains "core" RNA polymerase II, a subset of basal transcription factors (such as TFIIB, TFIIE, TFIIF, and/or TFIIH), nine SRB (surpressor of RNA polymerase B, *srb* defined as suppressors of the conditional growth defect associated with truncation of the RNA pol II carboxy-terminal repeat domain) proteins, as well as other known (such as GAL1, SIN4, RGR1, and ROX3) and unknown proteins (Koleske and Young, 1994; reviewed in Kadonaga, 1998). The existence of multisubunit complexes containing RNA pol II was independently demonstrated based on the association of RNA pol II with the mediator complex, required for transcriptional activation *in vitro* (Kim et al., 1994). The mediator complex was found to contain SRB proteins as well as GAL1, SIN4, RGR1, and ROX3.

Recent studies suggested that RNA polymerase II also associates with chromatin remodeling machines such as the SWI/SNF complex (pronounced "switch-

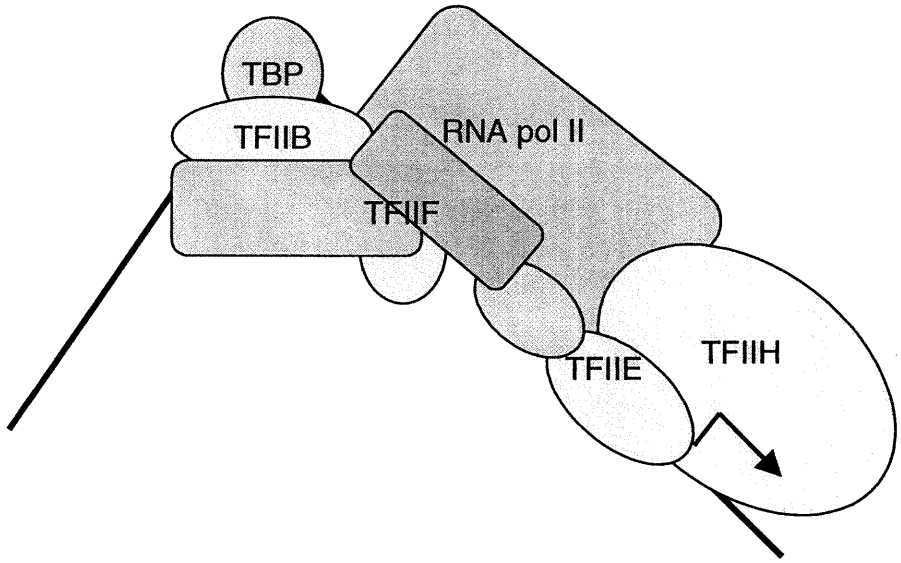

Figure 8. Schematic depiction of the transcription PIC.

PIC assembly is nucleated by TBP binding to the TATA box, including a sharp bend in the DNA template, followed by association of TFIIB, RNA pol II/TFIIF, TFIIE, and TFIIH. Each pattern denotes a distinct general transcription factor. Subunit composition is indicated, except for TFIIH (9 subunits) and RNA pol II (12 subunits). Although PIC assembly can occur by stepwise addition of the general transcription factors (GTFs) in vitro, the discovery of RNA pol II holoenzyme complexes that include GTFs suggests that stepwise assembly might not occur *in vivo* (from Hampsey, 1998).

sniff", *swi* stands for the mutants defective in mating-type switching; *snf* stands for mutants unable to ferment sucrose. The SWI/SNF complex, which contains 11 known subunits, was first characterized in yeast), because the SWI/SNF complex was found to be an integral and equimolar component of the RNA polymerase II holoenzyme and mediator (Wilson et al., 1996; reviewed in Kadonaga, 1998).

1.3 Current model of RNA polymerase II transcription initiation machinery

The model shown in figure 9 summarizes some of the protein complexes thought to be involved in transcription initiation in yeast cells. Mechanisms of transcription initiation are well conserved from yeast to human. It has been proven that yeast is a very valuable research tool to unravel the sophisticated mechanisms of control of transcription. Compared to figure 8, RNA polymerase II associates with Srb/Mediator complex, the Srb10 CDK complex, the Swi/Snf complex (chromatin remodeling complex), and general transcription factors. Most of these components can be purified from cells as a single complex called an RNA polymerase II holoenzyme (see above). Transcription of most genes is initiated by the holoenzyme form of RNA polymerase II (Holstege and Young, 1999). This current model is consistent with the hypothesis that ordered assembly of general transcription factors might not occur in vivo.

2. Chromatin modifying machines

In the eukaryotic cell nucleus, DNA is packaged by histones into nucleosomes, the repeating subunits of chromatin. Every nucleosome in a typical animal cell consists of two molecules of each of the four core histones: H2A, H2B, H3 and H4, about 180 bp of DNA and a single molecule of a linker histone H1. Each histone has

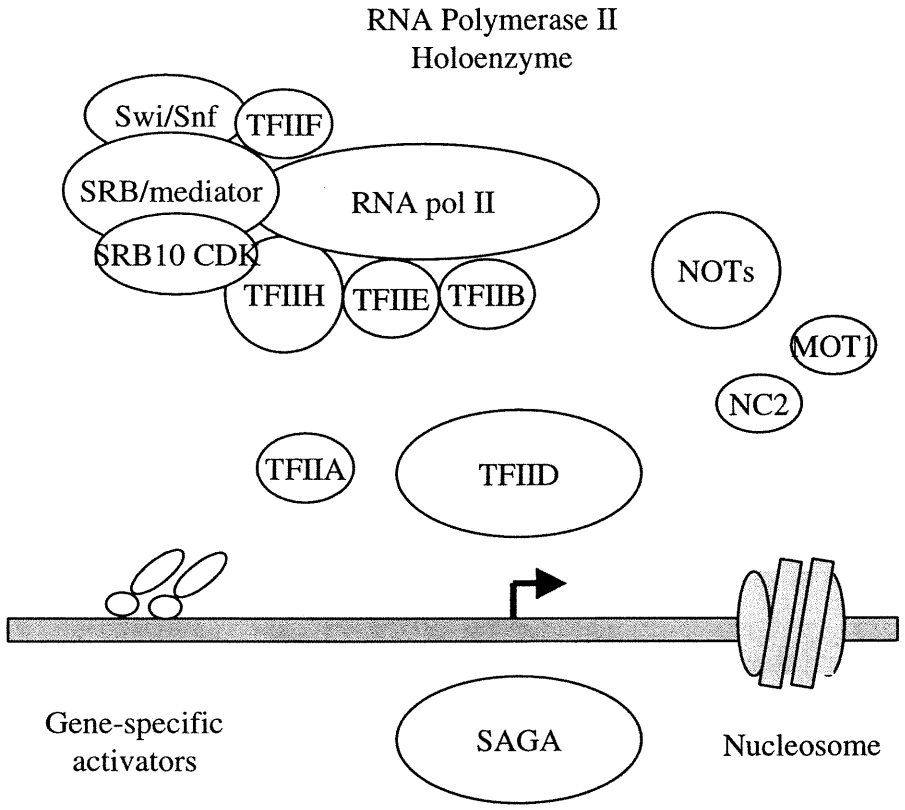

Figure 9. Current model of RNA polymerase II transcription initiation machinery in yeast

This model summarizes some of the protein complexes thought to be involved in transcription initiation in yeast cells. RNA polymerase II associates with Srb/Mediator complex, the Srb10 CDK complex, the Swi/Snf complex, and general transcription factors. Most of these components can be purified as a single complex from cells called an RNA polymerase II holoenzyme and transcription of most genes is initiated by the holoenzyme form of RNA polymerase II. This current model suggests that ordered assembly of general transcription factors might not occur in vivo. SAGA is one of the multisubunit native yeast histone acetyltransferase complexes recruited by transcriptional activators that regulate chromatin structure and the transcription apparatus. Nots, MOT1 and NC2 are general transcriptional repressors that target general transcription machinary (from Holstege and Young, 1999).

two domains, an N-terminal tail that faces solution at the outer surface of the nucleosome and a C-terminal histone fold domain that is involved in wrapping DNA. It has been known that the packaging of genes into chromatin represses basal transcription and that transcriptional activators function, at least in part, to counteract chromatin-mediated repression. Two different, yet highly conserved, mechanisms are found to relieve chromatin-mediated repression: 1) chromatin remodeling. 2) Post-translational modifications of chromatin components, in particular histone acetylation (reviewed by Kuo and Allis, 1998).

2.1 Chromatin remodeling complexes

Chromatin remodeling has been referred to as any sort of detectable change in chromatin or mononucleosome structure (Kadonaga 1998). The chromatin remodeling machines are multisubunit molecular machines, include the yeast SWI/SNF complex, yeast RSC (remodels the structure of chromatin) complex, Drosophila NURF (nucleosome remodeling factor), Drosophila CHRAC (chromatin accessibility complex), Drosophila ACF (ATP-utilizing chromatin assembly and remodeling factor, Drosophila BRM complex, and mammalian BRG-1 (brahma-related gene 1) or hbrm (brahma)-associated complexes. These different protein complexes are placed in the same general category because they each contain a closely related subunit (SWI2/SNF2 in the yeast SWI/SNF complex; STH1 in RSC; ISWI (imitation SWI) in NURF, CHRAC, and ACF; Drosophila BRM; and mammalian BRG1 or hbrm) that is a member of a family of ribonucleoside triphosphate (NTP)-binding proteins. The presence of this NTP-binding subunit in the different factors suggests that it may carry out a related biochemical function in each of the complexes. For instance, it has been

postulated that the NTP-binding subunit might act as a processive, ATP-driven DNA-translocating motor that transiently disrupts histone-DNA interactions.

Yeast SWI/SNF complex, purified as a 2-MDa, 11-subunit complex, is the most well characterized of the remodeling complexes,. Subunits include Swi1, Swi2/Snf2 (a connection between the SNF and SWI system was made when Swi2 and snf2 were found to be identical), Swi3, Snf5, Snf6, Snf11, Swp29, Swp59, Swp61, Swp73, and Swp82. To date, Swi2/Snf2 is the best-characterized component and, as a DNA-dependent ATPase, is the only subunit with known enzymatic activity.

2.2 Histone acetyltransferase and deacetylase complexes

Chromatin remodeling is not sufficient for productive RNA polymerase II-dependent transcription *in vitro*. Posttranslational modifications of the core histones within the nucleosome have also been linked to the transcriptional capacity of chromatin. The most extensively studied modification has been the acetylation of the highly conserved lysine residues in the N-terminal tails of all four core-histones. Correlations between transcription and acetylation are reinforced by studies demonstrating that active chromosomal domains are hyperacetylated, while inactive or heterochromatin domains are hypoacetylated (Bone et al., 1994; Jeppesen and Turner, 1993).

Histone acetyltransferase (HAT) proteins have been identified from various organisms from yeast to human. Four multisubunit native yeast HAT complexes have been identified. Two of these complexes, SAGA and Ada, preferentially acetylate nucleosomal histone H3 and contain Gcn5 as the catalytic histone acetyltransferase subunit. SAGA (for Spt-Ada-Gcn5-acetyl-transferase) is an 1,800K HAT complex

which contains Ada2, Ada3, Ada5/Spt20, Spt3, Spt7 and Gcn5. The 800K Ada (for alteration/deficiency in activation) complex contains Ada1, Ada2, Ada3, Ada5/Spt20, and Gcn5. Another two HATs, formally termed complex 2 which acetylates histones H4 and H2A, and complex 3, with a substrate preference for histone H3, were purified and named NuA4 (for nucleosomal acetyltransferase of histone H4) and NuA3, respectively (Utley et al., 1998; Wallberg et al., 1999).

Several previously characterized mammalian transcriptional coactivators are now known to possess intrinsic histone acetyltransferase activity, such as SRC-1, SRC-3, TAFII 250 (the largest TBP-associated factor), CBP/p300 and p/CAF. Moreover it has been demonstrated that three distinct HATs, SRC-1, CBP/p300 and PCAF, can form a multiprotein complex with nuclear receptor to facilitate transcriptional activity. But the relative contribution of each HAT to the overall transcriptional activation awaits further studies.

Histone deacetylases (HDACs) are transcriptional repressors that reduce histone acetylation levels to create localized regions of repressed chromatin. It is now known that at least six HDACs exist in yeast, encoded by the yeast genes *RPD3*, *HDA1*, *HOS1*, *HOS2*, *HOS3*, and *SIR2*. The small molecule trichostatin A (TSA) was instrumental in the initial characterization of HDACs. TSA is an inhibitor of HDAC, and TSA treatment also has been shown to induce a hyperacetylated state in yeast. Among the six identified HDACs, Rpd3p and Hda1p are sensitive to TSA. Hos3p and Sir2p are TSA-insensitive. *SIR2* is one of the silent information regulator (SIR) genes that mediate silencing (repression) at telomeres, mating type loci and ribosomal DNA. Little is known about Hos1p and Hos2p. Yeast genetics suggested that HDACs

function within multisubunit complexes to mediate transcriptional repression. For example, Rpd3p forms a complex with Sin3p (Sin3p serve as a corepressor bridging gene-specific repressors to HDACs) and Sap30p that is recruited to DNA by the Ume6p transcription factor to mediate transcription repression. (Bernstein et al., 2000).

Two families of HDACs, referred to as class-I and class-II HDACs, have been identified in mammals. Class-I HDACs (HDAC1, -2, and -3) are related to the yeast Rpd3p; Class-II HDACs (HDAC4, -5, -6, and -7) are related to another yeast HDAC, Hda1p. Mammalian HDACs also function in large corepressor complexes. As mentioned before, in the absence of hormone, type II nuclear receptors bind DNA and recruit corepressor N-CoR or SMRT to mediate suppression of basal transcriptional activity. One mechanism underlying the repression activity of N-CoR and SMRT is through their recruitment of a HDAC complex containg mSin3A and HDAC1 (Heinzel et al., 1997; Nagy et al., 1997). Direct interaction of NcoR or SMRT with the class II HDACs (HDAC4) independent of Sin3A provides yet another mechanism for NcoR and SMRT-mediated transcriptional repression (Huang et al., 2000). Upon hormone binding, the HDAC complex is replaced with a HAT complex that catalyzes histone acetylation, resulting in transcriptional activation (Collingwood et al., 1999).

3. The role of chromatin modification in glucocorticoid receptor transcription

As described above, eukaryotic transcription is an interlaced network of transcription factors and chromatin-modifying complexes. The glucocorticoid receptor (GR) has been the best studied receptor regarding how chromatin modification can contribute to transcription regulation.

Mechanisms of transcriptional activation are well conserved from yeast to human. Yeast activators function in mammalian cells and vice versa. Although yeast does not possess endogenous nuclear receptors (NRs), it has been shown that a number of NRs, including ERα, GR, RARs, RXRs, and TRs can function as ligand-dependent transactivators in yeast. When GR is expressed in yeast cells, several studies have shown that HAT complexes are important for GR-mediated gene activation in yeast. GR τ1 is dependent upon Ada2, Ada3, and Gcn5 for transactivation *in vitro* and *in vivo*. Moreover GR τ1 interacts directly with the native Ada containing HAT complex SAGA *in vitro*. Mutations in GR τ1 that reduce τ1 transactivation activity *in vivo* lead to reduced binding of τ1 to the SAGA complex and conversely, mutations increasing the transactivation activity of τ1 lead to increased binding of GR τ1 to SAGA. In addition, the Ada-independent NuA4 HAT complex also interacts with τ1 and stimulates τ1-mediated transcription. Further, it has been demonstrated that the human homolog of Ada2 also enhances GR transcription in mammalian cells (Henriksson et al., 1997; Wallberg et al., 1999). Taken together, these results indicate that chromatin modification by histone acetylation plays an important role in GR transcription both in yeast and in mammalian cells.

On the other hand, recruitment of the SWI/SNF chromatin remodeling complex has been shown to be a mechanism of gene activation by the GR. Previously Yaniv's group reported that hbrm (a human homologue of yeast SNF2/SWI2 and *Drosophila brm* genes) can potentiate GR activity in mammalian cells (Muchardt and Yaniv, 1993). Fryer and Archer demonstrated that chromatin remodelling by the glucocorticoid receptor requires the *Brahma*-related gene 1 (BRG-1) complex, another

mammalian SWI/SNF homologue (Fryer & Archer, 1998). Recently, Wallberg and coworkers showed that the SWI/SNF complex can potentiate the activity of GR through the N-terminal τ1 in both yeast and mammalian cells. GR τ1 can directly interact with purified SWI-SNF complex, and mutations in τ1 that affect the transactivation activity in vivo also directly affect τ1 interaction with SWI/SNF (Wallberg et al., 2000).

To summarize, both HAT complex and SWI/SNF complex can facilitate GR transcription in mammalian cells. The question is how the two distinct complexes function at a specific GR targeting promoter. Recently, Wallberg and coworkers show that the SWI/SNF and SAGA complexes represent independent pathways of τ1-mediated activation but play overlapping roles that are able to compensate for one another under some conditions (Wallberg et al., 2000). More recently, Workman's group found that histone acetyltransferase complexes (SAGA or NuA4) can stabilize SWI/SNF binding to promoter nucleosomes, illustrate a functional link between HAT complexes and the SWI/SNF chromatin remodeling complex and provide a mechanistic basis for the ordered recruitment of these complexes (Hassan et al., 2001).

III. SIGNALING OF MITOGEN ACTIVATED PROTEIN KINASE AND ACTIVATOR PROTEIN-1

An animal cell contains an elaborate system of proteins that enables the cell to respond to signals from other cells. The system includes cell surface and intracellular

receptor proteins, protein kinases, protein phophatases, GTP binding proteins, and the many intracellular proteins with which these signaling proteins interact.

Protein phosphorylation plays a cardinal role in regulating many cellular processes in eukaryotes. In particular, signal transduction pathways employ protein phosphorylation as a mechanism to transmit information to different cellular compartments to elicit distinct cellular responses. Protein kinases catalyze the addition of phosphate groups from ATP to specific amino acids within proteins, while protein phosphatases catalyze phosphate group removal by hydrolysis. In principle, the extent of phosphorylation at a particular site can be regulated by changing the activities of both protein kinases and protein phosphatases. The importance of protein kinases in regulating cellular activities is underscored by the large number of protein kinase genes that are present in eukaryotic genomes. It has been estimated that humans have as many as 2000 conventional protein kinase genes (Hunter, 1995).

Protein kinases and protein phosphatases can be divided into broad groups based upon the identity of the amino acid that they target (serine/threonine, tyrosine, lysine, and histidine). However, dual-specificity protein kinases and protein phosphatases that target both Tyr and Ser/Thr are also present in cells. A number of protein kinases are components of signal transduction cascades that are formed by the sequential phosphorylation and activation of protein kinases. One of the best-studied groups of protein kinases in recent years is the mitogen-activated protein (MAP) kinases.

1. Mammalian mitogen activated protein kinases

Mitogen activated protein (MAP) kinases are proline-directed serine/threonine kinases that are activated by dual phosphorylation on threonine and tyrosine residues in response to a variety of extracellular stimuli, including mating pheromones and osmotic stress in yeast, cell–cell interactions in *Drosophila*, and mitogens, cytokines, and UV irradiation and other stresses in vertebrates.

A major role for MAP kinases is to transmit extracellular signals to the nucleus, where the transcription of specific genes is induced. The transcription factor complex AP-1 (see below) has been identified as a target of MAP kinase signaling pathways. Most of the MAPK substrates remain uncharacterized. However, because of the ease of yeast genetic analysis, the functions and regulation of MAPK cascades are first best understood in yeast. Consequently the analyses of targeted mutations in mice, MAPK and MAPKK knockout mice, and the development of specific inhibitors have begun to shed light on MAP kinase functions in mammals. MAPKs have been found to regulate almost all cellular processes, such as gene expression, cell proliferation, cell survival and cell death, embryonic development, as well as cell and neuronal differentiation. The impact of MAP kinase pathways on the pathology of chronic inflammation, heart disease, stroke and cancer therapy, not to mention embryonic development, innate and acquired immunity, is profound. So it is not surprising that understanding MAP kinase pathways has attracted wide interest, and dramatic progress has been made in the past 10 years (Kyriakis et al., 2001).

1.1 Mammalian MAP kinase signaling cascades

Previously, three distinct MAPK cascades have been identified in mammalian cells, leading to activation of extracellular signal-regulated kinases (ERKs), the Jun N-terminal kinases (JNKs), and p38 proteins. MAPK cascades are composed of a MAPK, a MAPK kinase (MAPKK, MKK or MEK) and a MAPKK kinase (MAPKKK or MEKK). MAPK is activated by phosphorylation on Thr and Tyr by a dual specificity MAPKK, which in turn is activated by Ser/Thr phosphorylation by a MAPKKK. Recently, at least four mammalian MAPKs have been identified: ERK1/2, JNK1/2/3, p38α/β/γ/δ and ERK5. MAPKs are activated by specific MAPKKs: MEK1/2 for ERK1/2, MKK4/7 (JNKK1/2) for the JNKs, MKK3/6 for the p38, and MEK5 for ERK5 (as shown in figure 10). Each MAPKK, however, can be activated by more than one MAPKKK, increasing the complexity and diversity of MAPK signalling. Presumably each MAPKKK confers responsiveness to distinct stimuli. For example, activation of ERK1/2 by growth factors depends on the MAPKKK c-Raf, activation of ERK1/2 by cytokine depends on other MAPKKKs. But the situation is more complex with the JNK and p38 cascades, which respond to many stimuli and can be activated on overexpression of at least a dozen MAPKKKs. For example, at least four MAPKKKs are implicated in JNK activation by tumour necrosis factor (TNF) and interleukin (IL)-1, including ASK1 (MEKK5), TAK1, MLK and MEKK1 (Chang and Karin, 2001).

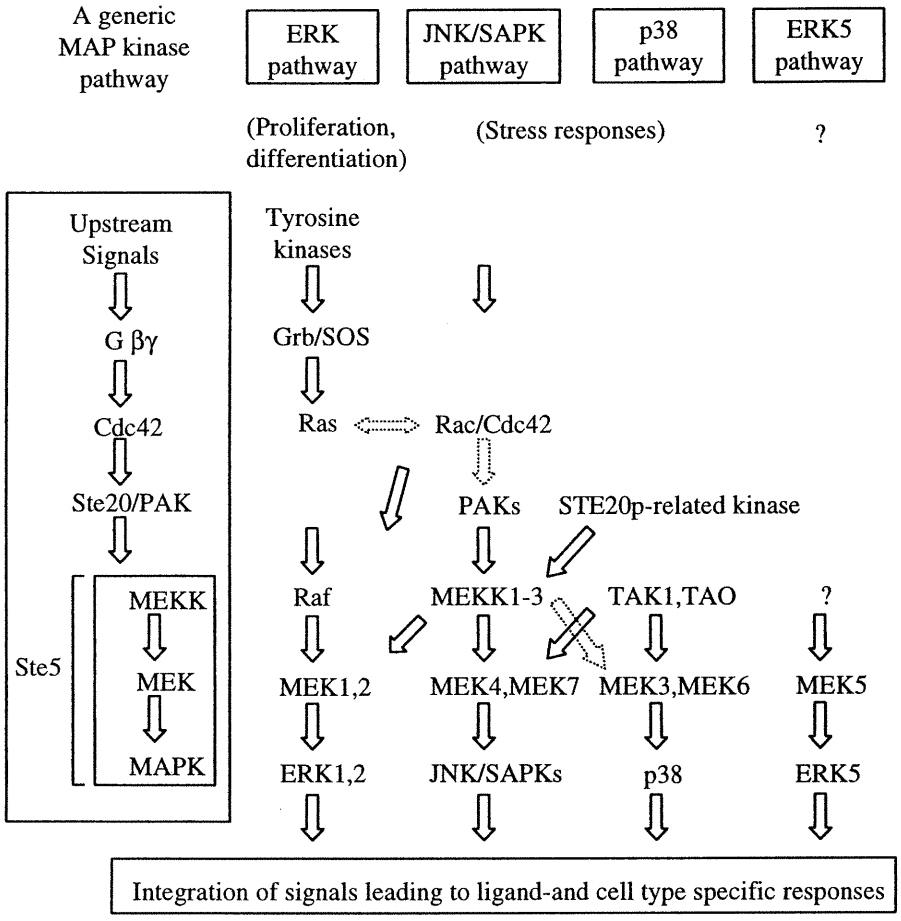

Figure 10. Schematic diagram of the currently known mammalian MAPK pathways

Mitogen activated protein kinase (MAPK) cascades consist of a three kinase module that includes a MAPK, which is activated by a MAPK kinase (MAPKK or MEK), which in turn is activated by a MEK kinase (MEKK or MAPKKK). The first and best characterized MAPK cascade consists of Raf isoforms, MEK1/2, and ERK1/2, and is regulated by Ras. Mitogenic signals can stimulate this pathway, and proliferation can be blocked by inhibiting it. JNK and p38 pathways mediate responses to cellular stress. Dotted arrows represent connections between pathways that are known to exist but for which not enough supporting data is available (adapted from Robinson and Cobb, 1997).

MAP kinases have two main characteristics. First, they are activated by dual phosphorylation upon Thr and Tyr within protein kinase subdomain VIII. The sequences of the tripeptide dual phophorylation motif are distinct: Thr-Glu-Tyr from ERKs, Thr-Pro-Tyr from JNKs, and Thr-Gly-Tyr from p38. Second, MAP kinases themselves are proline-directed Ser/Thr protein kinases with a minimum consensus target sequence of Ser/Thr-Pro (Whitmarsh and Davis, 1996).

1.2 The ERK MAP kinase signal transduction pathway

The best characterized signaling pathway involving mammalian MAP kinase is the activation of ERK signaling by growth factors and receptor tyrosine kinases, such as the epidermal growth factor (EGF) receptor, signaling through the small GTP binding protein Ras, leads to c-Fos induction through the SRE of the *c-fos* promoter (refer to figure 11). The binding of a growth factor to its specific receptor results in receptor tyrosine kinase activation, associated with autophosphorylation of multiple tyrosine residues within the cytoplasmic domain of the rceptor. These phosphorylated Tyr residues serve as binding sites for adaptor protein Grb2 (growth factor receptor-bound protein 2), which contains one Src-homology 2 (SH2) domain and two Src-homology 3 (SH3) domains. The Grb2 SH2 domain binds to autophosphorylated Tyr residues of EGF receptor. Even in quiescent cells, two SH3 domains of Grb2 bind to a proline-rich region in the carboxy-terminal tail of the guanine nucleotide exchange factor Sos (son of sevenless). The activated EGF receptor thus recruits a Grb2-Sos complex, leads to the translocation of Sos to the plasma membrane where Ras is

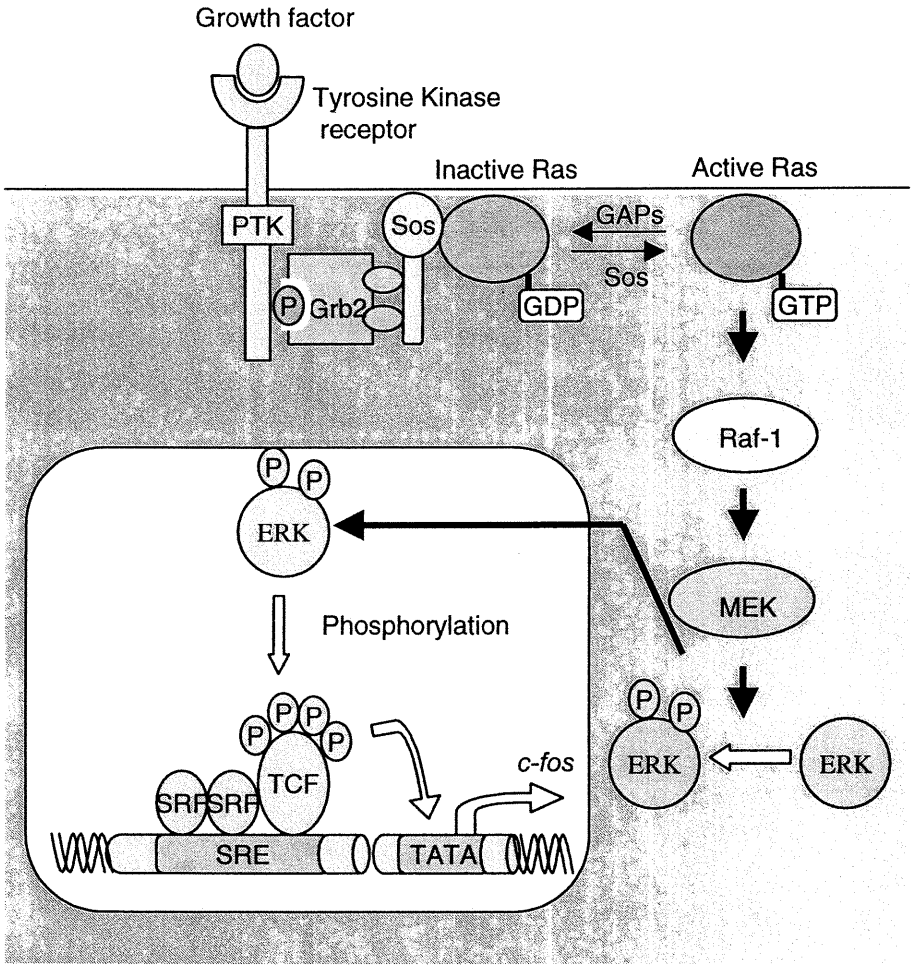

Figure 11. Scheme of the MAPK-mediated signaling pathway of growth factor

Ligand binding results in receptor autophosphorylation, followed by recruitment of Grb2 (an adaptor), leads to translocation of the Grb2-associated Sos (guanine nucleotide exchange factor) to the plasma membrane, where Ras is located, followed by Ras activation. Subsequently, activate Raf-1 (serine/threonine protein kinase), MEK (dual-specificity kinase), and ERK (serine/threonine kinase). ERK translocates to nucleus upon activation. In the nucleus, ERK phosphorylate transcription factor TCF, which is bound together with SRF to the SRE situated at the *c-fos* promoter. Phosphorylation of TCF stimulates its transactivation function, leads to activation of *c-fos* transcription (adapted from Karin and Hunter, 1995; Kato et al., 2000).

located , followed by Ras activation which is the exchange of GDP for GTP on Ras. Subsequently, the serine/threonine protein kinase Raf-1 binds to Ras and becomes activated, leading to phosphorylation and activation of the dual-specificity ERK-kinase, MEK. MEK activates the MAPKs ERK1 and ERK2, which translocate to the nucleus once activated. In the nucleus, the ERKs phosphorylate transcription factor TCF, which is bound together with SRF to the SRE of the *c-fos* promoter. Phosphorylation of TCF at a cluster of sites located next to its carboxyl terminus stimulates its transactivation function, probably by enhancing its ability to interact with the basal transcription machinery. This pathway leads to rapid activation of *c-fos* transcription (Karin and Hunter, 1995; Kato et al., 2000).

ERK1 and ERK2, often referred to as p44 and p42 MAP kinases, were first cloned in the early 1990s. ERK1 and ERK2 are nearly 85% identical overall, with much greater identity in the core regions involved in binding substrates. Both ERK1 and ERK2 are ubiquitously expressed. They are activated by serum, growth factors, cytokines, ligands for G protein coupled receptors and mitogens.

Only the knockout of ERK1 has been described. *Erk1*$^{-/-}$ mice are viable and appear normal, with only modest defects in T-cell development. It is likely that most ERK1 functions are equally served by ERK2. A similar and more marked defect is present in transgenic mice expressing dominant-negative MEK1 in thymocytes. In addition, *Mek1*$^{-/-}$ mice die *in utero*, exhibiting defective placental vascularization.

1.3 JNK MAP kinase signal transduction pathway

JNKs (46 and 54 kDa) were purified by affinity adsorption to a c-Jun fusion protein. Isolation of cDNAs encoding these enzymes and subsequent analysis of their

expression revealed three genes (*Jnk1/2/3)* encoding proteins with 10 or more alternatively spliced forms. Within the core catalytic domains, JNK1, JNK2, and JNK3 are more than 85% identical. JNK1 and JNK2 are widely expressed. In contrast JNK3 is expressed predominantly in the nervous system. JNKs are activated by exposure of cells to environmental stress and by the treatment of cells with cytokines. Studies of the physiological function of JNK have been facilitated by the molecular genetic analysis of JNK signalling in Drosophila and by the creation of mice with targeted disruptions of components of the JNK pathway. These studies demonstrate that the JNK pathway regulates AP-1 transcriptional activity *in vivo* and indicate that JNKs are required for embryonic morphogenesis (individual JNK knockout mice are viable, $Jnk1^{-/-}Jnk2^{-/-}$ double mutation results in lethality), the regulation of cellular proliferation and apoptosis, and the response of cells to immunological stimuli (Yang et al., 1997; Davis, 1999; Chang and Karin, 200; Pearson et al., 2001).

1.4 p38 MAP kinase signal transduction pathway

p38α MAP kinase was cloned in 1994 following the purification of a 38-kDa protein that is tyrosine phosphorylated in response to hyperosmolarity and endotoxic lipopolysaccharide (Han et al., 1994). Shortly thereafter, two human p38 homologs, termed CSBP1 (cytokine suppressive anti-inflammatory drug–binding protein) and CSBP2, were identified as the targets of an anti-inflammatory drug (pyridinyl imidazole drug SB203580, which is now defined as a specific p38 inhibitor), which inhibit the production of the cytokines Interleukin-1 (IL-1) and tumor necrosis factor α (TNFα) in stimulated monocytes (Lee et al., 1994). Cloning strategies rather than biological approaches were used to identify the other three genes that encode members

of the p38: p38β, p38γ, and p38δ. p38α and p38β are sensitive to pyridinyl imidazole inhibitors, but p38γ and p38δ are resistant to these drugs (Pearson et al., 2001). Similar to JNK, p38 MAP kinases are stress activated protein kinases, and repond to cellular stress (UV radiation, osmotic shock, heat shock, lipopolysaccharide, protein sysnthesis inhibitors), certain cytokines, and activation of G-protein coupled receptors (Whitmarsh and Davis, 1996; Pearson et al., 2001). The only p38 isozyme whose *in vivo* function has been examined genetically is p38α. Unlike deletions of individual *Jnk* loci or *Erk1*, inactivation of p38α results in embryonic lethality. It is not clear whether the lack of compensation by other isoforms is indicative of distinct biochemical functions or a marked difference in expression patterns. Consistent with the presence of two MAPKKs (MKK3/6) that activate p38 MAPKs, *Mkk3*$^{-/-}$ mice are viable without obvious abnormalities (Chang and Karin, 2001).

1.5 Specificity in MAPK activation and function

Scaffolding proteins: In yeast, the STE5 protein functions as a scaffold that organizes the three components of a pheromone-responsive MAPK cascade and its upstream activators into a specific module. A search for analogous mammalian scaffolds led to JIP1, which organizes JNK1/2, MKK7 and the MAPKKK MLK1 into a specific signalling cassette. MP1, another mammalian scaffold protein not related to JIP-1, interacts with ERK1 and MEK1, thereby potentiating ERK1 activation (Chang and Karin, 2001). So, eukaryotic cells have evolved scaffolding proteins that can simultaneously bind multiple components of a signaling pathway and thereby impose a degree of specificity and order on the highly interactive network of signaling proteins.

Substrate specificity of MAP kinases: While the ERKs phosphorylate TCF/Elk-1 and thereby induce c-Fos synthesis, they do not phosphorylate c-Jun, c-Fos or ATF2. The JNKs phosphorylate the N-terminal activation domains of c-Jun and ATF2, but do not phosphorylate c-Fos. The JNKs also phosphorylate TCF/Elk-1. p38 MAP kinases are capable of phosphorylating TCF/Elk-1 and ATF2 (Karin, 1995; Karin et al., 1997).

It is now clear that MAPKs are highly specific in their choice of substrates and do not phosphorylate just any Ser or Thr residue that is followed by a Pro, as previously assumed. The amino acids that surround these phosphoacceptor sites further increase the specificity of recognition by the catalytic pocket of the enzyme. The molecular mechanisms underlying the high degree of substrate specificity of MAP kinase are being explored with the Jun-JNK interaction as a paradigm. Efficient phosphorylation by the JNKs requires a docking site located between residues 30 and 60 of c-Jun. The presence of a JNK-docking site is essential, but not sufficient, for efficient c-Jun phosphorylation in response to JNK activation. Although not involved in kinase docking, efficient phosphorylation also requires certain specificity-conferring residues, such as a proline residue at the P+1 position flanking the phosphoacceptor site. Although JunB has an efficient JNK-docking site, it is not phosphorylated by JNK as it lacks this P+1 position proline. JunD, on the other hand, has the same specificity-conferring residues as c-Jun but lacks an efficient docking site. As a result, JunD is phosphorylated less efficiently than c-Jun in response to JNK activation. Most importantly, it was shown that the low-level phosphorylation of JunD most likely occurs through its heterodimerization with c-Jun or JunB, which have an

effective docking site. First, the kinase binds to one of these molecules, and then it phosphorylates the JunD partner. A mutant of JunD that forms more stable heterodimers with c-Jun or JunB is phosphorylated very efficiently by JNK, but only if it dimerizes with a docking-competent partner (Karin, 1995; Karin et al., 1997; Chang and Karin, 2001).

In the case of ERK1/2, besides the requirement of a proline at the P+1 position, substrates often also contain proline at the P-2 position, giving the motif PX(T/S)P, to confer the specificity of substrate recognition. Unfortunately, such specificity parameters have not been delineated for JNK and p38 MAP kinases (Pearson et al., 2001).

Recently, conserved motifs that mediate interactions of some MAP kinases with their substrates have been identified. The best characterized docking domain sequence is a cluster of basic residues, usually two or more, followed within a few residues by (L/I)X(L/I). The docking domain may appear at some distance from and in apparently any orientation with respect to the phosphoacceptor site in the substrate. Docking domains interact with ERK1/2, JNK, p38 family members, and perhaps other MAP kinases. Modest sequence differences may result in recognition by only one or two of these types of MAP kinases. Docking domains are present in numerous substrates including the transcription factors Elk-1, c-Jun and the MEF2 family (a family of transcription factors that regulate muscle-specific and immune cell gene expression), upstream activators such as MEK1/2, and phosphotyrosine phosphatases. Removing or mutating these domains within substrates markedly reduces their phosphorylation by MAP kinases (Pearson et al., 2001).

Another targeting motif that may be related to the docking domain is typified by the sequence LAQRR and its variants, and is present in several protein kinases that are MAP kinase substrates. LAQRR is thought to be recognized specifically by ERK1/2, and is found in Rsk isoforms (ribosomal S6 kinases, Rsk1 and Rsk2 also known as MAPK-activated protein kinase 1α and β) and Mnk2 (MAP kinase-interacting kinase). The related sequence LA(K/R)RR has been suggested to bind to both ERK1/2 and p38 and is found in the protein kinases MSK1 and Mnk1. A variation, LX(K/R)(R/K)RK is targeted by p38 and is found in several downstream protein kinases including MSK2, MAPK-activated protein kinase-2, and MAPK-activated protein kinase-3 (Pearson et al., 2001).

A distinct motif is the FXFP sequence that is thought to interact only with the ERK1/2. This motif is present in transcription factors such as LIN-1, SAP-1, and Elk-1, protein kinases such as A-Raf and some dual-specificity protein phosphatases such as MKP-1 and DUS4 (Pearson et al., 2001).

1.6 Inactivation of MAP kinases

The duration and amplitude of MAP kinase activation reflects the balance between activating signals and inactivating signals. ERK activity is tightly regulated through phosphorylation of tyrosine and threonine residues in the TEY motif mentioned above. ERK inactivation is mediated by dephosphorylation of these residues by tyrosine, serine/threonin, or dual-specificity phosphatases. A substantial number of dual-specificity Thr/Tyr phosphatases known as MAP kinase phosphatases (MKPs) are largely dedicated to the inactivation of MAP kinases. These dual specificity Thr/Tyr phosphatases include: MAP kinase phosphatase 1 (MKP1, also

termed 3CH134/CL100), MKP2 (also termed TYP-1/hVH2), hVH3, hVH5, PAC-1, and MAP-3 (also termed hVH6). The specificity of different MKPs for different MAP kinase family members has been suggested by *in vitro* and cellular studies that sometimes have different conclusions (Whitmarsh and Davis, 1996; Pearson et al., 2001).

2. Regulation of AP- 1 activity by mitogen activated protein kinases

12-O-tetradecanoyl-phorbol-13 acetate (TPA) is a tumor-promoting phorbol ester. TPA treatment leads to induction of proto-oncogenes (c-sis, c-fos, c-myc), genes encoding secreted proteases (collagenase, stromelysin), human metallothionein II_A, and SV40. Analysis of the promoter regions of TPA inducible genes has led to the identification of consensus sequences (TGAC/GTCA), called TPA-responsive elements (TREs). TREs are recognized by a common cellular protein: AP-1 (activator protein-1), and are for this reason also termed "AP-1 sites". AP-1 is thus at the receiving end of a complex pathway responsible for transmitting the effects of phorbol ester tumor promoters from the plasma membrane to the transcriptional machinery (Angel, et al., 1987; Chiu, et al., 1987).

The AP-1 family of transcription factors consists of homodimers and heterodimers of Jun (c-Jun, JunB and JunD), Fos (c-Fos, FosB, Fra1, and Fra2) or activating transcription factor (ATF2, ATF3, B-ATF) bZIP (basic region leucine zipper) proteins. Jun proteins form very stable heterodimers with Fos- and ATF-family members, and can also homodimerize among themselves. ATF proteins, but not Fos proteins, also form stable homodimers (Angel & Karin, 1991; Karin et al., 1997).

AP-1 activity was found to be induced by not only TPA, but also a number of other stimuli, including growth factors, cytokines, T cell activators, neurotransmitters, and UV irradiation. Mechanisms involved in induction of AP-1 activity can be classified as those that increase the abundance of AP-1 components and those that stimulate their activity by phosphorylation (Karin, 1995). Considerable progress has been made towards understanding the transcriptional and posttranslational regulation of AP-1 activity by MAPKs.

2.1 Induction of *c-fos and c-jun*

Induction of c-Fos: AP-1 components c-Jun and c-Fos behave as "immediate early" genes, whose transcription is rapidly induced, independent of *de nevo* protein synthesis, following cell stimulation. There are three major *cis* elements present in the *c-fos* promoter that are targeted by a diverse spectrum of extracellular stimuli: the cAMP response element (CRE), the serum response element (SRE), and the Sis-inducible enhancer (SIE). The coordinated action of each of these elements is required for correct spatial and temporal regulation of *c-fos* gene expression *in vivo* (figure 12A).

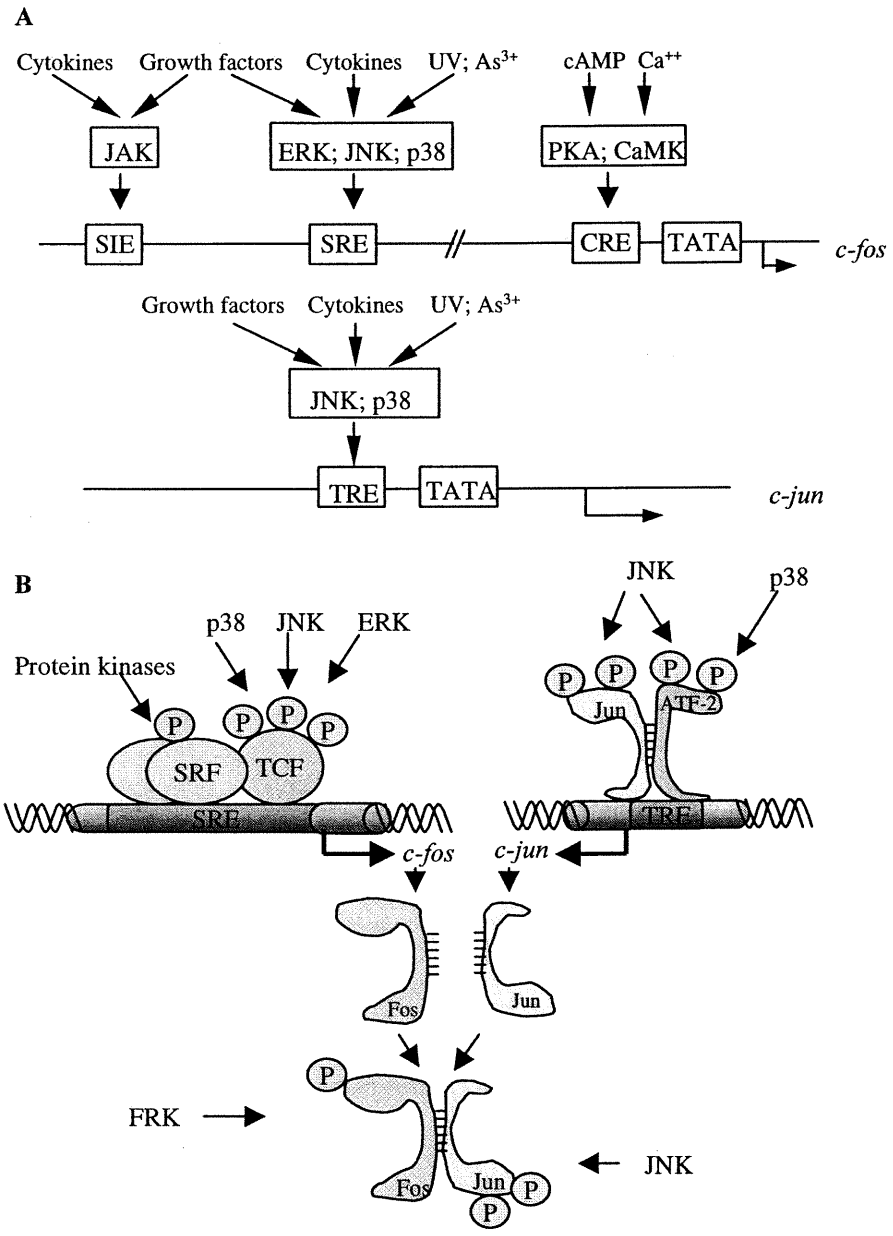

Figure 12. Regulation of AP-1 in response to extracellular stimuli

A. Regulation of *c-fos* and *c-jun* transcription in response to extracellular stimuli. The cis-acting elements in the *c-fos* and *c-jun* promoters that mediate their induction in response to extracellular stimuli are illustrated. The protein kinases that phophorylate the transcription factors that interact with these elements are also indicated. **B.** Regulation of AP-1 activity by MAP kinases (adapted from Karin, 1995).

Proximal to the c-*fos* TATA box is a cAMP reponse element (CRE), target of CREB (CRE-binding protein, another bZIP transcription factor) or ATF proteins, which all mediate c-*fos* induction in response to neurotransmitters and polypeptide hormones using cAMP or Ca^{2+} as second messengers. cAMP and Ca^{2+} activate protein kinase A (PKA) and calmodulin-dependent protein kinases (CaMK), respectively (Whitmarsh and Davis, 1996; Karin et al., 1997), which regulate CREB or ATF phosphorylation and activity.

The *c-fos* serum response element (SRE) was originally demonstrated to mediate serum induction of the *c-fos* promoter, but it is also induced by TPA, growth factors, cytokines, and other stimuli that activate MAPKs. The SRE is recognized by a dimer of the serum response factor (SRF) that recruits the monomeric ternary complex factors (TCFs) to form a ternary complex (as shown in figure 12B). TCF proteins belong to the ETS-domain family of DNA binding proteins that includes Elk-1, SAP-1, and SAP-2. TCFs are important mediators of c-*fos* induction by a large variety of extracellular stimuli. Mitogen-stimulated phosphorylation of Elk-1 is carried out by the ERK MAP kinases. As the ERKs are rapidly activated by most mitogens (such as TPA, growth factors), this mechanism may account for mitogen-induced c-*fos* transcription (as described before, also refer to figure 11). The sites phosphorylated by the ERKs reside in the COOH-terminal activation domain of Elk-1 and have a positive regulatory role (Marais et al., 1993). Similar phosphoacceptor sites are present in other TCFs whose phosphorylation and activity are also stimulated by the ERKs. However, the SRE also mediates c-*fos* induction by stimuli such as UV irradiation and IL-1, neither of which results in considerable ERK activation. Rather, these stimuli activate

JNKs and p38. JNKs and p38 are capable of phosphorylating the Elk-1 COOH-terminal activation domain and increase Elk-1 transcriptional activity and SRE mediated c-*fos* induction, as shown in figure 12A and figure 12B (Karin, 1995; Whitmarsh and Davis, 1996; Karin et al., 1997).

The third *cis* element that regulates *c-fos* transcription is a Sis-inducible enhancer (SIE) which is recognized by the STAT (signal transducer and activator of transcription) group of transcription factors. The STATs are activated and translocated to the nucleus in response to signals which activate the JAK (Janus kinase) group of tyrosine kinases (Karin et al., 1997).

Induction of c-Jun: The *c-jun* gene is expressed in many cell types at low levels, and its expression is elevated in response to many stimuli, including growth factors, cytokines and UV irradiation. Induction is usually mediated through the two TREs in the *c-jun* promoter (figure 12), which were proposed to be more efficiently recognized by c-Jun-ATF2 heterodimers than conventional AP-1 complexes. Exposure of cells to UV irradiation, proinflammatory cytokines or growth factors results in activation of the JNK and p38 groups of MAPKs. The JNKs were identified by their ability to specifically phosphorylate c-Jun at two positive regulatory sites residing within its amino-terminal activation domain (Ser 63 and Ser73). The JNKs can also phosphorylate and stimulate the transcriptional activity of ATF2. The same positive regulatory sites on ATF2 are also phosphorylated by p38, while the sites in the c-Jun activation domain are phosphorylated only by the JNKs (Karin, 1995; Whitmarsh and Davis, 1996; Karin et al., 1997).

2.2 Posttranslational regulation of AP-1 activity by MAP kinases

The activities of both pre-existing and newly synthesized AP-1 components are modulated through their phosphorylation. So far, this form of posttranslational control has been demonstrated for c-Jun, c-Fos, and ATF2, but it is likely that other Jun and Fos proteins are similarly regulated. In the case of c-Jun, phosphorylation of c-Jun at Ser-73 and Ser-63, located within its transactivation domain, potentiates its ability to activate transcription as either a homodimer or a heterodimer with c-Fos. These residues are phosphorylated by JNKs (among JNKs, JNK2 exhibits the highest affinity). Phosphorylation may potentiate c-Jun transcriptional activity through recruitment of CBP. CBP is postulated to connect the phosphorylated activation domains of CREB or c-Jun to the basal transcriptional machinery (Karin, 1995).

Interestingly, the sequence surrounding the N-terminal phosphoacceptor (Ser 73) of c-Jun is conserved in the C-terminal activation domain of c-Fos. However, Thr-232 of c-Fos is not phosphorylated by either JNK1 or JNK2 but by a novel 88-kDa MAPK termed FRK (Fos regulating kinase). Like the ERKs and the JNKs, FRK is a proline-directed kinase, whose activity is rapidly stimulated in response to Ha-Ras activation by growth factors. Phosphorylation at Thr-232 stimulates c-Fos transcriptional activity (Karin, 1995).

A similar situation may apply for c-Jun-ATF2 heterodimers, as ATF2 phosphorylation at Thr-63 and Thr-71 within its N-terminal activation domain was shown to stimulate its transcriptional activity. ATF2 is phosphorylated and activated by both the JNKs and p38 (Karin, 1995; Whitmarsh and Davis, 1996).

2.3 Biological function of AP-1

A powerful method for identifying the potential function of a specific protein is to examine the phenotype of the knockout mice. This approach has been successfully used to provide information concerning the functions of the AP-1 components c-Fos, FosB, c-Jun, and ATF2. Despite the striking induction of c-*fos* expression in response to growth factors and antisense RNA, suggesting that c-*fos* is essential for cell proliferation, c-*fos*$^{-/-}$ mice demonstrated that c-*fos* is not essential for the viability, proliferation and differentiation of most cell types, the exception being some cells that are involved in bone formation, gametogenesis and certain neuronal functions. Homozygous c-*fos*$^{-/-}$ mice, although viable, are growth-retarded and develop osteopetrosis, with deficiencies in bone remodeling and tooth eruption. The fact that c-Fos does not seem to be required for proliferation of most cells *in vivo* raises the question as to why expression of c-*fos* is so tightly linked to mitogenic stimulation. One possible explanation could be that other AP-1 proteins compensate for most of the c-Fos functions except those whose loss leads to bone and neuronal defects. Indeed, it was shown through microinjection of specific antibodies that none of the Fos proteins individually (i.e. c-Fos, FosB, Fra1 or Fra2) is essential for cell proliferation, but the neutralization of all four blocked cell proliferation. Correspondingly, *fosB*$^{-/-}$ mice, although defective in nurturing behavior, are otherwise normal. Additionally, mice deficient in both c-*fos* and *fosB* exhibit the same phenotype as c-*fos* null mice (Karin et al., 1997).

In contrast to *c-Fos, c-Jun* is essential for normal mouse development. Homozygous c-*Jun*$^{-/-}$ mouse embryos die after 12-14 days at mid-to-late gestation and

display morphological abnormalities of the liver and widespread edema. c-*Jun*$^{-/-}$ embryonic stem cells differentiate into functional germ and somatic cells but not into hepatocytes. Although this may suggest that c-Jun is not required in other cell types, it should be noted that primary fibroblasts from c-*Jun*$^{-/-}$ embryos are completely defective in their proliferation. c-Jun is probably the first transcription factor found to be absolutely required for fibroblast proliferation (Whitmarsh and Davis, 1996; Karin et al., 1997).

It should also be noted that overexpression of c-Fos in bone tissue resulted in development of osteosarcomas. Simultaneous overexpression of c-Jun and c-Fos in bone enhanced the rate of osteosarcoma formation, indicating *in vivo* cooperation between c-Jun and c-Fos in tumor development (Karin et al., 1997).

ATF-2 deficient mice have decreased postnatal viability and widespread abnormalities in skeletal and central nervous system development, including chondrodysplasia, ataxic gait, hyperactivity, and decreased hearing (Whitmarsh and Davis, 1996).

IV. CROSSTALK BETWEEN NUCLEAR RECEPTORS AND AP-1 SIGNALING PATHWAYS

It is an emerging notion that a limited number of signaling proteins interact in a combinatorial fashion to build intracellular networks that allow diverse cellular responses. A possible advantage of a signaling network with multiple intersecting pathways is to direct a coherent response to numerous, potentially conflicting signals. *In vivo* a single cell will be exposed to multiple stimuli: steroid hormones, growth factors, cytokines, osmotic stress etc. These different signals may act synergistically or may antagonize one another. Phosphorylation of proteins provides an important mechanism for crosstalk between signaling pathways.

1. Phosphorylation of nuclear receptors

1.1 Overview of nuclear receptor phosphorylation

Nuclear receptors, like many transcription factors, are phosphoprotein, and increasing evidence suggests that the activity of nuclear receptors be regulated by phosphorylation. Treatment of cells with activators or inhibitors of protein kinases or phosphatases affects the transcriptional activity of steroid receptors. In some cases, these treatments can activate the receptor in the absence of hormone. Phosphorylation of nuclear receptors provides an important mechanism for crosstalk between signaling pathways. Multiple kinase pathways have been implicated, including cAMP dependent protein kinase (PKA), casein kinase, glycogen synthase kinase (GSK), cyclin-dependent kinases (CDKs), JNK and ERK MAP kinases. Phosphorylation has been

shown to modulate the activity of many nuclear receptors, as shown in table 4 (Shao and Lazar, 1999). It has been suggested that phosphorylation might modulate hormone binding, influence nucleocytoplasmic shuttling of the protein, dimerization, or affect transcription by altering DNA binding or interaction with cofactors or the basal transcription machinery (Garabedian et al., 1998). Some of the effects of phosphorylation are listed in table 4. Intriguingly, phosphorylation of nuclear receptors can either positively or negatively modulate transactivation. For example, phosphorylation of human RXRα serine 260 by MAP kinase inhibits RXR and VDR ligand-dependent activation, while phosphorylation of ERα serine 118 by MAP kinase promotes both ligand-dependent and ligand-independent activation. In the case of rat GR, phosphorylation of serine 246 by JNK MAP kinase inhibits GR ligand-dependent transactivation, while phosphorylation of Serine 224 and 232 by CDKs enhances GR ligand-dependent transactivation.

1.2 Phosphorylation of glucocorticoid receptor

GR is phosphorylated (predominantly on serine, threonine residues at the N terminal) in both the presence and the absence of hormone, and is reportedly subjected to cell cycle-related changes. However, additional phosphorylation occurs in conjunction with agonist, but not antagonist, binding (Orti et al., 1989). Mouse and rat GR phosphorylation sites have been well characterized. Bodwell et al have identified seven phosphorylation sites in the mouse GR (Serines 122, 150, 212, 220, 234, and 315 and threonine 159) by direct sequencing of phosphorylated peptides that were

Table 4. Phosphorylation of nuclear receptors*

Receptor	Kinase	Site(s)	Effect of phosphorylation
AR	PKA	Ser641, 653	Promote ligand-dependent and ligand-independent activation
ER	PKA,	Ser236 (ERα)	Inhibit dimerization and DNA binding
	MAPK	Ser118 (ERα)	Promote ligand-dependent and ligand-independent activation
	MAPK	Ser106, 124 (ERβ)	Promote ligand-dependent and ligand-independent activation
	Receptor tyrosine kinase	Tyr537 (ERα)	Enhance ER interaction with SRC-1
GR	MAPK	Ser246	Inhibit ligand-dependent activation
	CDKs	Ser224, 232	Enhance ligand-dependent activation
	GSK-3	Thr171	Inhibit ligand-dependent activation
PR	CDK2	Ser162, 190, 400	Ligand-independent activation
	Casein kinase	Ser102, 294, 345	Regulate hormone-dependent activation
TR	Casein kinase	Ser474, 475 (TRα2)	Inhibit monomer DNA binding
	PKA	Ser28/29(TRα1)	Inhibit monomer DNA binding
	PKA	Ser16/17(v-erb A)	Inhibit monomer DNA binding
		Multiple sites (TRβ)	Tissue-specific stabilization Promote RXR heterodimerization
RAR	Cdk7/TFIIH	Ser77 (RARα1)	Increase AF1 Transactivation
	PKA	Multiple sites	RA-dependent activation
RXR	MAPK	Ser260	Inhibit RXR and VDR ligand-dependent activation
	PKA	Multiple sites	RA-dependent activation in muscle cells
PPARγ	MAPK	Ser112 (PPARγ2)	Decrease ligand-independent activation Decrease ligand-binding affinity
	JNK/SAPK	Ser82 (PPARγ1)	Decrease ligand-dependent activation
PPARα	Insulin-activated kinase	Not known	Increase ligand-dependent activation
SF-1	MAPK	Ser203	Promote cofactor recruitment of LBD
HNF4	PKA	Multiple sites	Promote DNA binding
Nurr77	PP90rsk	Ser354	Inhibit DNA binding

*From Shao and Lazar, 1999.

isolated from the receptor overexpressed in WCL2 cells, a chinese hamster ovary cell line. Serines 122, 150, 212, 220, and 234 and the sequences surrounding them are conserved in the homologous regions of the rat and human receptors, as shown in figure 13. Threonine 159 and serine 315 have no homologues in the human GR, although they are still conserved in the rat GR. Mouse GR Serines 212, 220 and 234 and threonine 159 are in consensus sequences for proline-directed kinases. Serine 122 is in a consensus sequence for casine kinase II, whereas serines 150 and 315 do not appear to be in any known kinase consensus sequence (Bodwell et al., 1991).

Consistently, Krstic et al have identified four major phosphorylation sites on rat GR expressed in hepatoma cells and in yeast that coincide with a subset of sites identified in mouse GR and correspond to T171, S224, S232, and S246. It has been shown that for the rat GR, phosphorylation of S224 and S232 increases in the presence of hormone agonists, whereas residues T171 and S246 appear to be phosphorylated constitutively. In each case, residues T171, S224, S232, and S246 are followed by proline, thus corresponding to a motif typically modified by cyclin-dependent kinases (CDK consensus = S/T(P)-P-X-R/K) or mitogen activated protein kinase (similar as introduced before, MAPK consensus = nonpolar-X-S/T(P)-P). Of the remaining potential GR phosphorylation sites, S134 is located in a consensus motif for casine kinase II (CK II consensus =S/T(P)-X-X-E/D), whereas the sequences surrounding S162 and S327 do not match any known kinase consensus motif (Krstic et al., 1997; Garabedian et al., 1998). Recently, the rat GR has also been shown to be

A.

```
mGR 107  MGNDLGYPQQGQLGLSSGETDFRLLEESIANLNRSTSRPENPKSSTPAAGCATPTEKEFP 166
rGR 119  ..............S.....................V.....S..S.T...T..... 178
hGR  98  ......F.....IS.S.....LK.............V.....S.AST.VS.A..... 157

mGR 167  QTHSDPSSEQQNRKSQPGTNG-GSVKLYTTDQSTFDI----LQDLEFSAGSPGKETNESP 221
rGR 179  K....A..........T....-......P.......L----.K.......S.S.D...S. 233
hGR 158  K....V.....HL.G.T....-.N.............----.......S.S.......S. 212

mGR 222  WRSDLLIDEN---LLSPLAGEDDPFLLEGDVNEDCKPLILPDTKPKIQDTGDTILSSPSS 278
rGR 234  ..........---..S.............NT................K............. 290
hGR 213  ..........C--..S.......S.....NS................K.N..LV.....N 270

mGR 279  VALPQVKTEKDDFIELCTPGVIKQEKLGPVYCQASFSGTNIIGNKMSAISVHGVSTSGGQ 338
rGR 291  .....................................S...................... 350
hGR 271  .T........E.................T.......P.A..................... 330
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B.

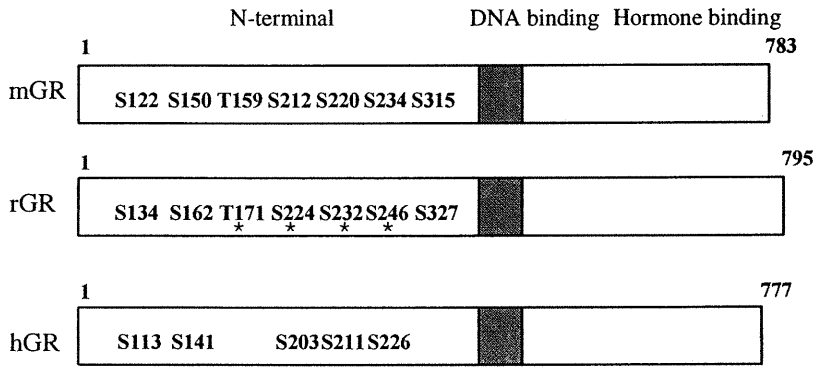

Figure 13. Residues phosphorylated on the glucocorticoid receptor

A. Sequence alignment of mouse, rat and human GR N-terminal. The latest versions of SwissProt sequences were used in this comparison. -, space; .,identity with mGR.

B. Schematic representation of the seven phosphorylation sites identifed by Bodwell et al on the mouse GR, five of the seven conserved phosphorylation sites in rat and human GR are also shown. The four major phosphorylation sites on rat GR identified by Krstic are marked with *.

phosphorylated *in vitro* at S527 by DNA-dependent protein kinase (DNA-PK) (Giffen et al., 1997).

2. Phosphorylation of coactivators

Recent evidence has suggested that the steroid receptor coregulator proteins may be targets of cellular signaling pathways. The activities of the corepressors N-CoR and SMRT and the coactivator CBP are regulated by cell signaling pathways. SRC-1 phosphorylation has been recently characterized by O'Malley's group. It was reported that ERK-2 phosphorylated threonine 1179 and serine 1185 *in vitro*. They also found that the protein kinase A (PKA) activator, 8-bromo-cAMP can enhance the ligand-independent activation of the chicken progesterone receptor, and that whole progesterone receptor phosphorylation was not altered, coactivator SRC-1 phosphorylation was increased 1.8 fold, phosphorylation was increased on two MAPK sites, threonine 1179 and serine 1185. PKA did not phosphorylate these sites *in vitro*, while 8-bromo-cAMP was found to activate ERK1/2. Mutation of threonine 1179 and serine 1185 to alanine resulted in a 50% decrease in coactivation during PR ligand-independent activation. This 50% decrease was ascribed, in part, to loss of functional cooperation between SRC-1 and CBP for coactivation of PR (Rowan et al., 2000).

In addition, Kushner's group found that EGF regulates the activities of the p160 GRIP1 through ERK MAP kinases, which phosphorylate GRIP1 at serine 736 *in vitro*. Mutation of S736 to alanine substantially reduces the ability of GRIP1 to enhance transcription of the EGF activated PR and of the estrogen and EGF potentiated ER (Lopez et al., 2001). Font de Mora et al demonstrated that AIB1 is a

phosphoprotein *in vivo* and can be phosphorylated *in vitro* by ERK-2 (Font de Mora et al., 2000). Ait-Si-Ali et al showed that CBP phosphorylation by ERK1 results in the stimulation of its histone acetyltransferase activity (HAT) *in vitro* (Ait et al., 1999). Taken together, these results indicate that coactivators are phosphoproteins *in vivo*, and that coactivator phophorylation modulation may affect nuclear receptor transcriptional activity via several mechanisms.

3. Mechanisms of GR repression of AP-1

More than a decade ago, our view of gene regulation by glucocorticoids and other steroid hormones underwent a dramatic change with the discovery of negative crosstalk (transcriptional interference) between the glucocorticoid receptor and transcription factor AP-1. It was initially observed that induction of the collagenase type I gene, which is mediated through activation of AP-1 by growth factors and inflammatory cytokines, is repressed by glucocorticoids. This repression was attributed to mutual negative interactions between AP-1 and GR. It has become clear that this and analogous interactions with other transcription factors (e.g. nuclear factor-κB) underlie the anti-inflammatory and immunosuppressive activity of glucocorticoids (Karin and Chang, 2001). But the exact molecular mechanism underlying this GR-dependent repression of NF-κB or AP-1 activity remains to be determined.

Several models for the role of GR have been suggested: (1) Direct protein-protein interaction: Analysis of the collagenase promoter revealed that the segment that is sufficient for mediating repression by glucocorticoids do not contain GREs,

while it contains a consensus AP-1 site. Furthermore repression is rapid and independent of induction of GR target genes. Protein-protein interactions between GR and the Jun and Fos components of AP-1 were detected, and mutual inhibition of DNA-binding activity upon mixing of GR with AP-1 proteins was demonstrated (Jonat etal., 1990; Schüle et al., 1990; Yang-Yen et al., 1990). These early results led to the suggestion that the basis for transcriptional interference in this case is formation of a physical complex between GR and AP-1 (Jun:Fos or Jun:Jun dimers), in which either participant is unable to recognize its cognate DNA-binding site. This simple model was quickly challenged by genomic footprinting experiments, which suggested that AP-1 remains bound to the collagenase promoter under conditions of glucocorticoid-mediated repression (Konig et al., 1992).

(2) Competition for limiting amounts of CBP/p300 in cells: The basis for this model is that GR and AP-1 proteins all directly interact with a common coactivator, CBP/p300. CBP/p300 is required for both GR and AP-1 transactivation. So it was suggested that GR and AP-1 compete for this common coactivator for their transactivation (Kamei et al., 1996). However, as the amounts of nuclear CBP/p300 seem to exceed those of AP-1 or GR and CBP/p300 is also a common target for many other sequence specific transactivators, such as NF-κB or STATs, which do not transrepress AP-1 activity, it is unlikely that simple competition for a limiting amount of CBP/p300 can explain the transrepression of AP-1 activity by GR (Karin and Chang, 2001). Recently, De Bosscher et al demonstrated that AP-1-targeted gene repression by glucocorticoids is refractory to increased amounts of nuclear coactivators CBP/p300 and SRC-1 (De Bosscher et al., 2001).

(3) GR inhibition of JNK: Muñoz's group has demonstrated that glucocorticoids can reduce JNK activity induced by TNFα by approximately 50% (Caelles et al., 1997). This effect was shown to be recepter dependent, but it is unlikely to be exerted by direct interaction between the activated GR, which translocated to the nucleus, and the bulk of JNK, which remains outside the nucleus even after it is activated. It is also not clear how a mere 50% reduction in JNK activity can account for the almost complete reprssion of AP-1 target genes, such as collagenase, by glucocorticoids (Karin and Chang, 2001). In agreement with these findings, a recent paper by Muñoz's group demonstrates that inhibition of JNK activity by glucocorticoids is greater within the nucleus than that observed in the cytoplasm, and that this inhibition does not affect JNK subcellular distribution (Gonzalez et al., 2000). Still the question of how the activated JNK (mostly outside the nucleus) is inhibited by the activated GR (inside the nucleus) remains to be answered.

4. Crosstalk between ER and AP-1

Cho and Katzenellenbogen (1993) reported that activators of protein kinase A and protein kinase C markedly synergize with estrogen in ER-mediated transcriptional activation. Later, Kato et al demonstrated that ER Ser118 is phosphorylated by mitogen-activated protein kinase *in vitro* and in cells treated with epidermal growth factor (EGF) and insulin-like growth factor (IGF) *in vivo*. The phosphorylation of ER Ser118 is required for full activity of the ER activation function 1 (AF-1). Therefore, ER activity is modulated not only by ligand but also by signals from tyrosine kinase-linked cell surface receptors, such as EGF and IGF (Kato et al., 1995). On the other

hand, several reports documented rapid activation of MAP kinases ERK1 and ERK2 by estradiol (Migliaccio et al., 1996; Filardo et al., 2000; Manthey et al., 2001; Wade et al., 2001). Umayahara et al. (1994) reported that an AP-1 site is essential to mediate estrogen stimulation in the insulin-like growth factor-I promoter. Kushner's group reported that estrogens and tamoxifen stimulated transcription of promoters regulated by AP-1 sites including the human collagenase gene promoter (-73 to +63) and constructs in which an AP-1 site is fused to the herpes thymidine kinase promoter (Webb et al., 1995).

5. Crosstalk between GR and AP-1

Crosstalk between glucocorticoid receptor and AP-1 was discovered more than 10 years ago. As mentioned previously, recent experiments have demonstrated that the crosstalk ability of glucocorticoid receptor is essential for mouse development. It has become clear that glucocorticoid receptor's repressive effect on AP-1 and other transcription factors (e.g. nuclear factor–κB) underlies the anti-inflammatory and immunosuppressive activity of glucocorticoids. However so far the exact molecular mechanisms underlying this transcriptional interference are not clear. Mechanisms involving direct protein-protein interactions and interference at the level of MAP kinase activity have been proposed. Conversely, there are conflicting reports concerning MAPK signaling pathway action on glucocorticoid signaling. Rogatsky et al (1998) reported that selective activation of JNK and ERK inhibits GR transcriptional activation, while Moyer et al (1993) demonstrated that protein kinase C

activators such as TPA enhance glucocorticoid-induced gene expression in T47D cells.

V. RESEARCH OBJECTIVES

Nuclear receptors are involved in the regulation of cellular growth in different cell types including epithelial cells. Transcriptional regulation by nuclear receptors binding to target response elements in the regulatory region of target genes can explain in part the proliferative or anti-proliferative effects of estrogen and glucocorticoids. However, an alternative pathway of transcriptional regulation is the crosstalk between nuclear receptors and activator protein-1 (AP-1), a signaling pathway that is rapidly induced by a number of growth factors and other signaling molecules.

AP-1 activity can be induced by estrogen and repressed by glucocorticoids, suggesting that this alternative pathway could contribute importantly to the mechanism of cell growth regulation by both hormones. Estrogen induces breast cancer growth, antiestrogens can block estradiol stimulation of cellular growth in breast cancer cells and in breast tumors. However, some antiestrogens like tamoxifen (TAM) can, when administered in the absence of estrogen, induce uterine cell growth. The mechanisms of this tissue-specific agonistic activity of tamoxifen (TAM) are poorly understood. It has been proposed that TAM may induce cell growth through AP-1 activation. To investigate the tissue-specific agonism of antiestrogens like tamoxifen, we have investigated whether the partial agonist activity of anti-estrogens in Ishikawa cells can be mediated at the level of regulation of gene expression by typical estrogen response elements and/or by AP-1 sites.

On the other hand, AP-1 activators, such as EGF and TPA exert their mitogenic effects through the MAP kinase ERK signaling pathway, which promotes proliferative responses in the cells. Whereas GR activation has potent anti-

proliferative effects in multiple cell types. Rogatsky et al reported that selective activation of either ERK or JNK inhibits GR-mediated transcriptional activation. It has been proposed that phosphorylation of GR by JNK or of a GR cofactor by ERK provides mechanisms to ensure the rapid inhibition of GR-dependent gene expression when it conflicts with mitogenic signals. However, literature reports on the effect of activation of the MAP/SAP kinase pathway on GR trancriptional activity are not in agreement. To address this issue, we first studied the effects of MAP kinase activators on GR transcriptional activity. Further we investigated and analyzed several potential mechanisms, such as whether AP-1 activators can regulate GR expression, GR phosphorylation, or nuclear receptor coactivator phosphorylation. Knowledge resulting from these studies will contribute to a better understanding of the mechanisms of crosstalk between nuclear receptors and the MAPK-AP-1 pathway, and of the regulation of cell proliferation by nuclear receptor ligands.

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Chapter II

REGULATION OF AP-1 BY STEROID HORMONES

Journal of Biological Chemistry. 1998 Jul 3;273(27):17138-46.

Estrogen Response Elements Can Mediate Agonist Activity of Anti-estrogens in Human Endometrial Ishikawa Cells

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THE JOURNAL OF BIOLOGICAL CHEMISTRY
Vol. 273, No. 27, Issue of July 3, pp. 17138–17146, 1998
Printed in U.S.A.

Estrogen Response Elements Can Mediate Agonist Activity of Anti-estrogens in Human Endometrial Ishikawa Cells*

(Received for publication, September 3, 1997, and in revised form, April 9, 1998)

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Anti-estrogens like hydroxytamoxifen (OHT) have mixed agonist/antagonist activities, leading to tissue-specific stimulation of cellular proliferation. Partial agonist activity of OHT can be observed *in vitro* in endometrial carcinoma cells like Ishikawa. Here, we have compared several anti-estrogens (including extensively characterized OHT and pure anti-estrogens such as ICI164,384 and RU58,668, which are devoid of uterotrophic activity) for their capacity to stimulate promoters containing estrogen response elements (EREs) or AP1-binding sites (12-*O*-tetradecanoylphorbol-13-acetate response elements, TREs), the two types of DNA motifs known to mediate transcriptional stimulation by estrogen receptors. Assays were performed in Ishikawa cells either by transient transfection or by using cell lines with stably propagated reporter vectors. In transient transfection experiments, none of the anti-estrogens displayed agonist activity on the promoters tested. In contrast, significant transcriptional stimulation was observed with low concentrations of OHT and RU39,411 in Ishikawa cells stably propagating reporter constructs containing a minimal ERE3-TATA promoter. In addition, micromolar concentrations of OHT, but not of RU39,411, stimulated stably propagated AP1-responsive reporter constructs. No transcriptional stimulation of ERE- or TRE-containing promoters was observed with the pure anti-estrogens ICI164,384 and RU58,668. These results indicate that the presence of estrogen response elements in promoters is sufficient to mediate cell-specific agonism of anti-estrogens at the transcriptional level, and that stimulation of AP1 activity may be restricted to a subset of anti-estrogens possessing agonist activity on EREs. In addition, our results suggest that transient transfections do not fully recapitulate *in vivo* conditions required to observe agonist activity of anti-estrogens.

The estrogen 17β-estradiol (E2)[1] regulates gene transcription by binding to the estrogen receptor (ER), which interacts with specific target DNA sequences known as estrogen response elements (EREs). When bound to DNA the ER stimulates transcription via two transcription activation domains, AF-1 and AF-2 (1–3). AF-1 is located in the poorly conserved N-terminal A/B domain of the ER (2, 4), whereas AF-2 is found in the C-terminus of region E, the hormone-binding region (5–8). Binding of E2 to the ER is thought to induce a conformational change in the hormone binding domain, stimulating its transactivation properties.

Different types of synthetic compounds have been developed that are capable of antagonizing ER action in reproductive tissues and, in particular, of blocking estradiol stimulation of cellular growth in breast and uterine tissues. These anti-estrogens act by competing with E2 for binding to the ER and block ER-mediated activation of transcription when co-administered with hormone (9). However, tamoxifen, one of the most widely used anti-estrogens in breast cancer treatment (10), can induce uterine cell growth *in vivo* in animal models (11) and in humans (12, 13). Hydroxytamoxifen also induces cellular proliferation (14, 15) and transcription of endogenous estrogen target genes such as the progesterone receptor (PR) gene in human endometria and cultured human endometrial carcinoma cells (15, 16). Other anti-estrogens, like ICI164,384 (17) or the more recently developed RU58,668 (18), were reported not to stimulate uterine cell growth and may therefore prove more appropriate for breast cancer therapy (17).

To better understand the mechanisms of tissue-specific estrogenic activity of anti-estrogens, we compared transcriptional activation of ERE-containing reporter constructs by full or partial anti-estrogens in the estrogen-dependent breast carcinoma cell line MCF7 and in the endometrial carcinoma cell line Ishikawa. We also assessed whether anti-estrogens may regulate transcription of target genes through AP1-binding sites (TPA response elements, TREs) rather than, or as well as, through EREs. Indeed, estrogenic stimulation of promoters containing TRE sites has been documented (19–22), a possible mechanism being direct interaction between ER and AP1 components (22). Assays used for investigating the contribution of EREs or TREs in transcriptional stimulation by anti-estrogens with partial agonist activity included, in addition to transient transfection assays in MCF7 or Ishikawa cells, direct hormonal stimulation of reporter vectors stably propagated as episomes in these cell lines. The latter assay was selected because of increasing evidence for mechanistic links between transcriptional stimulation and reorganization of chromatin structure.

* This work was supported by Grant MT-13147 from the Medical Research Council of Canada and grants from the Cancer Research Society Inc. and the Fonds pour la Formation de Chercheurs et l'Aide à la Recherche. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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[1] The abbreviations used are: E2, estradiol; EBV, Epstein-Barr virus; ER, estrogen receptor; ERE, estrogen response element; PR, progesterone receptor; FBS, fetal bovine serum; TAM, tamoxifen; OHT, 4-hydroxytamoxifen; ICI, ICI164,384; RUp, RU39,411; RUf, RU58,668; CAT, chloramphenicol acetyltransferase; tk, thymidine kinase; STR, rat stromelysin promoter; Vit, *Xenopus* vitellogenin A2 promoter; TPA, 12-*O*-tetradecanoylphorbol-13-acetate; TRE, TPA response element; RT, reverse transcriptase; PCR, polymerase chain reaction; DMEM, Dulbecco's modified Eagle's medium; GRE, glucocorticoid response element(s).

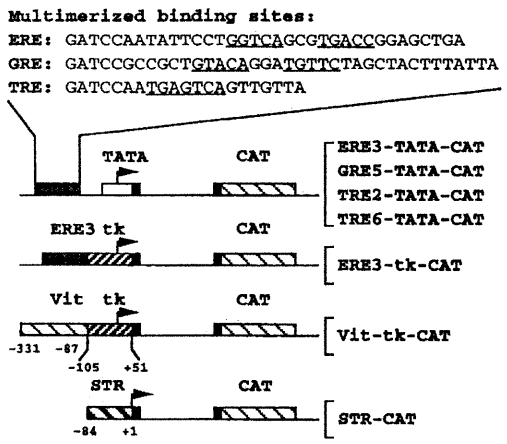

FIG. 1. **Transcription units of reporter plasmids.** Sequences of multimerized binding sites present in minimal estrogen-, glucocorticoid-, and AP1-responsive promoters are shown (see "Experimental Procedures" for details of plasmid constructions). Note that estrogen-responsive promoter/reporter constructs used for transient or stable transfections were inserted in EBV episomal vectors and are referred to in the text as ERE3-TATA-CAT/EBV. The glucocorticoid-responsive GRE5-TATA-CAT plasmid is identical to GRE5/CAT (26). AP1-responsive promoters were inserted either in non-episomal vectors derived from GRE5/CAT (TRE6-TATA-CAT) or in EBV vectors (TRE2-TATA-CAT/EBV and TRE6-TATA-CAT/EBV).

Comparison of the two types of assays and implications for the mechanism of cell-specific agonism by tamoxifen are discussed.

EXPERIMENTAL PROCEDURES

Chemicals and Materials—17β-Estradiol (E2) was purchased from Sigma; RU58,668 (RUf) and RU39,411 (RUp) were generous gifts from Dr. D. Philibert, Hoechst-Marion-Roussel, Romainville, France. ICI164,384 was kindly provided by Dr. T. Willson, Glaxo-Wellcome Research Institute, Research Triangle Park, NC. OHT and TAM were purchased from Sigma. Cell culture media, fetal bovine serum, G418, and hygromycin B were purchased from Life Technologies, Inc.

Plasmid Recombinants—Expression vectors pSG5, pSG5-HEG0, and reporter recombinants Vit-tk-CAT (23) and STR-CAT (equivalent to construct 84-CAT in Ref. 24) were kindly provided by Dr. P. Chambon (Illkirch, France). ERE3-tk-CAT was constructed by insertion of three copies of double-stranded oligonucleotides containing the 15-bp *Xenopus* vitellogenin A2 ERE sequence (25) flanked by *Hin*dIII and *Xba*I sites between the *Hin*dIII and *Xba*I sites of pBLCAT8+. ERE3-TATA-CAT was constructed in several steps from GRE5-CAT (26). First, the *Bgl*II site upstream of the CAT gene in GRE5-CAT was deleted by filling-in with Klenow, creating GRE5-CAT[-*Bgl*II]. A fragment containing the ERE3-TATA promoter was then excised from the vector ERE3-pAL10 (27) by digestion with *Asp*-718, end-filling with Klenow fragment, and digestion with *Bam*HI; this fragment was inserted into GRE5-CAT[-*Bgl*II] which had been digested with *Sac*I, treated with Klenow fragment, and then digested with *Bam*HI to remove the GRE5-TATA promoter. By taking advantage of the unique *Xho*I and *Bgl*II sites upstream and downstream from the three EREs, respectively, these motifs were removed and replaced by multimerized oligonucleotides containing a consensus TPA response element (TRE, Fig. 1*A*), creating TRE2-TATA-CAT and TRE6-TATA-CAT. ERE3-TATA-CAT/EBV, TRE2-TATA-CAT/EBV, and TRE6-TATA-CAT/EBV were obtained by removal of *Xba*I fragments containing the whole minimal promoter-CAT gene transcriptional unit from the parental vectors and insertion into GRE5-CAT/EBV also digested by *Xba*I (28).

Cell Culture and Transfections—MCF7 and Ishikawa cells were grown in α-minimum Eagle's medium supplemented with 10 and 5% fetal bovine serum (FBS), respectively, and switched to phenol red-free (29) DMEM supplemented with 5% charcoal-treated FBS 72 h before plating for transient transfections. Cells were divided into 10-cm plates (1.5 million cells/plate) and transfected using the calcium-phosphate coprecipitation method (30) with 15 μg of DNA (1 μg of expression vector where applicable, 2 μg of CAT reporter vector, 2 μg of internal standard vector RSV-LacZ, and Bluescribe M13+ to 15 μg). After 20 h, medium was changed twice to remove precipitate, and hormones were added for a further 24 h (as indicated in figure legends). Cells were harvested by scraping in 1 ml of phosphate-buffered saline 1×, followed by centrifugation at 2,500 rpm for 10 min. Extracts were prepared by three cycles of freeze-thawing in 0.25 M Tris-HCl, pH 8.0, and standardized for β-galactosidase activity. CAT activity was determined by incubation of protein samples for 1 h with 0.25 μCi of [^{3}H]chloramphenicol and *N*-butyryl-CoA (0.2 mg/ml), followed by extraction with xylene, and liquid scintillation counting (31). To test for regulation of progesterone receptor expression, cells maintained in phenol red-free medium supplemented with charcoal-treated serum were further incubated for 5 days in the absence or presence of estradiol or of anti-estrogens. Medium was changed every 2nd day, and hormones were added every day. On day 5, cells were plated (1.8 million cells in 10-cm dishes) and transfected in duplicate with 2 μg of GRE5-TATA-CAT plasmid (26), 1 μg of internal control RSV-LacZ, and Bluescribe M13+ as carrier DNA (total 15 μg). Progesterone was added 1 day later, after removal of calcium-phosphate precipitates by two consecutive washes. Cells were harvested 24 h later, and CAT activity was assayed as described above. All CAT assays were reproduced a minimum of three times.

COS-1 cells were grown in DMEM supplemented with 5% FBS. Cells were plated at a confluency of 1.5×10^6 cells/10-cm plate and transfected with 15 μg of pSG5 or pSG5-HEG0 expression vectors. Cells were harvested 36 h later, and extracts were prepared in gel retardation buffer 4× (20 mM Tris-HCl, pH 7.5, 20% glycerol, 400 mM KCl, 0.1 mM EDTA, pH 8.0, 2 mM dithiothreitol, supplemented with protease inhibitor mixture) by three cycles of freeze-thawing on ice. For gel retardation assays, extracts were incubated with 2 μg of poly(dI-dC) in gel retardation buffer 1× for 20 min on ice, followed by further incubation in the presence of hormone (10^{-8} M E2 or 10^{-7} M anti-estrogens) and of labeled ERE (20,000 cpm) for 1 h on ice, and then for 30 min at room temperature. Complexes were then resolved by 5% polyacrylamide gel electrophoresis (120 V, 4 °C for 4 h).

Generation and Hormonal Treatment of Stably-transfected Cell Lines Derived from Ishikawa and MCF7 Cells—Ishikawa or MCF7 cells were transfected with 15 μg of ERE3-TATA-CAT/EBV, TRE2-TATA-CAT/EBV, or TRE6-TATA-CAT/EBV (10-cm plates, 1.5 million cells). Forty eight hours after transfection, cells were passaged into 15-cm plates using medium containing 150 μg/ml hygromycin B and maintained in this medium for about 2 weeks until disappearance of all cells in control non-transfected plates (28). Surviving cells in each 15-cm plate were then pooled, propagated, and tested for estrogen or TPA induction of CAT activity. Different pools of cells carrying the same reporter plasmid were found to behave similarly. For generation of stable cell lines containing non-episomal TRE-based reporter vectors, Ishikawa cells were cotransfected with 15 μg of TRE6-TATA-CAT vectors and 1.5 μg of neomycin resistance gene expression vector Rc/RSV (Invitrogen). 48 h after transfection, cells were trypsinized and replated into selection medium (α-minimum Eagle's medium containing 5% FBS and 1 mg/ml G418). Two weeks later, individual clones were selected, expanded, and tested for stimulation of CAT activity by incubation with TPA (100 ng/ml) for 24 h. Established cell lines were subsequently maintained in medium containing half the concentration of antibiotic used for selection. For hormonal treatment, cells were preincubated for 72 h in medium without phenol red, supplemented with charcoal-treated serum. Incubation with estrogen or anti-estrogens was then carried out for 24 h except when indicated otherwise.

Reverse-transcription-PCR Amplification of Actin and CAT mRNAs—Ishikawa-ERE3/EBV cells maintained in phenol red-free DMEM, 5% charcoal-treated FBS were incubated with E2 (25 nM), OHT (100 nM), or ethanol for 8 h before harvesting and isolation of total RNA by CsCl gradient centrifugation. 3 μg of total RNA were precipitated, resuspended in 6 μl of 1× DNase I digestion buffer (Promega) containing 0.5 units of DNase I, incubated for 15 min at 37 °C and for 10 min at 75 °C, and then transferred on ice. DNase I-treated RNAs (2 μl) were reversed-transcribed using Superscript II RNase H reverse transcriptase (Life Technologies, Inc.) and 0.5 μM random hexamer in a 20-μl final volume of RT buffer (50 mM Tris-HCl, pH 8.3, 75 mM KCl, 3 mM $MgCl_2$, 5 mM dithiothreitol supplemented with 0.5 mM dNTPs) at 37 °C for 1 h, followed by 75 °C for 10 min. Aliquots of resulting cDNAs (2 μl) were amplified by PCR using *Taq* DNA polymerase (Amersham Pharmacia Biotech) in a 50-μl final volume of 1× *Taq* buffer supplemented with 0.2 mM dNTPs and 0.5 μM forward and reverse CAT or β-actin primers as follows: CAT forward, 5′-CCGCCTGATGAATGCTCATCCG-3′, and CAT reverse, 5′-GCATTCTGCCGACATGGAAGCC-3′; β-actin forward, 5′-GCTGTGCTATCCCTGTACGC-3′, and β-actin reverse, 5′-GCCATGGTGATGACCGGC-3′.

24 cycles of PCR (95 °C for 30 s, 56 °C for 1 min, and 72 °C for 25 s) were performed for amplification of β-actin cDNAs and 28 cycles were performed (95 °C for 30 s, 58 °C for 1 min, and 72 °C for 30 s) for amplification of CAT sequences, followed by a final elongation step

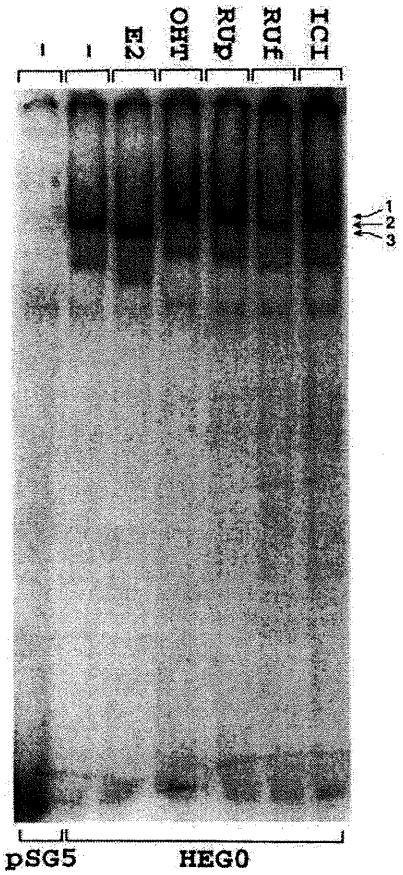

FIG. 2. **Effect of anti-estrogens on the electrophoretic mobility of ER·ERE complexes.** COS-1 cells were transiently transfected with 10 μg of either pSG5 (*lane 1*) or pSG5-HEG0 (*lanes 2–7*) expression vector. Whole cell extracts were prepared by three cycles of freeze-thawing and incubated with anti-estrogens or estrogen and labeled ERE for 1 h on ice, 30 min at 25 °C, and separated by non-denaturing polyacrylamide gel electrophoresis. The position of complexes containing ER liganded by partial anti-estrogens (*arrow 1*), full anti-estrogens (*arrow 2*), or estrogen (*arrow 3*) is indicated.

(72 °C for 10 min). The amplified products were then resolved on a 1.5% agarose gel.

RESULTS

Differential Effects of Anti-estrogens on the Electrophoretic Migration of Estrogen Receptor-DNA Complexes—Partial anti-estrogens such as hydroxytamoxifen are thought to induce a specific conformation of the estrogen receptor, which differs from both those of unliganded and estrogen-liganded ER. As a result, interaction between ER and estrogen or OHT differentially affects electrophoretic mobilities of ER·ERE complexes in gel shift assays. Full anti-estrogens like ICI164,384 induce yet another conformation, resulting in a migration that is closer to that of unliganded ER (32).

We used a gel shift assay to examine ER conformational changes induced by two other anti-estrogens, RU39,411 (RUp) and RU58,668 (RUf), which are both 11-β derivatives of estradiol and were previously reported to behave as partial and full anti-estrogens, respectively (18, 33). Extracts from COS-1 cells transiently transfected with an expression vector for wild-type estrogen receptor (pSG5-HEG0) were incubated with estrogen, anti-estrogens, or vehicle together with a labeled consensus estrogen response element. ER-containing complexes were then separated from free probe by 5% polyacrylamide gel electrophoresis. A specific complex could be detected in the absence of ligand (Fig. 2, *2nd lane*). Indeed, this complex was absent when using extracts from cells transfected with the parental expression vector pSG5 (Fig. 2, *1st lane*) or when HEG0-containing extracts were incubated with a glucocorticoid-response element instead of an ERE (data not shown). Estrogen treatment of the cellular extracts increased the electrophoretic mobility of the ER·ERE complex (Fig. 2, *E2, arrow 3*). OHT treatment, on the other hand, resulted in a slower migrating complex (Fig. 2, *OHT, arrow 1*), and RUp treatment generated a complex migrating at a position similar to that of OHT (Fig. 2, *RUp, arrow 1*). Complexes between ER and the anti-estrogen RUf migrated at a position intermediary between those of ER·E2 and ER·OHT (Fig. 2, *RUf, arrow 2*), and indistinguishable from that of the ER·ICI164,384 complexes (Fig. 2, *ICI, arrow 2*). Similar results were obtained using the ER mutant HE0, except that complexes were only observed in the presence of estrogen or of the various anti-estrogens and not in the absence of ligand (data not shown), consistent with the destabilizing effect of the G400V mutation on unbound ER (34, 35). In conclusion, the electrophoretic mobilities of ER liganded with RUp or RUf are consistent with their identification as partial and full anti-estrogens, respectively.

Differential Induction of Progesterone Receptor Gene Expression by Anti-estrogens in Ishikawa Cells—Hydroxytamoxifen was previously reported to induce expression of the estrogen target gene human progesterone receptor in Ishikawa cells (15, 16). We decided to investigate whether RUp, which similarly affects ER migration in gel shift assays, can also induce expression of PR in Ishikawa and MCF7 cells. Cells were pretreated with estrogen or anti-estrogens prior to transient transfection with GRE5-TATA-CAT/EBV (Fig. 1), followed by addition of progesterone for 24 h (see "Experimental Procedures"). No stimulation of CAT activity by progesterone could be observed in extracts of cells in the absence of treatment with estrogen or anti-estrogens (Fig. 3*A*, *lane 2*). In cells treated with estrogen or with the ER agonist moxestrol, the 11β-methoxy derivative of ethynyl estradiol (36, 37), CAT activity was induced over 20-fold in the presence of progesterone (Fig. 3*A*, *lanes 4* and *12*). In cells pretreated with OHT, progesterone-induced CAT expression levels were 12% of those obtained with estrogen pretreatment (Fig. 3*A*, *lane 6*). The anti-estrogen RUp induced PR expression to comparable levels (Fig. 3*A*, *lane 10*), whereas no stimulation could be detected following incubation with RUf (Fig. 3*A*, *lane 8*). Contrary to what was observed with Ishikawa cells, none of the anti-estrogens assayed detectably stimulated PR transcriptional activity in MCF7 cells using this assay (Fig. 3*B*, compare *lanes 5–10* to *lanes 1* and *2*). These observations confirm that partial agonist activity of anti-estrogens on expression levels of endogenous estrogen target genes can be observed in Ishikawa cells (15, 16).

Lack of Agonist Activity of Anti-estrogens in Transient Transfection Assays of ERE-containing Reporter Vectors—In order to analyze the mechanisms of transcriptional regulation by anti-estrogens with partial agonist activity in Ishikawa cells, we examined whether synthetic estrogen-responsive promoters can be stimulated by these anti-estrogens in transient transfection assays (Fig. 4). Three estrogen-sensitive reporter recombinants, ERE3-TATA-CAT/EBV, ERE3-tk-CAT, or Vit-tk-CAT (Fig. 1), were transiently transfected into Ishikawa cells in the absence of cotransfected estrogen receptor expression vector (Fig. 4*A*). Anti-estrogens did not detectably stimulate CAT expression from ERE3-TATA-CAT/EBV (Fig. 4*A*, *lanes 3–6*), ERE3-tk-CAT (Fig. 4*A*, *lanes 10–13*), or Vit-tk-CAT (Fig. 4*A*, *lanes 17–20*) under conditions where estradiol stimulated these reporter constructs 35-, 10-, and 25-fold, respectively (Fig. 4*A*, *lanes 2, 9,* and *16*). Similar results were obtained when CAT assays were repeated with 10 times more extract

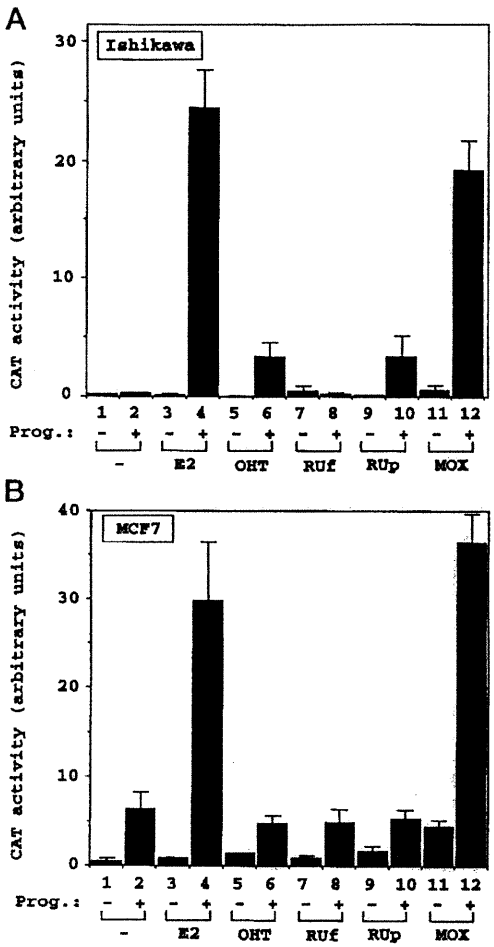

FIG. 3. **Differential regulation of progesterone receptor gene expression by anti-estrogens in Ishikawa and MCF7 cells.** Ishikawa cells (*A*) or MCF7 cells (*B*) were pretreated for 5 days with vehicle (−), estradiol (*E2*) (25 nM), moxestrol (*MOX*) (25 nM), or anti-estrogens (100 nM) as indicated, before being transiently transfected with the GRE5-TATA-CAT reporter plasmid ("Experimental Procedures"; the GRE motif is a response element for the progesterone receptor as well as for the glucocorticoid receptor). Progesterone (*Prog.*) (100 nM) was added 24 h before harvesting the cells. Note that similar results were obtained with 4 or 10 days incubation with anti-estrogens (data not shown).

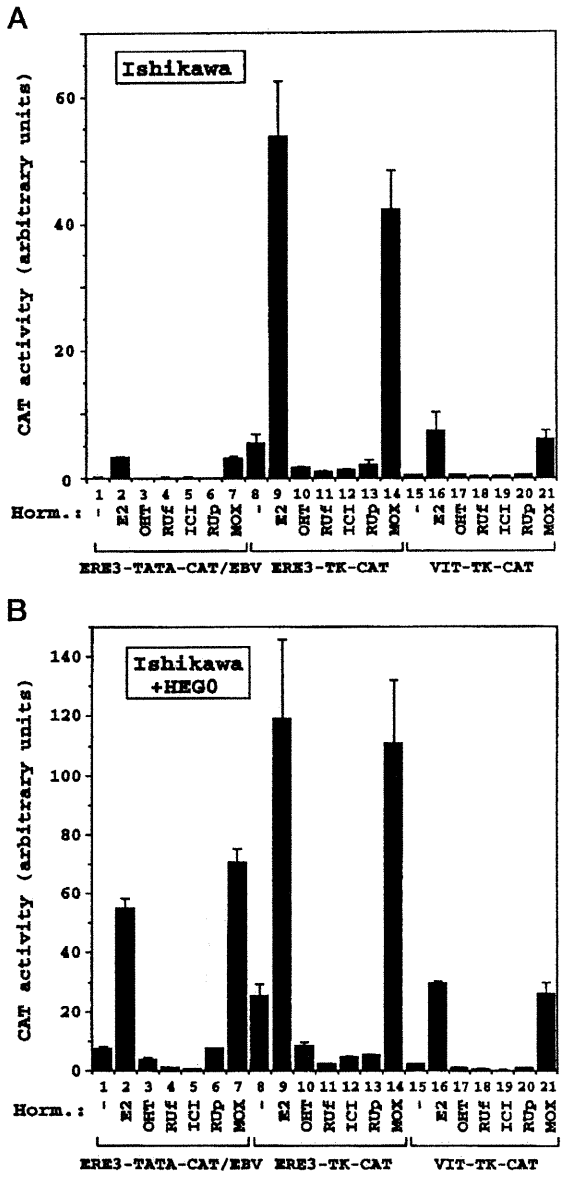

FIG. 4. **Estrogen, but not anti-estrogens, stimulates expression from ERE-containing reporter vectors transiently transfected in Ishikawa cells.** *A*, transient transfections were performed in Ishikawa cells with ERE3-TATA-CAT/EBV, ERE3-tk-CAT, or Vit-tk-CAT (23) reporter constructs as described under "Experimental Procedures." *B*, transient transfections in Ishikawa cells as above but with cotransfected pSG5-HEG0 expression vector (1 μg). Hormone concentrations used were 25 nM for estradiol (*E2*) and moxestrol (*MOX*) and 100 nM for all anti-estrogens.

than in Fig. 4*A* to confirm that none of the anti-estrogens tested stimulated CAT activity higher than vehicle (data not shown).

Cotransfection of an expression vector for wild-type ER (pSG5-HEG0) along with ERE3-TATA-CAT/EBV, ERE3-tk-CAT, or Vit-tk-CAT resulted in increased background levels in the absence of hormonal treatment (Fig. 4*B, lanes 1, 8,* and *15*). Treatment with anti-estrogens did not increase CAT expression levels compared with vehicle alone (Fig. 4*B, lanes 3–6, 10–13,* and *17-20*), although CAT levels in the presence of OHT or RUp were slightly higher than with other anti-estrogens. Background CAT expression in the absence of hormonal treatment could result from binding of residual estrogens in media, which would be competed out by incubation with anti-estrogens. Alternatively, unliganded ER may be a weak transcriptional activator under the conditions of this assay.

Results similar to those observed in Ishikawa cells were obtained by transient transfection of MCF7 cells (data not shown). In both cell lines, the concentrations of anti-estrogens used (100 nM) were sufficient to totally repress stimulation by 1 nM estrogen, demonstrating that estrogen receptors are fully saturated by these anti-estrogens under the conditions used to assay for agonist activity (data not shown). In conclusion, agonist activity of anti-estrogens could not be detected in transient transfection assays of ERE-containing reporter vectors either in Ishikawa or in MCF7 cells.

Detection of Cell-specific Agonist Activity of Anti-estrogens Using a Stably Propagated ERE3-TATA-CAT Episomal Reporter Vector—The failure to detect significant transcriptional activation by OHT or other anti-estrogens in transient transfections raised the possibility that *in vivo* conditions required for agonist activity of OHT are not fully reconstituted in this assay. Therefore, we established Ishikawa and MCF7 cell lines stably propagating the ERE3-TATA-CAT/EBV episomal plasmids, selecting pools of transfected cells by addition of hygromycin B in cell culture medium (see "Experimental Procedures"; note that for brevity cell lines stably propagating

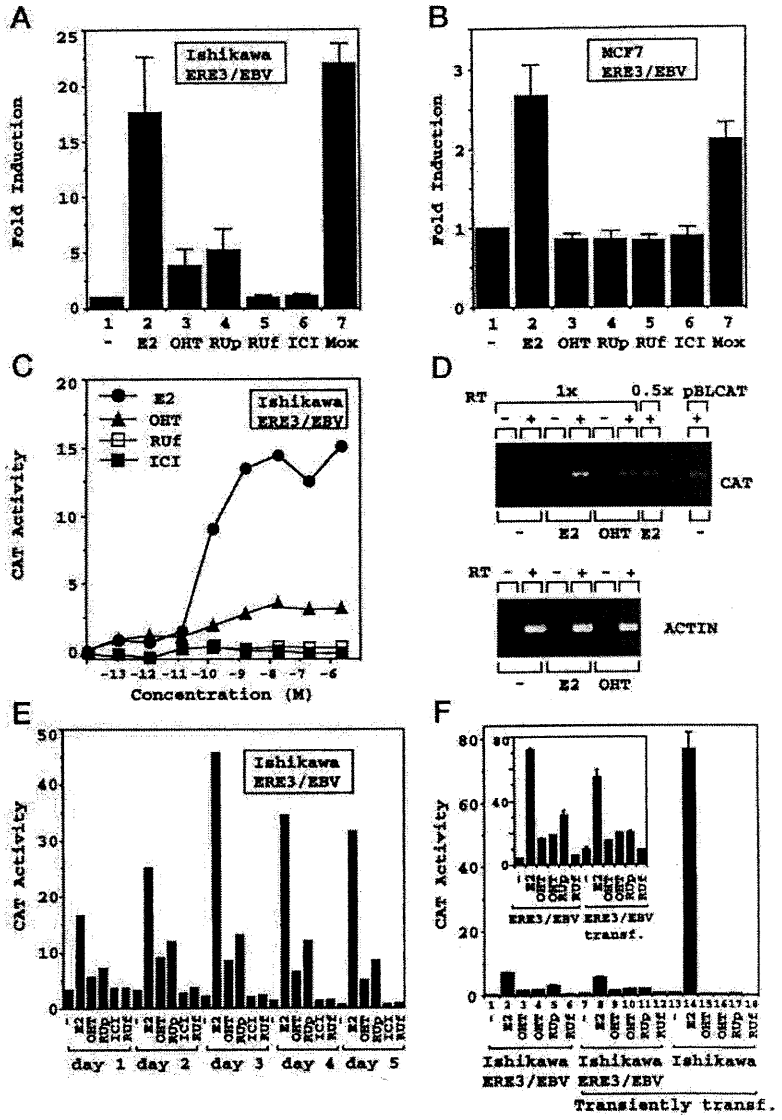

FIG. 5. **Agonist activity of anti-estrogens in Ishikawa, but not in MCF7 cell lines stably propagating ERE3-TATA-CAT/EBV.** *A* and *B*, CAT activity in extracts of Ishikawa (*A*) or MCF7 (*B*) cells carrying ERE3-TATA-CAT/EBV (Ishikawa-ERE3/EBV) after treatment with E2 (25 nM, *lane 2*), moxestrol (*Mox*; 25 nM, *lane 7*), or anti-estrogens (100 nM OHT, RUp, RUf, or ICI, *lanes 3–6* as indicated) for 24 h. *C*, CAT activity in extracts of Ishikawa cells treated with increasing concentrations of estrogen or anti-estrogens for 24 h. *D*, levels of CAT and β-actin mRNA after stimulation by estrogen or OHT for 8 h measured by semi-quantitative RT-PCR. The same amount of cDNA was used for PCR amplification in all lanes (*1×*) except in the lane marked *0.5×* where half the amount of cDNAs from E2-treated cells was used. Positive control obtained by PCR amplification using the same primers and the pBLCAT 8+ plasmid as template is shown in the *last lane* of the *top panel*. *E*, CAT activity in extracts of Ishikawa cells after treatment with estrogen or anti-estrogens for 1–5 days. *F*, CAT activity in extracts of Ishikawa-ERE3/EBV cells (*lanes 1–6*), Ishikawa-ERE3/EBV cells which were transiently transfected with 15 μg of Bluescribe M13+ (*lanes 7–12*), or Ishikawa cells which were transiently transfected with 2 μg of ERE3-TATA-CAT/EBV and 13 μg of Bluescribe M13+ (*lanes 13–18*). Cells were treated with 25 nM E2 (*lanes 2, 8,* and *14*), 5 μM OHT (*lanes 3, 9,* and *15*), 100 nM OHT (*lanes 4, 10,* and *16*), 100 nM RUp (*lanes 5, 11,* and *17*), or 100 nM RUf (*lanes 6, 12,* and *18*). The *inset* (*lanes 1–12*) represents CAT levels obtained with Ishikawa-ERE3/EBV cells after adjustment of E2-induced CAT levels (*lane 2*) to those obtained in transient transfection assay in the presence of E2 (*lane 14*).

ERE3-TATA-CAT/EBV episomal plasmids will be indicated by the suffix -ERE3/EBV). Stimulation with estrogen (25 nM, 24 h) of Ishikawa-ERE3/EBV cells led to a marked stimulation of CAT expression levels (~17-fold, Fig. 5*A*, *lane 2*). No stimulation was observed with the pure anti-estrogens RUf or ICI (100 nM, Fig. 5*A*, *lanes 5* and *6*). In contrast, OHT and RUp stimulated CAT activity to ~22 and 30% of the levels obtained with estrogen, respectively (Fig. 5*A*, *lane 3* and *4*). These results were obtained with different pools of Ishikawa-ERE3/EBV cells generated by two independent rounds of selection. On the other hand, neither the partial anti-estrogens OHT and RUp nor the full anti-estrogens ICI or RUf stimulated CAT expression more than background in MCF7-ERE3/EBV cells; note, however, that the fold stimulation by estrogen was lower in stably transfected MCF7 cells than in Ishikawa-derived cell lines (Fig. 5*B*, *lanes 1–7*).

Agonist activity of OHT was dose-dependent, and stimulation of CAT activity could be detected with concentrations as low as 1 nM, whereas ICI or RUf did not induce levels of CAT activity above basal levels at any of the concentrations tested (Fig. 5*C*). Note that none of the anti-estrogens were capable of inducing CAT expression in a cell line derived from Ishikawa cells by stable transfection of an episomal vector containing five glucocorticoid response elements (GRE5) instead of three EREs (GRE5-TATA-CAT/EBV, Ref. 28), demonstrating that the presence of EREs is required for transcriptional activation by anti-estrogens (data not shown).

The levels of CAT mRNAs after stimulation by estrogen and OHT for 8 h were measured by semi-quantitative RT-PCR in order to confirm that the anti-estrogen increased CAT mRNA levels. CAT mRNA levels obtained after OHT stimulation were ~40% of those obtained after incubation with estrogen, whereas actin mRNA levels did not vary between the different samples (Fig. 5*D*). These results are in good agreement with those obtained by measuring levels of CAT enzyme activity. The agonist effect of OHT and RUp persisted when incubations were performed for longer periods than 24 h. Treatment for 2–5 days with OHT or RUp consistently generated increased CAT expression compared with treatment with full anti-estrogens or vehicle (Fig. 5*E*).

In order to rule out the possibility that differences in the protocols used for transient transfection assays and experiments with stable cell lines might be the source of the discrepancy in the results observed with partial anti-estrogens (*i.e.* no stimulation of CAT activity in transient transfection *versus* stimulation in stable cell lines), we repeated these experiments in parallel using Ishikawa and Ishikawa-ERE3/EBV cells, with or without mock transfection of Ishikawa-ERE3/EBV cells (*i.e.*

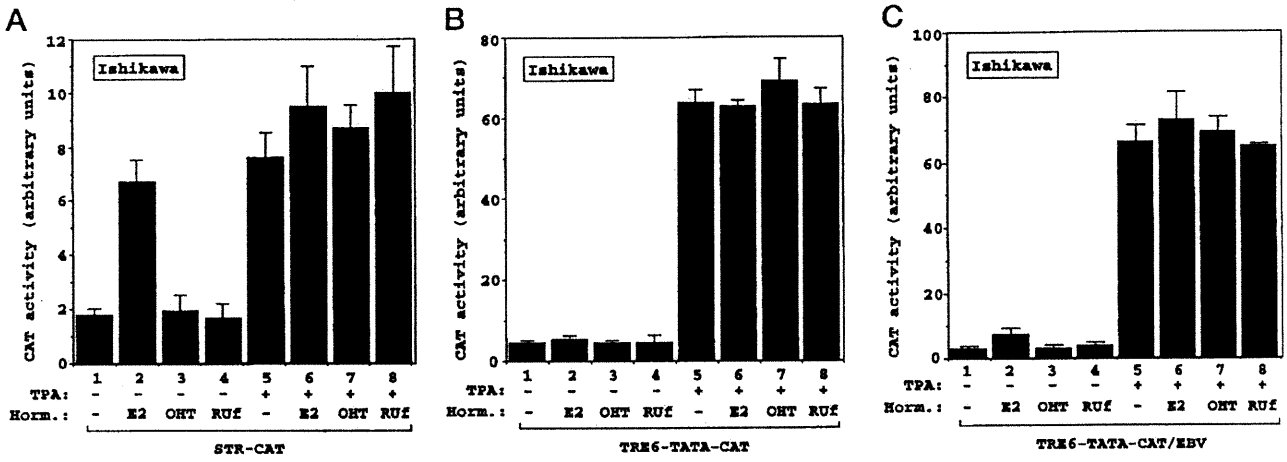

FIG. 6. **Estrogen regulation of AP1 activity is promoter context-dependent.** Reporter vectors STR-CAT (*A*), TRE6-TATA-CAT (*B*), or TRE6-TATA-CAT/EBV (*C*) were transiently transfected in Ishikawa cells. CAT activity was assayed after stimulation for 24 h with estrogen (25 nM) or anti-estrogens (100 nM), as indicated.

transfection with carrier DNA only). Estrogen stimulation of CAT activity was much higher in the transient transfection assay than with the stable cell line (55- and 10-fold, respectively), whereas background levels of CAT activity were similar (Fig. 5*F*, compare *lanes 1* and *2*, and *13* and *14*). Despite the high levels of stimulation seen with E2, CAT activities observed with transient transfections performed in the presence of OHT or RUp were lower than those obtained with vehicle (compare *lanes 15–17* with *lane 13*) and lower than those observed with OHT or RUp in Ishikawa-ERE3/EBV cells (compare *lanes 15–17* with *lanes 3–5*; see also *inset*). Mock transfection of Ishikawa-ERE3/EBV cells slightly reduced stimulation with both estrogen and RUp while increasing background, thereby blunting the stimulation by anti-estrogens but not suppressing it. Therefore, differences in the results observed using the two assays seem to be due at least in part to the status of reporter vectors within cells, *i.e.* number of copies per cell and/or integration into chromatin.

Taken together, these results suggest that anti-estrogens with partial agonist activity, but not full anti-estrogens, can stimulate transcription directed by endogenous levels of estrogen receptors bound to minimal ERE-containing promoters in Ishikawa cells, even though this activity is undetectable in transient transfection assays using the same reporter vectors.

Minimal Promoters Containing AP1-binding Sites Are Not Activated by Estrogen or Anti-estrogens in Transient Transfection Assays of Ishikawa Cells—Previous reports have described induction of AP1 activity by estrogen treatment in several cell lines (19–22) and by anti-estrogens in transient transfection assays of Ishikawa cells (22). To test whether TRE motifs are sufficient to mediate stimulation by estrogen and anti-estrogens in Ishikawa cells, we transiently transfected reporter vectors containing a minimal promoter composed of six TRE motifs upstream of a TATA box (Fig. 1) or a reporter vector containing the AP1-responsive rat stromelysin promoter, STR-CAT (Fig. 1, see also Ref. 24). Stimulation of expression from the STR-CAT reporter vector could be observed in the presence of estrogen (3-fold, compare *lane 2* to *lane 1* in Fig. 6*A*) but not in the presence of anti-estrogens. TPA-stimulated CAT expression levels 5–10-fold, and no additional increase was obtained in the presence of estrogen or anti-estrogens (Fig. 6*A*, *lanes 5–8*). No stimulation of the minimal TRE6-TATA-CAT reporter constructs could be observed after incubation of the transiently transfected cells with either estrogen or anti-estrogens (Fig. 6*B*). When the same promoter was incorporated into an episomal vector, a lower basal activity was observed (10-fold lower, data not shown) and a weak stimulation by estrogen (2.5-fold) could be detected in the absence but not in the presence of TPA (Fig. 6*C*, compare *lanes 2* and *6* to *lanes 1* and *5*). Anti-estrogens had no effect on CAT expression levels directed from this reporter construct (Fig. 6*C*, *lanes 3* and *4*, and *7* and *8*).

From these experiments, we conclude that stimulation of expression from promoters containing TRE motifs by estrogen is dependent on the promoter context. Although we did not observe transcriptional activation by anti-estrogens using these promoters, we cannot rule out the possible existence of promoter-specific effects.

Micromolar Concentrations of OHT, but Not of Other Anti-estrogens, Can Stimulate Transcription from Minimal Promoters Containing TRE Motifs in Stably Transfected Ishikawa Cells—To investigate further the potential role of anti-estrogens in stimulation of the AP1 signaling pathway, cell lines were derived from Ishikawa cells by stable transfection of TRE6-TATA-CAT vectors (clonal selection by integration into the cellular genome) or of the episomal vectors TRE6-TATA-CAT/EBV or TRE2-TATA-CAT/EBV.

Two clones obtained by selection for integration of the TRE6-TATA-CAT reporter vector responded to TPA stimulation (100 ng/ml) by an increase in CAT expression (3–7-fold; Fig. 7*A*, compare *lane 5* to *lane 1* for *Ishikawa-TRE6 #29*). Ishikawa-TRE6 clone 29 was further used to investigate whether estrogen and/or anti-estrogens can stimulate CAT activity under the same tissue culture conditions as used for the Ishikawa-ERE3/EBV cell lines (*i.e.* phenol red-free DMEM supplemented with 5% charcoal-treated FBS). Treatment with estradiol or anti-estrogens did not significantly modulate CAT expression in these cells, either in the presence or in the absence of TPA (Fig. 7*A*, *lanes 2–4* and *6-8*). Similar results were obtained with the other clone (data not shown). Note that estrogen did stimulate expression from an ERE3-hsp68-LacZ reporter vector transiently transfected in Ishikawa-TRE6 cells, demonstrating that lack of induction of the AP1 pathway in these cell lines was not due to loss of ER function (data not shown).

Pools of cells were selected for propagation of the TRE6-TATA-CAT/EBV vector, generating the Ishikawa-TRE6/EBV cell line (CAT activity was stimulated ~5-fold by TPA in these cells, data not shown). Treatment of Ishikawa-TRE6/EBV cells as well as of Ishikawa-TRE6 clone 29 cells with estrogen or anti-estrogen did not lead to detectable stimulation of CAT activity (Fig. 7*B*, *lanes 1–10*), whereas in the same assay strong agonist activity could be observed using the Ishikawa-ERE3/EBV cells (Fig. 7*B*, *lanes 11–15*). Similar results were obtained

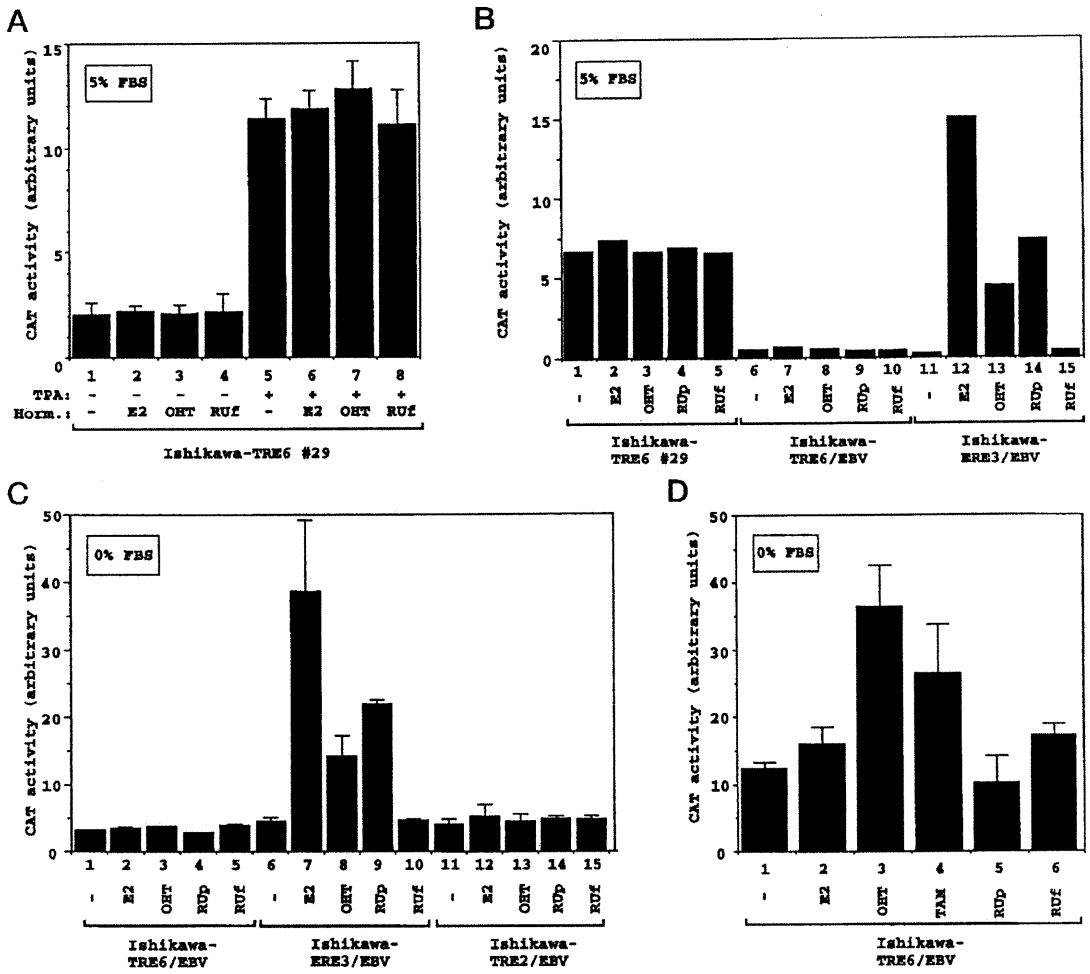

FIG. 7. **Micromolar concentrations of tamoxifen or hydroxytamoxifen, but not of other anti-estrogens, stimulate stably propagated AP1-responsive promoters.** *A,* Ishikawa cells stably transfected with TRE6-TATA-CAT (*Ishikawa-TRE6 #29*) were plated in phenol red-free DMEM supplemented with 5% charcoal-treated FBS and treated with estrogen (25 nM), anti-estrogens (100 nM), and TPA (100 ng/ml) as indicated. *B,* Ishikawa-TRE6 clone 29 (*lanes 1–5*), or Ishikawa cells stably transfected with TRE6-TATA-CAT/EBV (Ishikawa-TRE6/EBV, *lanes 6–10*), or ERE3-TATA-CAT/EBV (Ishikawa-ERE3/EBV, *lane 11–15*) were plated at a density of 1.5×10^6 cells per 10-cm plate in DMEM containing 5% charcoal-treated FBS and supplemented with estrogen (25 nM) or anti-estrogens (100 nM) as indicated (a representative experiment is shown). *C,* Ishikawa-TRE6/EBV cells (*lanes 1–5*), Ishikawa-ERE3/EBV (*lanes 6–10*), or Ishikawa cells stably transfected with TRE2-TATA-CAT/EBV (Ishikawa-TRE2/EBV; *lanes 11–15*) were plated in phenol red-free DMEM (0% FBS) and incubated with estrogen (25 nM) or anti-estrogens (100 nM) as indicated for 24 h. *D,* Ishikawa-TRE6/EBV cells plated in phenol red-free DMEM (0% FBS) were treated with estrogen (100 nM) or anti-estrogens (5 μM) for 24 h.

in the presence of TPA, except that levels of CAT activity were higher in the presence of TPA in the cells containing AP1-responsive promoters but not in the Ishikawa-ERE3/EBV cell lines (data not shown).

Because the conditions used for tissue culture in the above-described experiments (phenol red-free DMEM containing 5% charcoal-treated FBS) may mask stimulation of AP1 activity by estrogen or anti-estrogens, we performed these assays again in the absence of serum. CAT activity was induced by estrogen (10-fold) and anti-estrogens (3–5-fold) in the Ishikawa-ERE3/EBV cells (Fig. 7*C, lanes 6–10*) but not in Ishikawa-TRE6/EBV cells (Fig. 7*C, lanes 1–5*) or in Ishikawa-TRE2/EBV cells (Fig. 7*C, lanes 11–15*), which propagate EBV episomal vectors containing only two TPA response elements. However, when higher concentrations of anti-estrogens were used (5 μM instead of 0.1 μM), small but reproducible stimulations of CAT activity were observed with OHT (3-fold) or with tamoxifen (2-fold) (Fig. 7*D*, compare *lanes 3* and *4* to *lane 1*) but not with estrogen or with other anti-estrogens (Fig. 7*D, lanes 2, 5* and *6*). Similar results were observed using Ishikawa-TRE6 or Ishikawa-TRE2/EBV cells (data not shown).

These results indicate that TRE elements together with a TATA box can only mediate transcriptional stimulation by high concentrations of OHT (or TAM) in Ishikawa cells, whereas estrogen response elements can be activated at lower concentrations by both OHT and RUp under the same conditions.

DISCUSSION

Mechanisms underlying the partial agonist activity of anti-estrogens are still poorly understood (9). In this report, we have investigated whether the partial agonist activity of anti-estrogens in Ishikawa cells can be mediated at the level of regulation of gene expression by typical estrogen response elements and/or by TPA response elements, which can mediate estrogen stimulation in some promoters (19–22). Although transcriptional activation of ERE-containing promoters by OHT has been documented using transient transfection assays in a number of cell lines, including HeLa cells and chicken embryo fibroblasts (3), agonist activity of OHT and RU39,411 was not observed in transiently transfected Ishikawa cells using minimal promoters containing EREs (ERE3-TATA) or more complex promoters (ERE3-tk, Vit-tk). Failure to detect transcrip-

tional activation of ERE-containing promoters by OHT in transiently transfected Ishikawa cells is in agreement with previous observations (22). Contrary to results obtained with transient transfections, we observed significant levels of transcriptional activation by OHT and RUp in Ishikawa, but not in MCF7 cells, when the ERE3-TATA-CAT/EBV reporter vector was stably propagated as an episome. These results correlate well with the observed agonist effect of these two anti-estrogens on expression levels of the endogenous progesterone receptor (note that the human progesterone receptor upstream sequences contain a half-palindromic TGACC motif but no consensus EREs, Ref. 38). In addition, OHT and RUp, although structurally unrelated, induced similar shifts in mobility of ER·ERE complexes in gel retardation assays. The migration of these complexes was found to be distinct from those formed in the presence of the full antagonists RUf and ICI.

Differences observed in transcriptional activity in the presence of anti-estrogens in transient transfection assays and using "reporter cell lines" may reflect the different status of the reporter vectors, which are present at a lower copy number when maintained as episomes (generally less than 50 copies per cell, Ref. 39) and are incorporated into chromatin to a higher degree (40) compared with transiently transfected reporter plasmids. These results suggest that stimulation of ERE-mediated transactivation by anti-estrogens requires cofactor(s) limiting in amounts or availability in transient transfection assays in Ishikawa cells, whereas estrogen-liganded ER may recruit other, non-limiting cofactors (41, 42). Of interest is the fact that capacity to remodel chromatin structure via histone acetyltransferase or deacetylase activities has been attributed to an increasing number of nuclear receptor co-activators and co-repressors (42–49), demonstrating that incorporation of target promoters into chromatin is an integral part of the mechanism of transcriptional activation by nuclear receptors. It is not clear at present whether ER can interact with these cofactors *in vivo* when bound by anti-estrogens with partial agonist activity. It is possible that cofactors specific to OHT-bound ER, such as the newly described co-activator L7/SPA (50) may mediate the agonist activity of this anti-estrogen. Future functional characterization of cofactors interacting with ER in the presence of OHT and RUp should provide insights into the molecular mechanisms of action of anti-estrogens with partial agonist activity.

Low concentrations of OHT or RUp, which were sufficient for activation of ERE3-TATA promoters in stably propagated vectors, did not yield detectable transcriptional stimulation of promoters containing TRE sites inserted upstream of a TATA box either in transient transfection assays or using stably propagated vectors. Others have previously documented transcriptional activation of the TRE-containing collagenase promoter by anti-estrogens in transiently transfected Ishikawa cells (22). Discrepancy between these and our results could be due to the promoter context of TRE elements. Along the same line, our results indicate that promoter context influences stimulation of AP1-responsive promoters by estrogen in transient transfection assays. Alternatively, differences in cell lines or transfection methods could be the source of this discrepancy. Side-by-side comparison of cell lines carrying episomal reporter vectors whose promoters differed only by the response elements present in the minimal synthetic promoters confirmed that transcriptional stimulation by anti-estrogens like RUp or OHT could be mediated by estrogen response elements but not TPA response elements at low concentrations of anti-estrogens. Stimulation of AP1-responsive promoters could only be observed in serum-free medium using 5 μM OHT or TAM but not estrogen or other anti-estrogens. Whether this effect is mediated by estrogen receptors or is initiated at the cellular membrane remains to be investigated.

In conclusion, our results suggest that transcriptional stimulation by anti-estrogens can be mediated by consensus EREs in Ishikawa cells, this observation being consistent with the absence of TRE sites in the promoters of genes whose expression can be induced by hydroxytamoxifen in the uterus (38, 51). In addition, TREs are also capable of mediating transcriptional stimulation by anti-estrogens, although in a manner that is restricted by both the nature and the concentration of the anti-estrogen. Finally, while transient transfection has proven to be a powerful tool for analyzing intracellular signaling pathways, our study emphasizes that this assay only partially recapitulates the conditions required for initiation of transcription *in vivo*.

Acknowledgments—We are grateful to Dr. D. Philibert (Roussel-Uclaf, Romainville, France) and to Dr. T. Willson (Glaxo-Wellcome, Research Triangle Park, NC) for the gift of reagents and to Dr. P. Chambon (Illkirch, France) for providing reporter plasmids and ER expression vectors. We also thank Dr. J. White (McGill University, Montreal, Canada) for critical reading of the manuscript.

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OTHER RELATED RESULTS

Regulation of AP-1 acivity by glucocorticoids is cell-specific

We did not detect the activation of AP-1 by estradiol in Ishikawa cells using stably propagated episomal reporter vectors containing a minimal promoter composed of six AP-1 sites (also called TPA response elements, TREs) upstream of a TATA box, although we observed activation by TPA as a control. To examine whether lack of response is due to the reporter vector or the cells, we stably transfected Hela cells with the same reporter vector to test whether CAT activity can be repressed by dexamethasone, since it has been reported that AP-1 activity can be repressed by glucocorticoids in Hela cells (Schüle et al., 1990). In addition, stably transfected Hela cells that propagate AP-1 reporter vectors would provide a tool to screen for glucocorticoid derivatives with selective activity on AP-1.

In response to TPA, AP-1 mediated transcriptional activity is significantly induced 6- to 7-fold in Hela cells (figure 8, compare lane 2 to lane 1). In the presence of increasing concentrations of dexamethasone, TPA-induced AP-1 activity is gradually repressed (figure 8). Repression correlates with the concentration required to bind and activate the glucocorticoid receptor, suggesting that this effect is modulated by GR. Our results indicate that physiological concentrations of glucocorticoids can repress AP-1 activity, consistent with the literature reports. To rule out the possibility of clonal effects, we performed similar experiments in another two clones, and our results indicate that the repressive effect of dexamethasone on AP-1 activity is reproducible in other clones (figure 9, clone#12 and #14, compare lane 4 to lane 2). We next investigated whether repression of AP-1 by dexamethasone can be observed

in Ishikawa cells as well as in Hela cells. Using stably transfected Ishikawa cells that carry the same AP-1 reporter vector 6×TRE-TATA-CAT(clone #29, #46) or a reporter vector with only 2 copies of the TPA response element 2×TRE-TATA-CAT (clone #8), we investigated the effect of dexamethasone on AP-1. AP-1 mediated CAT expression can be induced up to 5- to 6 – fold in response to TPA in all three clones (figure 9B, clone #8, compare lane 2 to lane1; clone #29, compare lane 2 to lane 1; clone #46, compare lane 2 to lane 1). Levels of CAT expression in response to dexamethasone treatment were comparable to background levels of unstimulated transcription. Intriguingly, dexamethasone did not repress TPA-induced AP-1 transcriptional activity (figure 9B, clone #8, compare lane 4 to lane2; clone #29, compare lane 4 to lane 2; clone #46, compare lane 4 to lane 2). Therefore our results indicate that AP-1 is not sensitive to GR repression in Ishikawa cells.

Since GR transrepression was shown to involve a physical interaction between GR and AP-1, we investigated whether the differential effect is due to a different expression ratio of GR versus AP-1. Overexpression of GR in Ishikawa cells should thus restore GR transrepression. So we transiently transfected the stably transfected cell line Ishikawa-6×TRE-TATA-CAT/EBV with increasing amounts of GR. AP-1 mediated CAT expression in response to TPA can be induced more than 20-fold (figure 10, compare lane 2 to lane 1). Increasing amounts of GR expression did not alter the AP-1 transcriptional activity induced by TPA either in the absence (figure 10, compare lane 6, 10, 14, 18, 22 to lane 2) or in the presence of dexamethasone (figure 10, lane 8, lane 12, lane 16, lane 20, lane 24). Therefore our results indicate that overexpression of GR in Ishikawa cells cannot reestablish the repressive effect. To

further analyze the divergence between Hela and Ishikawa, we may compare the AP-1 components between these two cell lines. It has been demonstrated that GR transrepression on AP-1 is due to direct protein-protein interaction between GR and c-Jun or c-Fos (schüle et al., 1990). If c-Jun or c-Fos is not the major components of AP-1 in Ishikawa cells, we may fail to detect GR transrepression on AP-1. In addition, It has been reported that GR transrepression of AP-1 is due to repression of JNK activity (Caelles et al., 1997). If GR fails to repress JNK activity in Ishikawa cells, GR transrepression function may not be established in Ishikawa cells.

Figure 8. Dexamethasone represses AP-1 transcriptional activity in Hela cells.

Cells (clone #5) obtained by stable transfection of Hela cells with the pTRE×6-TATA-CAT reporter vector were treated with vehicle, TPA (10 ng/ml), increasing concentrations of dexamethasone or TPA plus dexamethasone for 24 hours. CAT assay was performed after standardization for protein concentration.

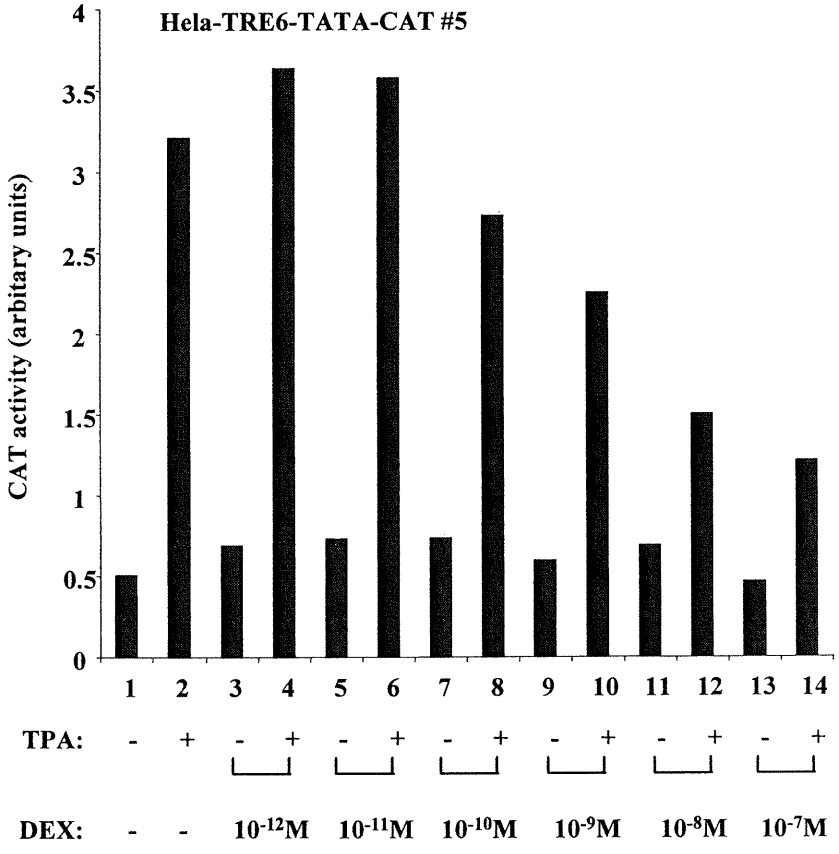
Hela-TRE6-TATA-CAT #5
CAT activity (arbitary units)
4
3.5
3
2.5
2
1.5
1
0.5
0
1 2 3 4 5 6 7 8 9 10 11 12 13 14
TPA: - + - + - + - + - + - + - +
DEX: - - 10-12M 10-11M 10-10M 10-9M 10-8M 10-7M

Fig 8.

Figure 9. Dexamethasone represses AP-1 in Hela cells, but not in Ishikawa cells.

A. Cells from three different clones (#5, #12, #14) obtained by stable transfection of Hela cells with the pTRE×6-TATA-CAT reporter vector were treated with vehicle, TPA (10 ng/ml), dexamethasone (25 nM), or TPA plus dexamethasone for 24 hours. CAT assay was performed after standardization for protein concentration. B. Cells from three different clones obtained by stable transfection of Ishikawa cells with the pTRE×6-TATA-CAT reporter vector (#29 and #46) or pTRE×2-TATA-CAT reporter vector (#8) were treated with vehicle, TPA (10 ng/ml), dexamethasone (25 nM), or TPA plus dexamethasone for 24 hours. CAT assay was performed as described previously.

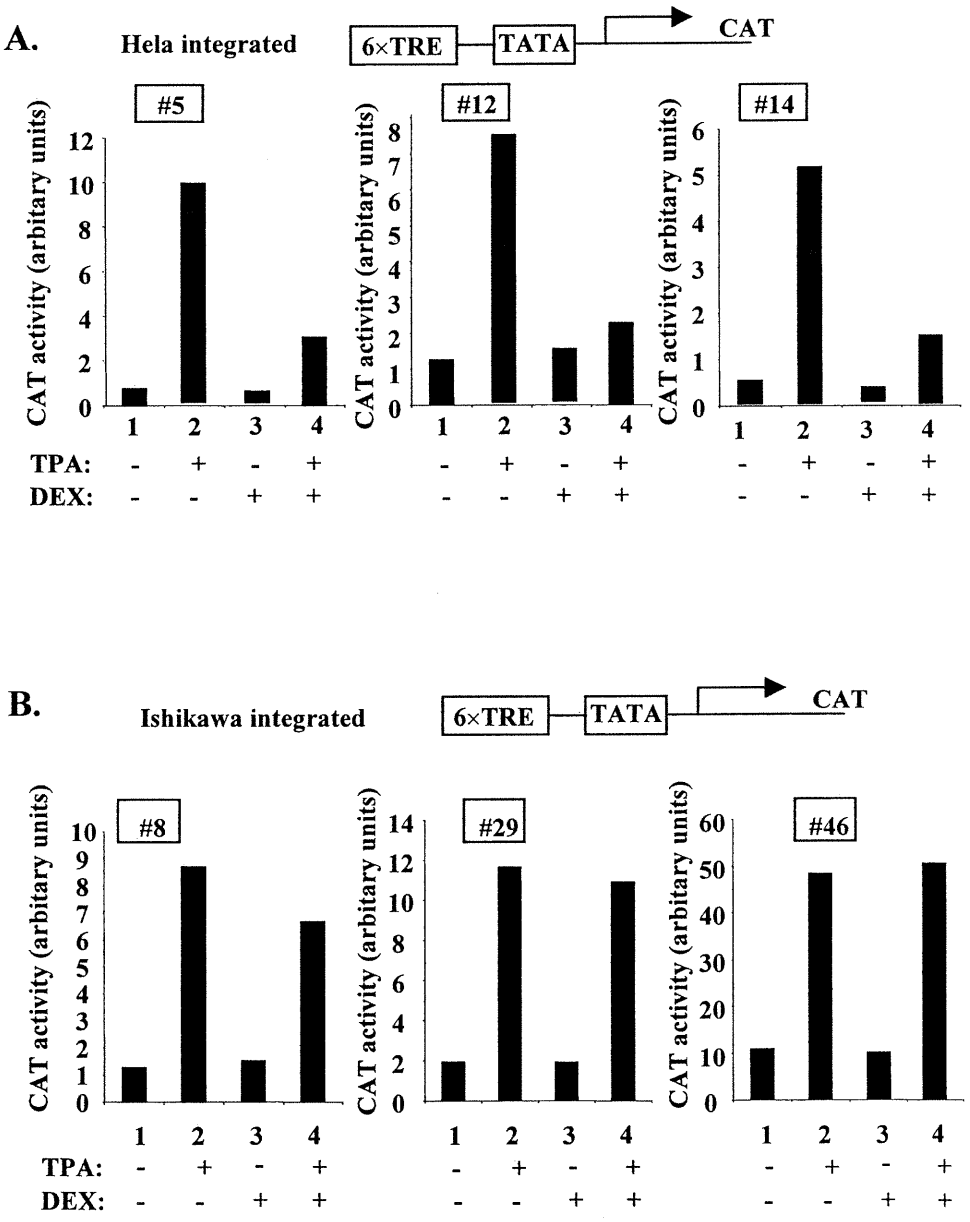
A.
Hela integrated
6×TRE
TATA
CAT
#5
#12
#14
CAT activity (arbitary units)
TPA:
DEX:
B.
Ishikawa integrated
6×TRE
TATA
CAT
#8
#29
#46
CAT activity (arbitary units)
TPA:
DEX:

Fig 9.

Figure 10. Overexpression of GR does not establish dexamethasone repressive effect on AP-1 in Ishikawa cells.

Cells obtained by stable transfection of Ishikawa cells with the pTREx6-TATA-CAT/EBV reporter vector were transiently transfeced with increasing concentrations of GR expression vector pSG5-hG0 as indicated, then treated with vehicle, TPA (10 ng/ml), dexamethasone (25nM) or TPA plus dexamethasone for 24 hours. CAT assay was performed as previously described.

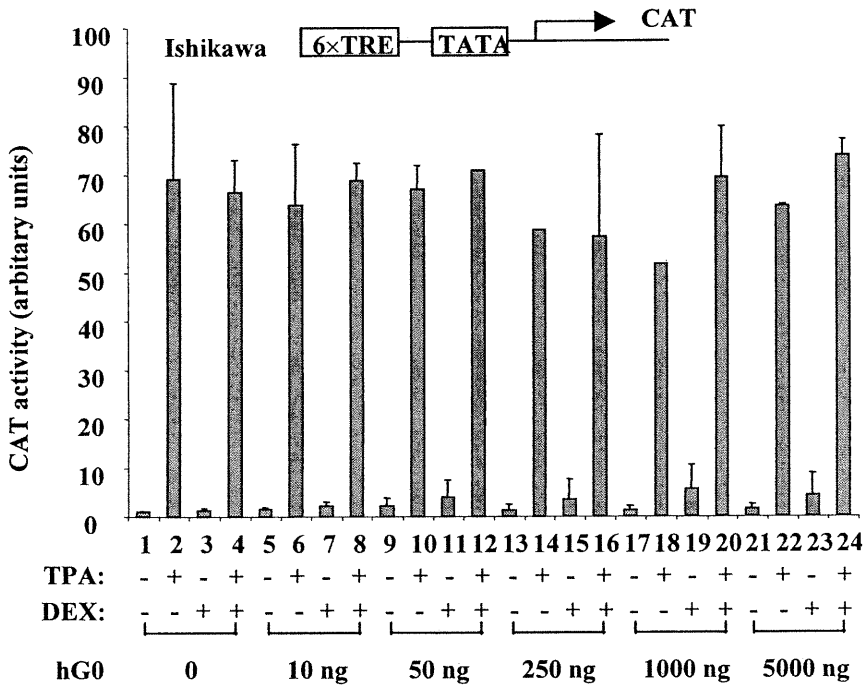
Ishikawa
6×TRE
TATA
CAT
CAT activity (arbitary units)
100
90
80
70
60
50
40
30
20
10
0
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24
TPA: - + - + - + - + - + - + - + - + - + - + - + - +
DEX: - - + + - - + + - - + + - - + + - - + + - - + +
hG0
0
10 ng
50 ng
250 ng
1000 ng
5000 ng

Fig10.

Chapter III

Regulation of glucocorticoid-dependent transcription by MAP kinase activators

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Abstract

The transcriptional activity of nuclear receptors can be regulated both positively and negatively by post-transcriptional modifications such as phosphorylation. The effect of activation of the MAP kinase signaling pathway on glucocorticoid receptor (GR) transcriptional activity remains however controversial. Here we have investigated the effect of MAP kinase inducers TPA and EGF on GR-dependent transcription in HeLa cells and in Ishikawa cells, two epithelial cell lines that express glucocorticoid receptors. Both TPA and EGF were found to positively modulate the transcription of reporter vectors containing minimal promoters composed of multimerized glucocorticoid response elements and a TATA box. This effect was more marked when using stably integrated or episomal vectors than with transiently transfected reporter vectors, possibly because transient transfection of culture cells itself stimulates MAP kinase signaling. The effect of TPA and EGF is early, specific to GR in Ishikawa cells, and can be inhibited by the ERK pathway inhibitor PD 98059. Our results indicate that GR expression is not regulated by TPA *in vivo*. Although phosphorylation of the GR AB region by Erk could be observed *in vitro*, overall phosphorylation levels of GR were not altered by TPA treatment *in vivo*. Furthermore, point mutants of GR phosphorylation sites and deletion of the τ1 or AB region did not abolish the effect of TPA. Finally, while the integrity of the ligand binding domain was necessary to observe enhancement by TPA, characterization of chimeric ER-GR proteins indicates that the GR DNA binding domain cannot be replaced by that of ER without losing TPA effect. These results suggest that the effect of TPA on GR-dependent transcription may be mediated by cofactors of GR that interact with the DNA binding domain, or that the DNA binding domain may allosterically regulate the activity of GR cofactors recruited via the ligand binding domain.

Introduction

The glucocorticoid receptor (GR) is a member of the nuclear receptor superfamily of ligand-dependent transcription factors (Wright et al., 1993). Ligand binding triggers dimerization, translocation to the nucleus, binding to the glucocorticoid response element (GRE), and transcriptional activation. Like other nuclear receptors, GR has a modular structure with principal functions (transactivation, DNA binding, and ligand binding) being localized to specific domains. The glucocorticoid binding domain is at the C-terminal end of the molecule (residues 501-777), whereas the DNA binding domain (residue 390-500), comprising two zinc finger motifs, is located in the middle of the protein. The N-terminal τ_1 domain (residues 77-262) is involved in cofactor binding and transcriptional transactivation of genes. In human GR another transactivating domain (τ_2, residues 526-556) is located just C-terminally of the DNA-binding domain (DBD). This region is also important for the nuclear translocation of the receptor (Wright et al., 1993; Wärnmark et al., 2000).

Protein kinases, such as mitogen activated protein kinases (MAPKs), are involved in the regulation of nuclear receptor signaling (Shao and Lazar, 1999). MAP kinases are a family of proline-directed serine–threonine protein kinase (Pearson et al., 2001). At least four mammalian MAPKs have been identified: ERK1/2, JNK1/2/3, p38α/β/γ/δ and ERK5. MAPK cascades are composed of a MAPK, MAPK kinase (MAPKK) and a MAPKK kinase (MAPKKK) (Chang and Karin, 2001). MAPK is activated by phosphorylation on Thr and Tyr by a dual specificity MAPKK, which in turn is activated by Ser/Thr phosphorylation by a MAPKKK. MAP kinases are activated by a variety of extracellular stimuli. The ERK pathway can be activated by mitogenic signals such as phorbol ester TPA (12-O-tetradecanoyl-phorbol-13 acetate), or growth factors like EGF. The JNK and p38 family members can be activated by stresses, such as osmotic stress, UV irradiation and by the treatment of cells with cytokines (Pearson et al., 2001).

GR is a target of phosphorylation by MAP/SAP kinases. Bodwell et al (1991) have identified seven phosphorylation sites in the mouse GR, Serines 122, 150, 212, 220, and 234 and the sequences surrounding them are conserved in the homologous regions of the rat and human receptors. Consistently, Krstic et al (1997) has identified four major phosphorylation sites on rat GR: T171, S224, S232, and S246. Residues T171 and S246 are followed by proline, thus corresponding to a motif typically modified by mitogen activated protein kinase [MAPK consensus = nonpolar-X-S/T(P)-P]. S246 is conserved in human, the corresponding site being S226.

There are conflicting reports in the literature regarding MAP kinase effects on GR signaling. Rogatsky *et al* demonstrated that selective activation of either ERK or JNK *in vivo* inhibits GR-mediated transcriptional activation in Hela cells via a GR reporter vector containing two consensus GREs upstream of chloramphenicol acetyltransferase (CAT) gene (Rogatsky et al.,1998). On the other hand, Moyer *et al* observed that TPA cotreatment enhances GR transcriptional activity in T47-D cells via an MMTV reporter vector (Moyer et al., 1993). Vacca *et al* reported that activation of PKC by TPA treatment reduced dexamethasone-mediated activation of an MMTV-CAT reporter gene transiently transfected into NIH-3T3 cells. Later, Maroder *et al* demonstrated that this effect is cell specifc, as TPA augmented dexamethasone-induced transcriptional activation of the MMTV LTR in several T cell lines but was inhibitory in NIH-3T3 fibroblasts. Here, we have used GR-positive Hela and Ishikawa cells stably transfected with a simple and sensitive reporter vector composed of five consensus GREs upstream of an adenovirus minimal promoter TATA box to study the effect of MAPK activators on GR transcriptional activity. Our results demonstrate that activation of MAPK/SAPK can positively modulate GR transcriptional activity in Hela and Ishikawa cells. Possible mechanisms underlying this effect are: 1) upregulation of GR expression by TPA. 2) upregulation of GR phosphorylation by TPA. 3) upregulation of GR

coactivator phosphorylation by TPA. 4) Protein-protein interactions between GR and TPA-induced Jun or Fos leading to enhanced GR transcriptional activity. Our results obtained from Northern and Western blotting indicate that GR mRNA and protein expression are not regulated by TPA. Our *in vivo* labeling data demonstrate that TPA does not change the overall phosphorylation levels of GR, and GR phosphorylation mutant S226E and S226A did not alter GRE-mediated transcriptional activity in COS-7 and Jurkat cells.

GR domains necessary for stimulation by TPA were mapped to the DNA binding and ligand binding regions. However, while exchanging the ligand binding domain (LBD) for that of estrogen receptor (ER) did not prevent stimulation by TPA, replacing the GR DBD region by that of ER led to a functional chimeric receptor whose activity was not further stimulated by MAP kinase. Possible mechanisms for this regulation are discussed.

EXPERIMENTAL PROCEDURES

Materials

Dexamethasone, 12-O-tetradecanoylphorbol 13-acetate (TPA), sorbitol, 17β-estradiol, diethyl-aminoethyl dextran were purchased from Sigma Aldrich. MAP kinase inhibitors PD98059 and SB203580 were from Calbiochem. EGF was from Upstate Biotechnology. Cell culture media, fetal bovine serum (FBS), geneticin (G418 sulfate) were purchased from Life Technologies. Hygromycin were from Gibco.

Plasmids

Human wild type (pKcR2-hG0) and mutant (pKcR2-hG2, pKcR2-hG4, pKcR2-hG8, pKcR2-hG11) GR expression vector, Gal and GR(A/B)-Gal expression vector, chimeric receptor expression vector pKcR2-GRERCAS, truncated (pSG5-TIF2.1) and full length coactivator TIF2 (pSG5-TIF2 fl) expression vector was kindly provided by Dr. Pierre Chambon (Strasbourg, France). GR τ1-deletion mutant (pRS-hGR Δ77-262) was obtained from Dr. André Tremblay (Saint Justine hospital research center, Canada). Luciferase reporter vector GALRE×5-ptk-luc was generously provided by Dr. John White (McGill University, Canada). pGRE5-TATA-CAT, pGRE5-TATA-CAT/EBV, pERE3-TATA-CAT, pKcR2-ERGRCAS, pSG2-HE61, pSG2-HE62 were described previously (Mader et al., 1993; White et al., 1994; Barsalou et al.,1998; Mader et al., 1989).

Cell culture, transfections and reporter assay

Hela and COS-7 cells were maintained in Dulbecco modified essential medium (DMEM) supplemented with 5% fetal bovine serum (FBS). Cells were plated at a density of 1.5 million per 100-mm dish, and transfected with 15 μg of DNA using calcium-phosphate coprecipitate method. After 20 h, medium was changed twice to remove precipitates, cells were incubated in serum-free DMEM for 24h before *in vivo* labeling. Other transfections, after precipitates

were washed, cells were incubated in serum-free DMEM for 5 h, then treated as indicated for 20 h before harvesting. Protein extracts were prepared for Bradford and CAT assay. The CAT assay was carried out as previously described (Seed and Sheen, 1988).

Jurkat cells were cultured in RPMI 1640 medium supplemented with 10% FBS. Transfection of Jurkat cells was carried out by diethyl-aminoethyl (DEAE) dextran method. 10 million cells were washed in serum-free RPMI medium for each transfection, then resuspended in 1 ml serum-free medium. Concomitantly, 9 μg DNA was mixed with 150 μl DEAE-dextran (5 μg/μl, prepared with 1M Tris [pH 7.4] and filtered with 0.22 μm filter to sterilize) and diluted with 2 ml serum-free medium. Cells were then added on top of diluted DNA/DEAE-dextran mixture, followed by incubation for 1 h at 37°C, gently shaken once after 0.5 h. Cells were collected by centrifugation, washed once with medium containing 10% FBS, and resuspended in 10 ml RPMI medium with 10% FBS. 24 h after transfection, cells were treated with the agent as indicated in the figure legends. After a further 20 h incubation, cells were harvested. Cell extracts were prepared for Bradford assay, CAT assay or luciferase assay. Luciferase activity was measured using a luciferase assay kit according to the manufacturer's instructions (Promega Luciferase Reporter 1000 Assay System).

Generation of stably-transfected cell line

Hela-GRE5-TATA-CAT/EBV, Hela-GRE5-TATA-CAT, Ishikawa-GRE5-TATA-CAT/EBV and Ishikawa-ERE3-TATA-CAT/EBV cell lines were generated and maintained as described before (Barsalou et al, 1998).

RNA purification

Hela-GRE5-TATA-CAT/EBV cells were plated at a density of 12×10^6 cells/150-mm plate and grown in DMEM supplemented with 10% FBS. The second day medium was replaced

with serum-free DMEM for 24 h. Cells were then treated with or without 10 ng/ml TPA and incubated for additional 1, 2, 3, 6, 12, 24 h respectively. Total RNA was isolated by TRIZOL Reagent (Gibco BRL), poly(A)-enriched mRNA was purified from total RNA by Oligotex mRNA Kit (Qiagen) according to manufacturer's instructions.

Northern analysis

2 μg Poly(A)-enriched mRNA was resolved on 1.0 % agarose, 6% formaldehyde gel and transferred to Hybond^{TM}-XL membrane (Amersham Pharmacia Biotech). A 0.24-9.5 Kb RNA ladder (Gibco BRL) was used for size determination. GR cDNA probe was a 746 bp EcoRI fragment purified from pSG5hG0, β-actin cDNA probe was a 350 bp EcoRI fragment purified from pCRII β-actin. GR and β-actin cDNA probe was labeled with [α-^{32}P] dCTP (NEN^{TM} Life Science Products, Inc.) by Random Primed DNA Labeling Kit (Boehringer Mannheim) according to manufacturers' instructions. Incorporated probes were purified by NICK^{TM} Column (Pharmacia Biotech). Membrane was prehybridized in hybridization solution (5 × SSPE, 5 × Denhardt's solution, 50% formamide, 5% dextran sulfate, 0.05 M sodium phosphate, 0.1 mg/ml denatured sheared salmon sperm DNA [5 Prime → 3 Prime Inc.]) at 42°C for 3 h. Hybridization was carried out in hybridization solution containing ^{32}P-labeled probes (GR cDNA probe 5 × 10^6 cpm/ml hybridization solution, β-actin cDNA probe 1× 10^6 cpm/ml hybridization solution) at 42°C overnight. Membranes were washed serially 2 × 5 min with 2×SSC, 0.5% SDS at room temperature; 2× 15 min with 2×SSC, 0.5%SDS at 65 °C; 2× 15 min with 0.5×SSC, 1%SDS at 65 °C. Autoradiographs were obtained by exposure to Kodak BIO MAX film with an intensifying screen for 1-3 days at -80°C. Relative quantitation of bands on autoradiographs was performed using a scanning densitometer, analyzed by NIH image software.

Western blotting

Hela-GRE5-EBV-CAT cells were plated in 15 cm plates at a density of 12×10^6 cells /plate, one day later, cells were serum starved for 24 h. Cells were then treated with or without 10 ng/ml TPA for 1, 2, 3, 6, 12, 24 hour, harvested in PBS, pelleted and resuspended in extraction buffer (600 mM KCl; 20 mM Tris-HCl, PH 7.5; 2 mM DTT; 0.1 mM EDTA; 20% glycerol [vol/vol]; 1mM phenylmethylsulfonyl fluoride [PMSF]; protease inhibitors leupeptin, pepstatin, aprotinin, trypsin inhibitor). Whole-cell extracts (WCE) were prepared by three cycles of freezing (-80°C) and thawing (0°C), and centrifugation was done at 10,000 × g for 20 min at 4°C. The supernatants were collected and protein concentrations of the WCE were determined by Bradford assay (Bio-Rad). Protein extracts were fractionated on 8% SDS/polyacrylamide gel, transferred to Hybond-P membrane (Amersham Pharmacia Biotech), and probed with polyclonal (rabbit) anti-human glucocorticoid receptor (aa 346-367) antibody (5 μg/ml, Catalog Number PA1-511, Affnity BioReagents Inc.) or mouse monoclonal TIF2 antibody (obtained from Dr. Pierre Chambon), followed by horseradish peroxidase-conjugated anti-rabbit (New England Biolab) or anti-mouse (Sigma) antibodies, and signals were visualized by enhanced chemiluminescence (NEN Life Science Products) as recommended by the manufacturers.

***In vitro* kinase assay**

Affinity purified GST fusion proteins (4μg) were incubated with active ERK-2 and [γ-^{32}P]ATP (10μCi) in kinase reaction buffer (20 mM Hepes, PH 7.5; 10 mM $MgCl_2$; 1 mM DTT; 10 mM pNPP), in a final volume of 40μl. The reaction mixture was incubated for 20 min at 30 °C, and the reaction was terminated by addition of 40μl SDS-gel loading buffer. Reaction products were separated by SDS/PAGE, stained with Coomassie blue and autoradiographed.

***In vivo* labeling and immunoprecipitation of phosphorylated protein**

Transfected and serum-starved Hela and COS-7 cells were washed twice with 5 ml phosphate free MEM (Bio media) supplemented with 1 mg/ml BSA, refed with 4 ml phosphate free medium. 2 mCi of [^{32}P] phosphoric acid (ICN) was added to each dish. Cells were labeled for 5h. 15 min or 1h before harvest, cells were treated with vehicle, dexamethasone (25 nM), TPA (10 ng/ml) or dexamethasone (25 nM) plus TPA (10 ng/ml). Medium containing [^{32}P] phosphoric acid was then removed and radiolabeled cells were washed twice with PBS and lysed in 0.9 ml of ice-cold lysis buffer (50 mM Tris pH 7.4, 100 mM NaCl, 50 mM NaF, 5mM EDTA, 40mM β-glycerolphosphate, 1% Triton X-100, 1mM Vanadate, 0.1 mM PMSF, 1 μg/ml leupeptine, 1 μM Pepstatin A) at 4°C for 30 min. Cell extract was collected by centrifugation at 10,000 × g for 20 min at 4°C. Meanwhile, 2 μl (0.5 μg/μl) polyclonal (rabbit) anti-human GR antibody (Cat. # PA1-511, Affnity BioReagents Inc.) or 1μl monoclonal (mouse) anti-TIF2 antibody (Asc 3Ti 3Fi, kindly provided by Dr. Pierre Chambon, France) was incubated with 60 μl protein A-sepharose beads (Amersham Pharmacia) in 400 μl TNET/BSA buffer (50 mM Tris [pH 7.4], 100 mM NaCl, 5 mM EDTA, 0.5% Triton X-100, 1% BSA) for 2 h at 4°C. Immunoprecipitations were carried out by incubation of cell extracts with quick spin obtained Ab/beads complex for 4 h at 4°C. The beads were washed three times with cold lysis buffer, immunoprecipitates were eluted by boiling the beads in 70 μl 2×SDS loading dye for 5 min and supernatants were resolved on SDS-7.5% polyacrylamide gels. After electrophoresis, proteins were transferred to PVDF membrane, protein amount and transfer efficiency were monitored by ponseau red staining. Immunoprecipitated and phosphorylated proteins were visualized by autoradiography. Following autoradiography, the membrane was subjected to Western blotting with anti-GR

antibody or anti-TIF-2 antibody as described above, to further confirm that equal amount of proteins were loaded among samples.

RESULTS

TPA positively modulates glucocorticoid-induced transcription in Hela cells

In order to characterize the effects of activation of MAP kinases on GR transcriptional activity, we stably transfected GR-positive Hela cells with a reporter vector containing a minimal promoter composed of five glucorticoid response elements upstream of a TATA box. Using a cellular clone obtained by integration of this reporter vector into the chromosomes, we measured glucocorticoid-induced CAT activity in the presence or absence of TPA treatment. In the absence of treatment, transcriptional activity was undetectable (Fig. 1A, lane 1). Dexamethasone (25 nM) induced CAT expression (Fig. 1A, lane 2), and this activation was increased by 3- to 4- fold in the presence of TPA (10 ng/ml). TPA alone had no detectable effect on transcription in the absence of Dex. These results suggest that TPA can potentiate Dex-induced transcription in HeLa cells. However, it remains possible that synergy between TPA and Dex may result from the presence of sites for TPA-regulated transcription factors in sequences surrounding the reporter vector incorporation site.

To rule this possibility out, we examined the effect of TPA on Dex-induced transcription in populations of HeLa cells transduced with a retroviral vector containing the GFP gene under control of the same minimal promoter. Similar results were obtained, although background levels of unstimulated transcription were higher and stimulation by TPA was weaker (about 2-fold, see Fig. 1B). However, when using a reporter vector that can be stably propagated in the absence of integration, basal levels of transcription in the absence of Dex were undetectable and stimulation by TPA was significant (about 5-fold, Fig. 1C, compare lanes 2 and 4). Note that while the minimal promoter was identical in all three vectors, backbone sequences were different, ruling out contribution of motifs outside the minimal promoter.

Finally, the potentiation of Dex-dependent transcription by TPA was also observed when the pGRE5-TATA-CAT reporter vector was transiently transfected in HeLa cells (data not shown but see also Figures 8 and 9), although the effect of TPA was reduced (about 2-fold). To investigate whether the effect of TPA was attenuated by the transient transfection procedure, we mock-transfected HeLa cells stably propagating the pGRE5-TATA-CAT vector. Transactivation by dexamethasone was significantly increased (about 2-fold) by transient transfection of carrier DNA, while transcription in the presence of both TPA and Dex was unaffected (Fig. 1D, compare lanes 8 and 4). This observation suggests that transient transfection results in activation of signaling pathways that mimic the effect of TPA on GR-dependent transcription. Accordingly, we observed that AP1-dependent transcription was induced by a factor 2 to 3 by transient transfection using the calcium-phosphate method (data not shown).

Together, these results suggest that GR dependent transcription is potentiated by TPA treatment in epithelial HeLa cells. To analyze whether the effect of TPA is direct or not, we performed time course studies of Dex-induced transcription in the presence or the absence of TPA. The stimulatory effect of TPA was observed as early as dexamethasone transactivation could be detected (4h, Fig. 2A, lanes 3-4). Thus, the effect of TPA is not likely to involve induction of protein expression.

Since the previous experiments were performed using saturating concentrations of ligand, it is unlikely that the effect of TPA is to increase the affinity of GR for Dex. Nevertheless, we examined the effect of TPA at different concentrations of Dex (Fig. 2B). TPA treatment potentiated GR-dependent transcription at all concentrations, but did not affect the apparent affinity of GR for Dex, suggesting that TPA does not affect GR ligand binding.

Stimulation of GR-dependent transcription by MAP-SAP kinase activators can be inhibited by ERK and p38 pathway inhibitors.

To investigate whether the effect of TPA on GR-dependent transcription is mediated by MAP kinase activators, we treated Hela cells stably propagating GRE5-TATA-CAT episomes by TPA alone or in combination with MAP/SAP kinase inhibitors PD98059 or SB203580. While TPA treatment induced GR transcriptional activity ~11 fold (figure 3, compare lane 6 to lane 2), cotreatment with the ERK inhibitor PD98059 blocked this effect (about 70% inhibition, lane 7). On the other hand, the p38 inhibitor SB203580 had no significant effect, and the effect of SB203580 together with PD98059 was similar to that of the ERK inhibitor alone. These effects are consistent with the hypothesis that activation of ERK by TPA leads to the potentiation of GR-dependent transcription. Note that PD and SB did not have any effect on GR-dependent transcription when added alone (lane 20-21). To further analyze the role of MAP/SAP kinase on regulation of GR dependent transcription, we treated cells with EGF, which stimulate ERK; or with sorbitol, which activates ERK, JNK, and p38. EGF treatment also induced GR transcriptional activity~ 4-fold (figure 3, compare lane 11 to lane 2). Similar to what was observed with TPA, treatment with the ERK inhibitor PD98059 inhibited EGF induced GR activity (lane 12), p38 inhibitor SB203580 had no significant effect (figure 3, lane 13), and the effect of PD98059 plus SB203580 was similar to that of PD98059 alone (figure 3, lane 14). In contrast, while Sorbitol treatment induced GR transcriptional activity to the same degree as EGF ~5 fold (figure 3, compare lane 16 to lane 2), both ERK inhibitor PD98059 and p38 inhibitor SB203580 inhibited sorbitol effect (figure 3, lane 18 versus lane 16). The effect of PD98059 plus SB203580 was more striking than

those of either compound alone, leading to complete inhibition of GR-dependent transcription (figure 3, compare lane 19 to lane 2). This striking effect is due to Sorbitol activation of both ERK and p38 signaling pathway. Taken together, these data suggest that activation of both ERK and p38 can lead to potentiation of GR-dependent transcription.

MAP kinase activators increase glucocorticoid-induced transcription, but not estrogen-induced transcription in Ishikawa cells.

To assess whether the effect of TPA and EGF on GR-dependent transcription can be observed in other epithelial cell lines, we investigated then in uterine carcinoma Ishikawa cells, which also express endogenous GR. We stably transfected Ishikawa cells with an episomal reporter vector: pGRE5-TATA-CAT-EBV. Similar to what was observed in Hela, TPA stimulated GR transcription in the presence, but not the absence of dexamethasone (figure 4A, compare lane 4 to lane 2). Since Ishikawa cells also express estrogen receptors (ERs), we investigated whether TPA and EGF also upregulated ER-dependent transcription. Using Ishikawa cells stably propagating ERE3-TATA-CAT/episome, we observed that neither TPA nor EGF had any significant effect on ERE-mediated transcription (figure 4B, compare lane 10 and 12 to lane 8). These results indicate that the effect of TPA and EGF are specific to GR in Ishikawa cells.

Levels of GR mRNA and protein are not up-regulated by TPA treatment.

The stimulatory effect TPA on GR transcription could result from upregulation of GR mRNA and protein expression. The human GR promoter has been characterized and contains a putative AP-1 site (TGAC<u>A</u>CA), which differs by one nucleotide (underlined) from the

consensus AP-1 site (TGAC/GTCA). TPA may induce GR expression through this putative AP-1 site. To test this hypothesis, we monitored GR mRNA levels in response to TPA by Northern blotting. As shown in figure 5A, two GR mRNA species could be detected (around 5 kb and 7 kb). GR mRNA levels were not upregulated by TPA treatment at various times (figure 5A). These results suggest that GR mRNA is not regulated by TPA, and that the potential AP-1 site in the human GR promoter is not functional under our assay conditions. Further, we performed Western blot to check whether TPA can upregulate GR protein level. Hela cells were treated with TPA for different times, a single band around 95 kDa could be detected by Western blot using an anti-human GR polyclonal antibody, corresponding to the expected molecular weight of GR. The specificity of the antibody was confirmed using whole cell extracts of COS-7 cells transiently transfected with wt GR or with deletion mutants (data not shown), further confirming that the single protein band we detected represents GR. We did not observe modulation of GR protein expression levels following TPA treatment (figure 5C). All these data indicate the effect of TPA on GR-dependent transcription is not mediated via increases in GR mRNA or protein expression.

TPA does not increase the overall GR phosphorylation levels in Hela cells.

MAP kinase activation has been demonstrated to result in phosphorylation of human, mouse, and rat GR, we want to test whether TPA effect is mediated via activation of ERK pathway which phosphorylate GR. Mouse and rat GR phosphorylation sites have been characterized (Bodwell et al., 1991; Krstic et al., 1997). It has been reported that MAPK phosphorylate rat GR threonine 171 and serine 246 (Krstic et al., 1997). Serine 246 is conserved in human, the corresponding site being serine 226. We performed *in vitro* kinase assay to asses whether MAP kinase can also phosphorylate human GR Ser226. Affinity purified GST-GR AB fusion proteins were incubated with active ERK-2. GST alone is the

negative control (figure 6A, lane 1), wild type GR-GST fusion protein is phosphorylated *in vitro* (figure 6A, lane 2 and 4), where as the serine 226 mutant GST-$GR_{218-235}$ S226A is not phosphorylated (figure 6A, lane 3), implying that Ser 226 is phosphorylated by ERK-2 *in vitro*. We next examined whether TPA can upregulate GR phosphorylation *in vivo*, therefore we performed in vivo labeling experiments to monitor the phosphorylation status of GR in response to TPA treatment. TPA treatment alone did not change the overall phosphorylation of GR (compare lane 7 to lane 5 and 3 to 1; note that the increase in overall phosphorylation levels in lane 3 is not reproducible). Treatment with TPA in the presence of dexamethasone did not lead to increase in GR phosphorylation levels either (figure 6B, compare lane 4 to lane 2, and lane 8 to lane 6). Western blot confirmed that equal amount of protreins were loaded. Whole cell extracts of Cos-7 cells either mock transfected or transfected with GR expression vector were loaded to confirm the specificity of the antibody (figure 6, lane 9–10). Our results demonstrate that GR phosphorylation levels are not regulated *in vivo* by TPA stimulation. To further investigate the role of aa 246 in activation of GR by MAP kinase activators, we tested whether mutation of this position can affect GR transcriptional activity. GR-negative COS-7 and Jurkat cells were transfected with wild type GR and GR mutant S226A and S226E. As shown in figure 7A, dexamethasone transactivation is not altered by GR mutant S226A and S226E (compare lane 6 and lane 10 to lane 2). TPA effect in the presence of dexamethasone is not altered as well (compare lane 8 and lane 12 to lane 4). Similar experiments were performed in Jurkat cells, note that the effect of TPA on GR-transcription is quite striking, more than 10-fold. GR mutants S226A and S226E mediate similar striking effect on GR-transcription in response to TPA. Collectively, we conclude that human GR mutants S226E and S226A do not alter GRE-mediated transcriptional activity in COS-7 cells, and in Jurkat cells.

The DNA-binding domain of GR is crucial to mediate potentiation by TPA.

We next examined whether TPA can modulate transcriptional activity of the GR A/B region, whether GR A/B region is sufficient to mediate TPA effect. Expression vectors for the chimeric transcription factor GR(A/B)-Gal, which is composed of GR A/B transactivation domain fused to yeast transcription factor Gal4 DNA binding domain, were cotransfected in Hela cells with the reporter vector GalRE×5-ptk-luc, which has five Gal4 response elements upstream of the thymidine kinase promoter region. GR (A/B)-Gal had a stronger transcriptional activity than Gal alone (figure 8A, compare lanes 1-2 and 3-4). However, we did not observe transactivation activity by TPA on this chimeric transcription factor (Fig 8A, compare lane 2 to lane 1). Similar results were observed in Jurkat cells (data not shown). We conclude that the activity of the A/B region is not induced by TPA, at least in the absence of the rest of the GR receptor.

We further mapped the GR domains that are responsible to TPA effect in GR negative Jurkat cells. We transiently transfected in these cells a series of GR deletion mutants (figure 8B). GR τ_1 or AB region deletion mutants were still capable of mediating TPA effect (figure 8C, compare lane 12 and lane 16 to lane 8). Whereas TPA effect is not detectable with GR ligand binding domain deletion mutants (figure 8C, compare lane 20 and lane 24 to lane 8). These results indicate that the ligand binding domain, but not the AB region of the GR is required to mediate TPA effect.

To analyze the role of GR DNA-binding domain, we used chimeric receptors ER-GR CAS, which is an estrogen receptor containing the GR DNA binding domain, and GR-ER CAS, which is a glucocorticoid receptor containing the ER DNA binding domain. Indeed, results obtained in Ishikawa cells suggested that TPA has no effect on estrogen-induced transcription of the minimal ERE3-TATA-CAT promoter. This was confirmed in transient transfection of Hela cells with an ER expression vector and this same reporter. While TPA

increased activity in the absence of estrogen, no additional effect of TPA in the presence of estrogen was observed (figure 9A, lanes 13-16). Note that both chimeras were active on reporter genes bound by their respective DNA binding domains (figure 9A, lanes 6 and 18). The effect of TPA on transcription mediated by ER-GR CAS were comparable to those on wt GR (Fig 9A, compare lane 8 to lane 4); while TPA had no effect on GR-ER CAS (Fig 9A, compare lane 20 to lane 18). We conclude that the GR DNA binding domain is crucial to mediate TPA effect. Similar experiments were performed in Jurkat cells to further confirm above results (figure 9B). Note that the effect of TPA is more striking in Jurkat cells than in Hela cells, which suggest that tissue-specific factors such as nuclear receptor coactivators are involved in this regulation. Like in Hela cells, stimulation by TPA was observed with ER-GR CAS, but not GR-ER CAS. Taken together, these results indicate that GR τ1 or AB region is not essential to mediate TPA effect, that the presence of a ligand binding domain of the ER or GR is required and that the GR DNA-binding domain is crucial.

DISCUSSION

Conflicting literature reports

Literature reports regarding the effect of activators of MAP kinase on GR transcriptional activity have led to totally different conclusions (Rogatsky et al 1998; Moyer et al., 1993; Maroder et al., 1993; Vacca et al., 1989). Rogatsky et al demonstrated that selective activation of either ERK or JNK *in vivo* inhibits GR-mediated transcriptional activation in Hela cells using transiently transfected reporter vectors containing two consensus GREs upstream of a TATA box (Rogatsky et al.,1998). On the other hand, Moyer et al observed that TPA cotreatment enhances GR transcriptional activity in T47-D cells via a stably transfected MMTV reporter vector (Moyer wt al., 1993). Vacca et al reported that activation of PKC by TPA treatment reduced dexamethasone-mediated activation of an MMTV-CAT reporter gene transiently transfected into NIH-3T3 cells. Later, Maroder et al demonstrated that this effect is cell specifc, as TPA augmented dexamethasone-induced transcriptional activation by the MMTV LTR in several T cell lines but was inhibitory in NIH-3T3 fibroblasts. To address this issue, we used Hela and Ishikawa cell lines stably propagating GR reporter vectors to study effects of MAP kinase activators on endogenous GR transcriptional activity. We used a reporter vector composed of only five GREs upstream of an adenoviral minimal promoter TATA box. Because complex promoter such as MMTV contain other cis-regulatory elements which may mediate transcriptional effects. In addition, use of stable cell clones avoids complicating effects on cell signalling due to transient transfection procedures. Our results indicate that TPA and other MAP kinase inducers stimulate GR dependent transcription in Hela cells and in Ishikawa cells as well as in Jurkat cells. These results, while in agreement with the observation that MMTV-driven transcription is stimulated by TPA in breast cells and in lymphocyte, contrast with those of Rogatsky et al.,

also obtained in Hela cells. The divergence between ours and these results may be due to the different ways used to stimulate MAP/SAP kinase activity. While we used signalling molecules or osmotic stress, these authors stimulated MAP/SAP kinase activity by transfection of constitutively active upstream components of these signalling pathways. Alternatively, clone differences in Hela cells used for these studies cannot be rule out.

GR DNA-binding domain is crucial to mediate TPA effect.

Our results indicate the GR DNA-binding domain is important to mediate TPA effect, as the ER-GR CAS chimera, which contains the GR DBD, is stimulated by TPA effect, but not the GR-ER CAS construct, which contains the ER DBD. In addition, an estrogen receptor which contains GR first zinc finger is able to mediate TPA effect in Jurkat cells as well (data not shown). Sequence analysis of GR DBD and ER DBD shows that one Serine residues (Ser 425) is present in the first zinc finger of GR but not in that of ER. Thus a possible mechanism of the effect of TPA may be the phosphorylation of Ser 425 in the GR DNA binding domain *in vivo*, although it is unlikely that GR is the direct target of MAP kinase, as the ser 425 surrounding sequences do not resemble to the MAP kinase consensus sequences.

In addition, the GR DNA binding domain has been shown to be important to mediate GR transrepression (Schüle et al., 1990). Direct protein-protein interactions between GR and c-Jun or c-Fos have been reported (Yang-Yen et al.,1990). The ER DNA binding domain does not interact with c-Jun or c-Fos. Therefore one potential mechanism of the effect of TPA on GR transcription may be protein-protein interaction between TPA-induced Jun or Fos with GR DNA-binding domain. Maroder et al reported that TPA augmented dexamethasone-induced transcriptional activation of the MMTV LTR in several T cell lines. The mechanism was ascribed to cooperation of TPA-induced Jun proteins with GR. This

hypothesis was supported by transfection of c-jun, jun-B, or junD expression vectors, which synergized with GR to activate GRE (Maroder et al., 1993). On the other hand, it was also reported that c-jun and jun-B inhibit GR-dependent GRE activity in Hela cells (Shemshedini et al.,1991). Similar to this report, we did not observe that overexpression of cJun, JunB, JunD and cFos induced GR activity (data not shown), implying that enhancement of GR transcriptional activity by TPA is not mediated through induction of cJun, JunB, JunD and cFos. Still we cannot rule out that other AP-1 members such as Fra-1, Fra-2, ATF-2, may mediate the effect of TPA on GR transcription.

Finally, it is possible that TPA may regulate the phosphorylation of GR-specific cofactors interacting with the DNA binding domain of GR, although such cofactors have not been characterized to date.

In conclusion, our results demonstrate that activation of MAP kinases stimulates GR-dependent transcription in epithelial cells as well as in lymphocytes. Thus the synergy between the two signalling pathways appears to be less cell specific than previously believed. It will be interesting in the future to test the effects of MAP kinase activation on a wide arrange of GR target genes.

Acknowledgement

We are grateful to Dr. J. Galipeau (McGill University, Montreal, Canada) for providing retrovirus vSINGRE5 transduced Hela cells.

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Figure Legends

Figure 1. TPA treatment potentiates GRE-mediated transcription in Hela .

(A) (C) Hela cells stably transfected with GRE5-TATA-CAT/EBV or GRE5-TATA-CAT reporter vectors were treated with dexamethasone (25 nM), TPA (10 ng/ml) or both for 24 hours. CAT was performed after protein quantitation using a Bradford assay in whole cell extracts. (B) Hela cells transduced with retrovirus vSINGRE5 were exposed to dexamethasone (25 nM), TPA (10 ng/ml) or both for 24 hours. GFP expression was detected by FACS analysis. (D) Stably transfected Hela-GRE5-TATA-CAT cells were transfected with Bluescribe M13+ (15 μg) by the calcium phosphate coprecipitate method. 24 h later, transfected or non-transfected cells were treated with dexamethasone (25 nM) in the presence or absence of TPA (10 ng/ml) as indicated for 20 h. Then cells were harvested and CAT assay was performed as described previously.

Figure 2. Stimulation of GRE-mediated transcription by TPA is rapid, and does not affect the apparent affinity for dexamethasone.

(A) Hela cells stably transfected with GRE5-TATA-CAT were treated with dexamethasone (25 nM) in the presence or absence of TPA (10 ng/ml) for 4h, 8h, 12h, 16h and 20h as indicated. (B) Hela cells stably transfected with GRE5-TATA-CAT/EBV reporter vectors were treated with increasing concentrations of dexamethasone in the presence or absence of TPA (10 ng/ml) for 24h as indicated. CAT was performed as described previously.

Figure 3. Stimulation of GRE-mediated transcription by MAPK/SAPK activators is blocked by ERK and p38 pathway inhibitors.

Hela cells stably transfected with GRE5-TATA-CAT/EBV reporter vectors were pretreated with PD98059 (50 μM), or SB203580 (10 μM) for 1h, then treated with dexamethasone (25

nM) in the presence or absence of TPA (10 ng/ml), EGF (50 ng/ml), or Sorbitol (0.4M) as indicated. Cells were harvested after a further incubation of 24 hours and CAT activity was measured in whole cell extracts.

Figure 4. TPA and EGF treatment stimulates GRE-mediated, but not ERE-mediated transcription in Ishikawa cells.

(A) Ishikawa cells stably transfected with GRE5-TATA-CAT/EBV were treated with dexamethasone (25 nM), TPA (10 ng/ml) or both for 24 hours. (B) Ishikawa cells stably transfected with GRE5-TATA-CAT/EBV or ERE3-TATA-CAT/EBV reporter vectors were treated with dexamethasone (25 nM) or estrogen (25 nM), in the presence or absence of TPA (10 ng/ml) or EGF (50 ng/ml) as indicated. CAT assay was performed as described previously.

Figure 5. GR mRNA and protein levels are not regulated by TPA treatment.

(A) Hela cells were exposed to vehicle or TPA (10 ng/ml) for 1h, 2h, 3h, 6h, 12h, 24 h respectively. Poly A^+ RNA (2 μg each) were separated on an agarose/formaldehyde gel. Northern blot analysis was performed to determine GR and β-actin mRNA expression levels. (B) Quantitative analysis of total GR mRNA/β-actin mRNA. (C) Hela cells were exposed to vehicle or TPA (10 ng/ml) for 1h, 2h, 3h, 6h, 12h, 24 h respectively. Whole cell extracts were separated on SDS-PAGE. Western blot was performed using a specific GR antibody. Experiments were reproduced 3 times, one typical experiment is shown.

Figure 6. *In vitro* and *in vivo* phosphorylation of GR

(A) Affinity purified GST fusion proteins (4μg) were incubated with active ERK-2 and [γ-^{32}P]ATP (10μCi). Reaction products were separated by SDS/PAGE, stained with Coomassie

blue and autoradiographed. (B) Hela cells (1.5×10^6/100-mm dish) were serum starved for 24 h, then incubated with [^{32}P] phosphoric acid (2 mCi/dish) for 5h. 15 min or 1h before harvest, cells were treated with vehicle, dexamethasone (25 nM), TPA (10 ng/ml) or dexamethasone plus TPA. Immunoprecipitation was performed with anti-hGR antibody, immunoprecipitates was then eletrophoresed on an 8% SDS-PAGE gel. Proteins were transferred to a PVDF membrane, and the membrane was exposed to film for autoradiography for 5h. (C) Following autoradiography, the membrane was subjected to Western blotting with anti-hGR antibody. COS-7 cells non-transfected or transiently transfected with GR expression vector pSG5-hG0 are negative and positive controls, respectively. Results are representative of three independent experiments.

Figure 7. Mutations S226A and S226E do not alter GR transcriptional activity.

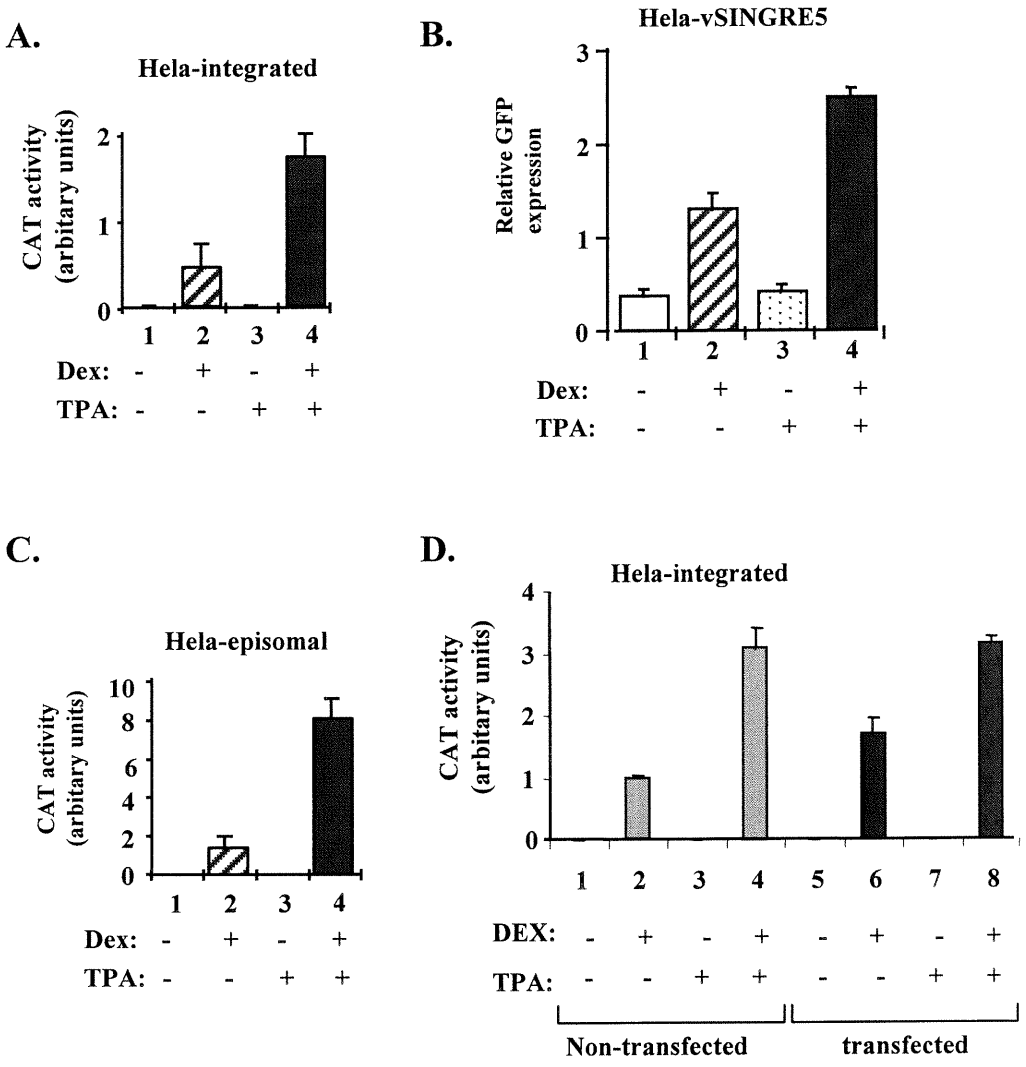
(A) Cos-7 cells were transfected with wild type human GR expression vector pSG5-hG0 or GR mutants S226A or S226E (3 μg/100 mm-dish), along with reporter vector pGRE5-TATA-CAT/EBV (2 μg/100 mm-dish). 24 h posttransfection, cells were incubated with dexamethasone (25 nM) in the presence or absence of TPA (10 ng/ml) for 20 h. CAT assay was performed as described previously. (B) Jurkat cells were transfected with wild type human GR expression vector pSG5-hG0 or GR mutants S226A or S226E (10 μg/100 mm-dish), along with reporter vector pGRE5-TATA-CAT (4 μg/100 mm-dish). Using the DEAE-dextran method, 24 h posttransfection, cells were incubated with dexamethasone (25 nM) in the presence or absence of TPA (10 ng/ml) for 20 h. CAT assay was performed as described previously.

Figure 8. Mapping of GR functional domains that mediate TPA effect

(A) Hela cells were transfected with GR(AB)Gal or Gal expression vector (3 μg/100-mm dish) along with reporter vector pGalRE×5-ptk-luc (2 μg/100-mm dish). 24 h posttransfection, cells were incubated with or without TPA (10 ng/ml) as indicated for 20 h. Then cells were harvested and luciferase assay was performed as described under "Experimental Procedures". In parallel, Hela cells were transfected with pGRE×5-TATA-CAT as control experiment. (B) Schematic representation of wild type GR and GR deletion mutant expression vectors used in (C). (C) Jurkat cells were transiently transfected with wild type GR or GR deletion mutant expression vectors Δ77-262, hG8, hG2, or hG11 (5 μg/100-mm dish) as shown in (C), along with reporter vector pGRE5-TATA-CAT/EBV (4 μg/100-mm dish). 24 h posttransfection, cells were incubated with dexamethasone (25 nM) in the presence or absence of TPA (10 ng/ml) as indicated for 20 h. Cells were then harvested and CAT activity was measured.

Figure 9. The GR DNA-binding domain can not be replaced by that of ER to mediate TPA effect

(A) Hela cells were transiently transfected with expression vectors for wt ER, ER-GR CAS, or GR-ER CAS (3 μg/100-mm dish) along with reporter vector pGRE5-TATA-CAT or pERE3-TATA-CAT (2 μg/100-mm dish). 24 h posttransfection, cells were incubated with dexamethasone (25 nM) or estradiol (25 nM) in the presence or absence of TPA (10 ng/ml) as indicated for 20 h. Then cells were harvested and CAT assay was performed as described previously. (B) Similar experiments were performed in Jurkat cells, except that expression vectors (5 μg/100-mm dish) and reporter vectors (4 μg/100-mm dish) were transfected using the DEAE-dextran method. 24 h posttransfection, cells were incubated for 20 h with dexamethasone (25 nM) or estradiol (25 nM) in the presence or absence of TPA (10 ng/ml), as indicated. Then cells were harvested and CAT assays were performed.


A.
Hela-integrated
CAT activity (arbitary units)
0
1
2
1 2 3 4
Dex: - + - +
TPA: - - + +
B.
Hela-vSINGRE5
Relative GFP expression
0
1
2
3
1 2 3 4
Dex: - + - +
TPA: - - + +
C.
Hela-episomal
CAT activity (arbitary units)
0
2
4
6
8
10
1 2 3 4
Dex: - + - +
TPA: - - + +
D.
Hela-integrated
CAT activity (arbitary units)
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1
2
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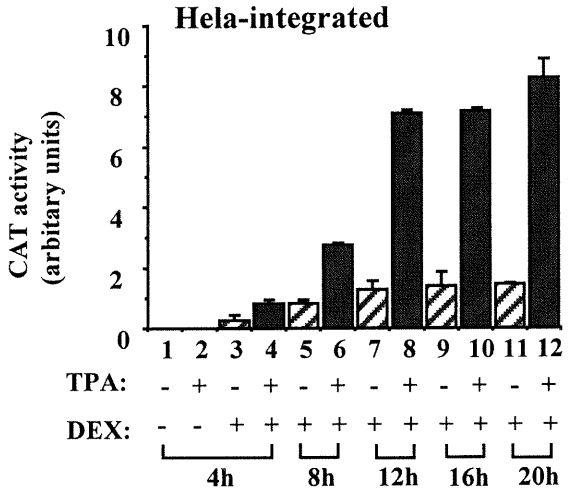
1 2 3 4 5 6 7 8
DEX: - + - + - + - +
TPA: - - + + - - + +
Non-transfected
transfected

Fig 1.

A.


Hela-integrated
CAT activity (arbitary units)
0
2
4
6
8
10
1 2 3 4 5 6 7 8 9 10 11 12
TPA: - + - + - + - + - + - +
DEX: - - + + + + + + + + + +
4h
8h
12h
16h
20h

B.

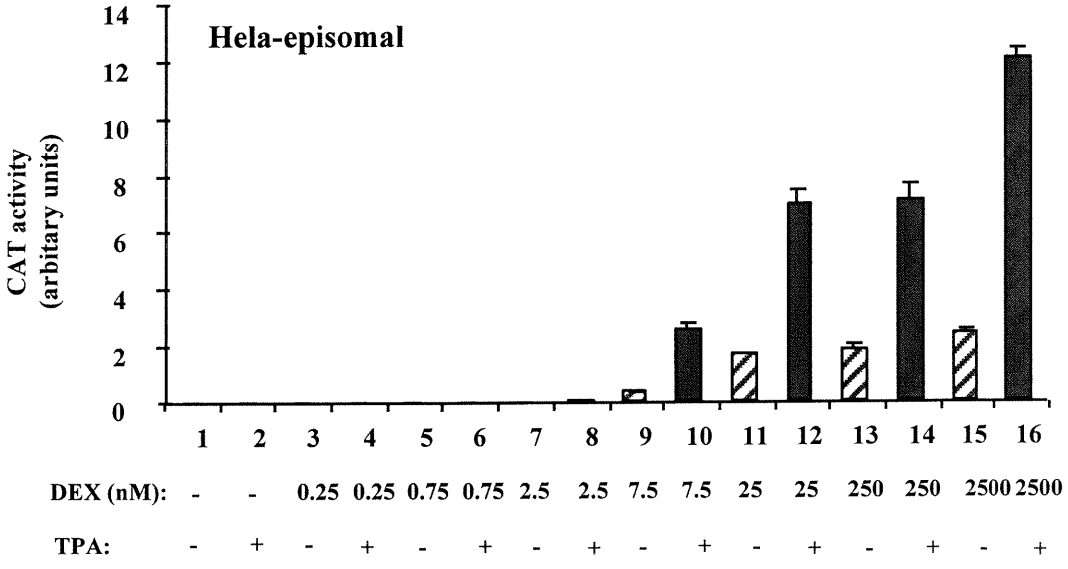

Hela-episomal
CAT activity (arbitary units)
0
2
4
6
8
10
12
14
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16
DEX (nM): - - 0.25 0.25 0.75 0.75 2.5 2.5 7.5 7.5 25 25 250 250 2500 2500
TPA: - + - + - + - + - + - + - + - +

Fig 2.

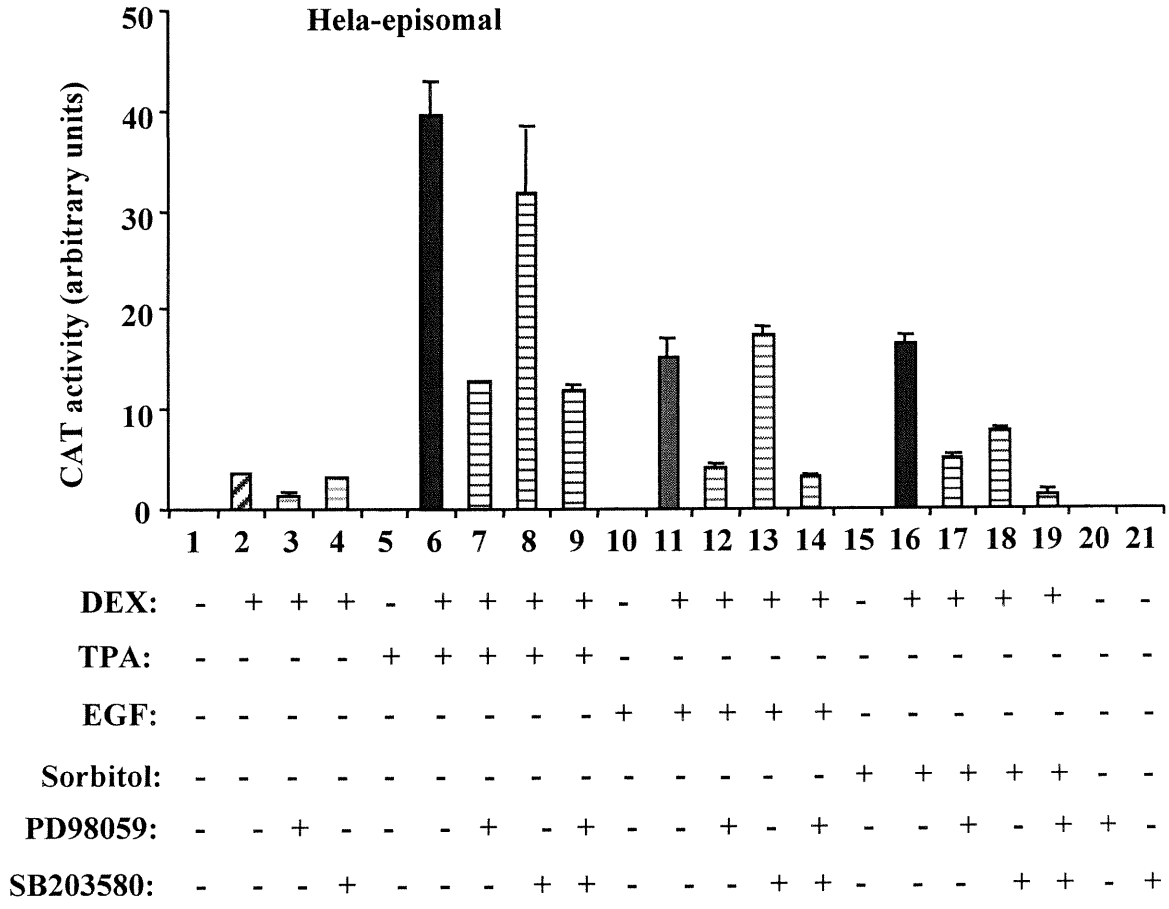

Hela-episomal
CAT activity (arbitrary units)
50
40
30
20
10
0
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21
DEX: - + + + - + + + + - + + + + - + + + + - -
TPA: - - - - + + + + + - - - - - - - - - - - -
EGF: - - - - - - - - - + + + + + - - - - - - -
Sorbitol: - - - - - - - - - - - - - - + + + + + - -
PD98059: - - + - - - + - + - - + - + - - + - + + -
SB203580: - - - + - - - + + - - - + + - - - + + - +

Fig 3.

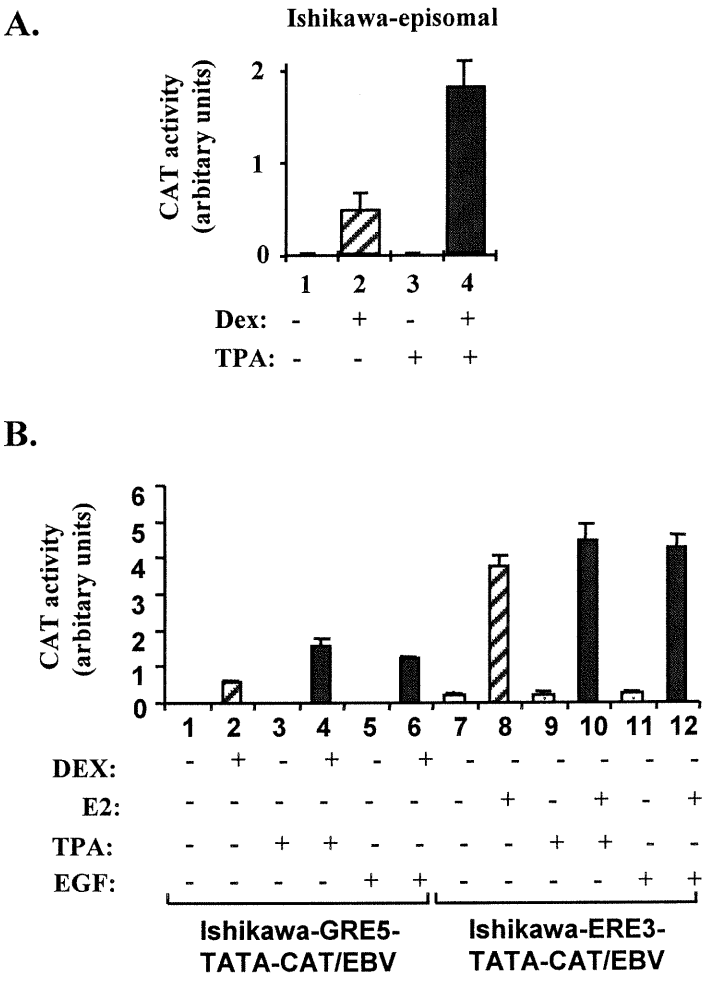

A.
Ishikawa-episomal
CAT activity (arbitary units)
2
1
0
1 2 3 4
Dex: - + - +
TPA: - - + +
B.
CAT activity (arbitary units)
6
5
4
3
2
1
0
1 2 3 4 5 6 7 8 9 10 11 12
DEX: - + - + - + - - - - - -
E2: - - - - - - - + - + - +
TPA: - - + + - - - - + + - -
EGF: - - - - + + - - - - + +
Ishikawa-GRE5-TATA-CAT/EBV
Ishikawa-ERE3-TATA-CAT/EBV

Fig 4.

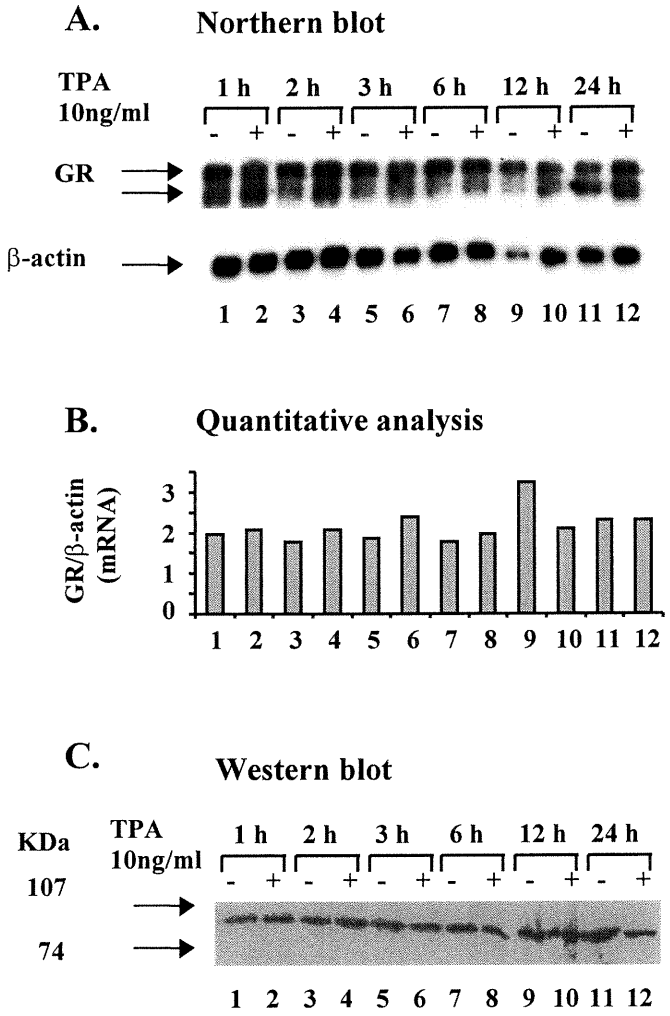

A.
Northern blot
TPA
10ng/ml
1 h
2 h
3 h
6 h
12 h
24 h
- + - + - + - + - + - +
GR
β-actin
1 2 3 4 5 6 7 8 9 10 11 12
B.
Quantitative analysis
GR/β-actin
(mRNA)
3
2
1
0
1 2 3 4 5 6 7 8 9 10 11 12
C.
Western blot
KDa
TPA
10ng/ml
1 h
2 h
3 h
6 h
12 h
24 h
- + - + - + - + - + - +
107
74
1 2 3 4 5 6 7 8 9 10 11 12

Fig 5.

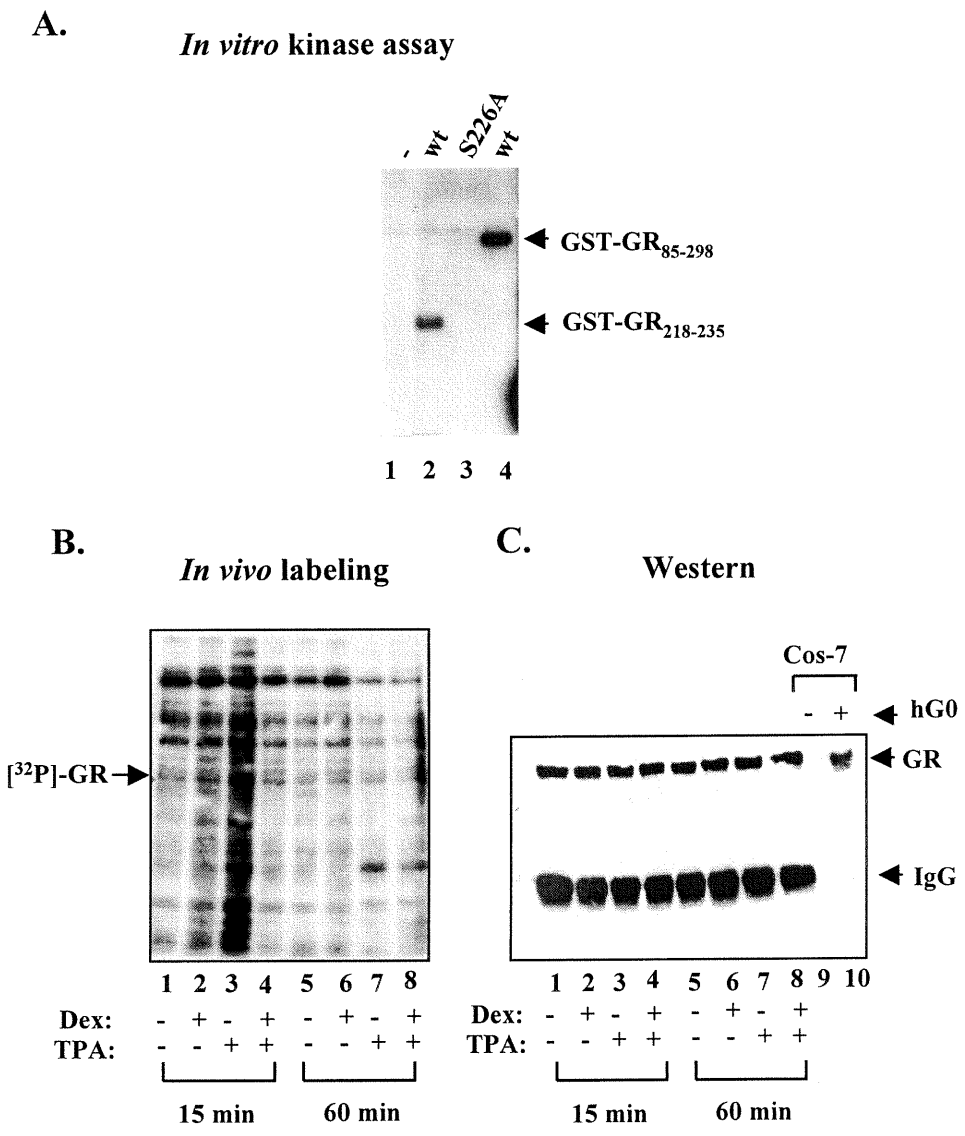

A.
In vitro kinase assay
-
wt
S226A
wt
GST-GR85-298
GST-GR218-235
1 2 3 4
B.
In vivo labeling
[32P]-GR
1 2 3 4 5 6 7 8
Dex: - + - + - + - +
TPA: - - + + - - + +
15 min
60 min
C.
Western
Cos-7
- +
hG0
GR
IgG
1 2 3 4 5 6 7 8 9 10
Dex: - + - + - + - + - +
TPA: - - + + - - + + - +
15 min
60 min

Fig 6.

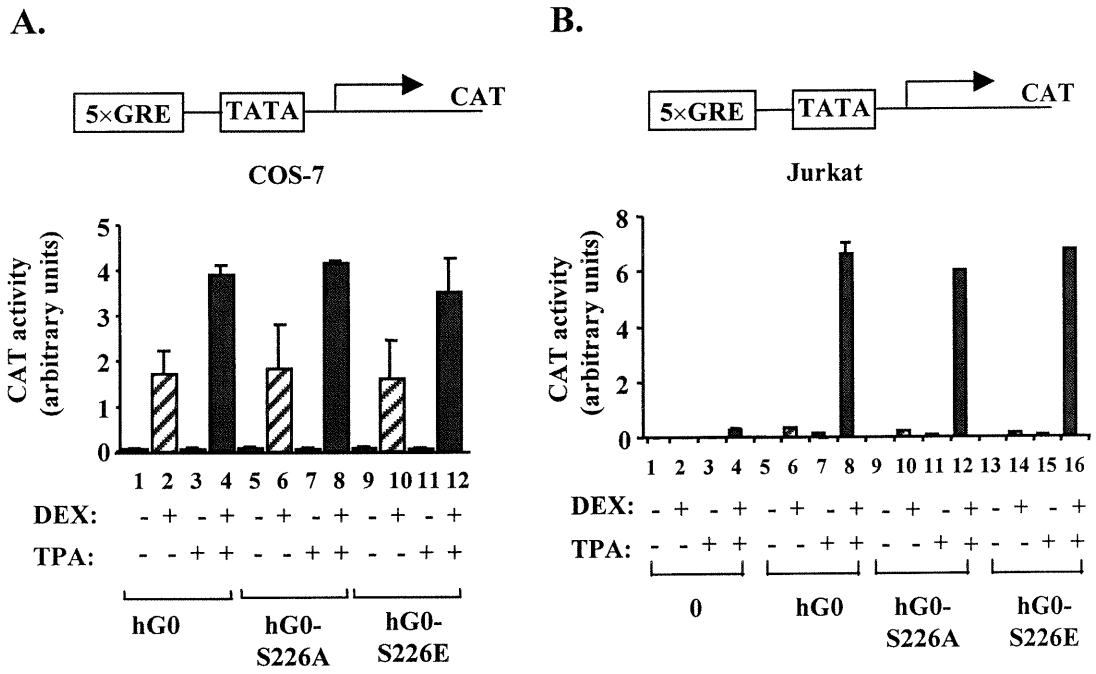

A.
5×GRE
TATA
CAT
COS-7
CAT activity (arbitrary units)
0
1
2
3
4
5
1 2 3 4 5 6 7 8 9 10 11 12
DEX: - + - + - + - + - + - +
TPA: - - + + - - + + - - + +
hG0
hG0-S226A
hG0-S226E
B.
5×GRE
TATA
CAT
Jurkat
CAT activity (arbitrary units)
0
2
4
6
8
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16
DEX: - + - + - + - + - + - + - + - +
TPA: - - + + - - + + - - + + - - + +
0
hG0
hG0-S226A
hG0-S226E

Fig 7.

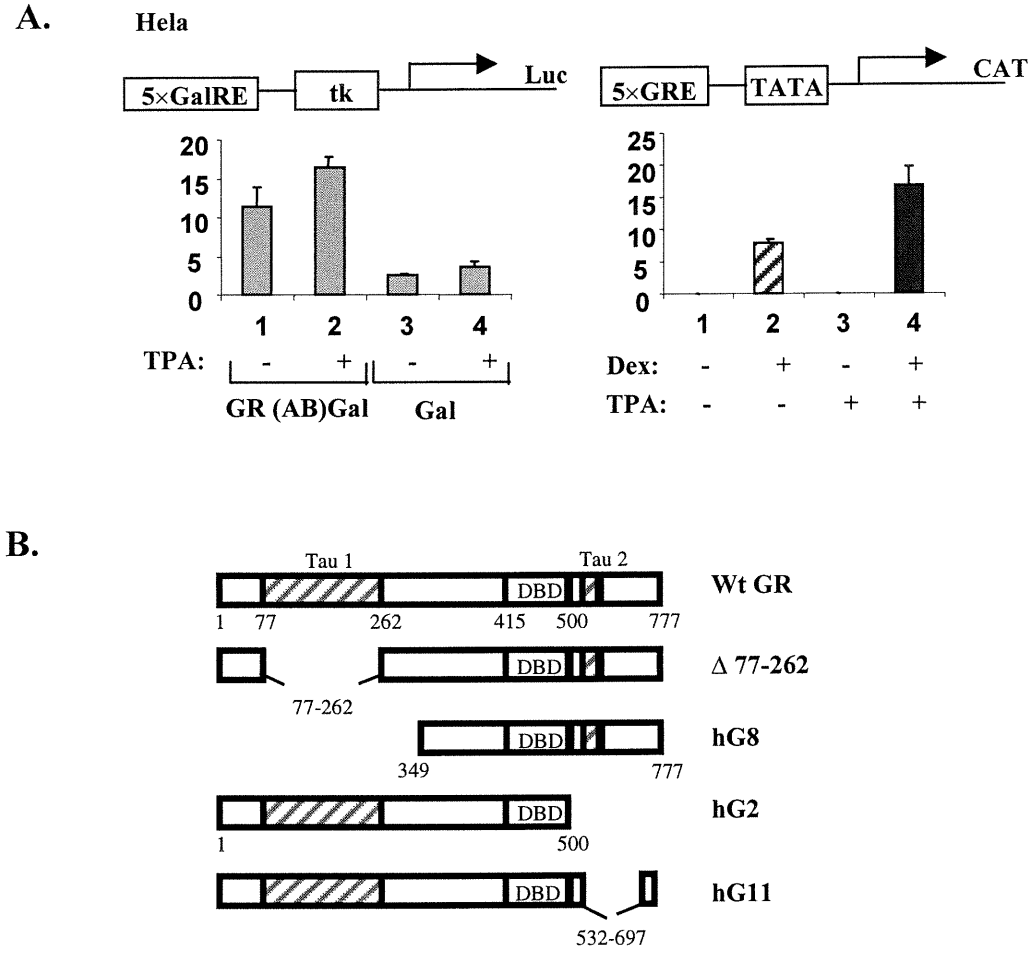
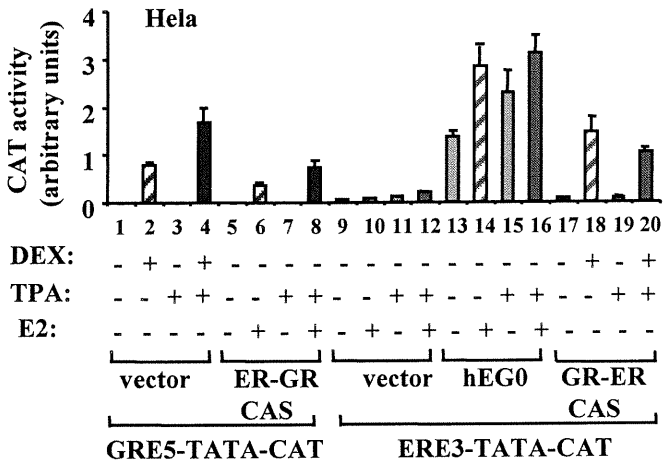

A.
Hela
5×GalRE
tk
Luc
5×GRE
TATA
CAT
TPA:
GR (AB)Gal
Gal
Dex:
TPA:
B.
Tau 1
Tau 2
DBD
Wt GR
1 77 262 415 500 777
Δ 77-262
77-262
hG8
349 777
hG2
1 500
hG11
532-697
C.
Jurkat
Reporter:
5×GRE
TATA
CAT
CAT activity (arbitrary units)
DEX:
TPA:
0
wt GR
Δ 77-262
hG8
hG2
hG11

Fig 8.

A.


Hela
CAT activity (arbitrary units)
0
1
2
3
4
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20
DEX: - + - + - - - - - - - - - - - - - + - +
TPA: - - + + - - + + - - + + - - + + - - + +
E2: - - - - - + - + - + - + - + - + - - - -
vector
ER-GR CAS
vector
hEG0
GR-ER CAS
GRE5-TATA-CAT
ERE3-TATA-CAT

B.

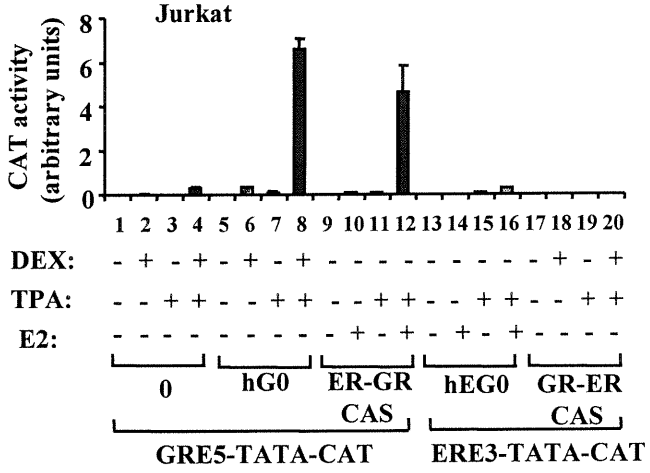

Jurkat
CAT activity (arbitrary units)
0
2
4
6
8
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20
DEX: - + - + - + - + - - - - - - - - - + - +
TPA: - - + + - - + + - - + + - - + + - - + +
E2: - - - - - - - - - + - + - + - + - - - -
0
hG0
ER-GR CAS
hEG0
GR-ER CAS
GRE5-TATA-CAT
ERE3-TATA-CAT

Fig 9.

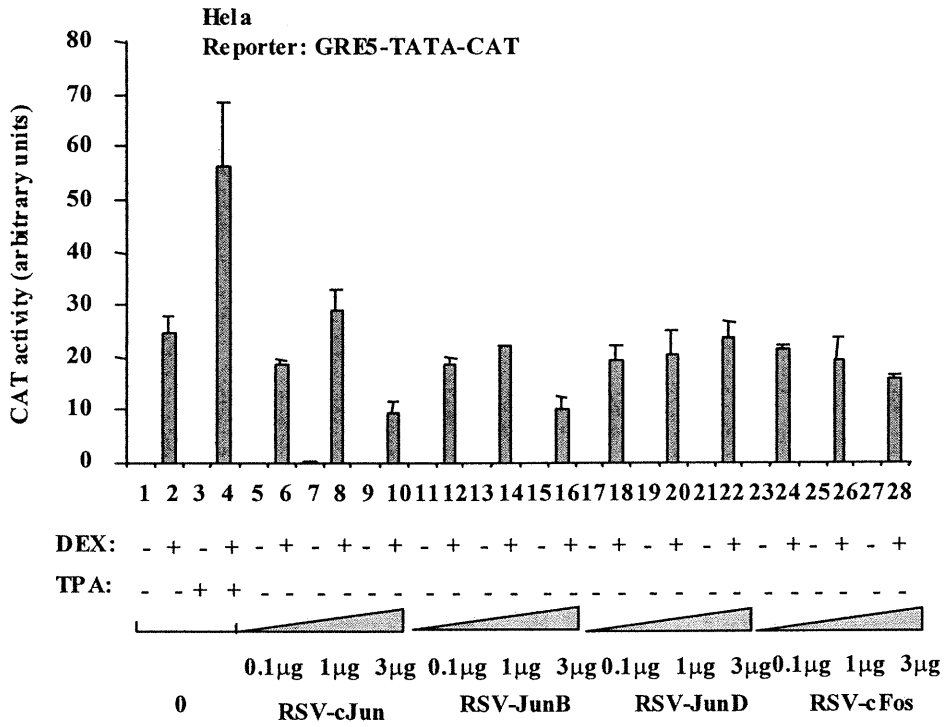
OTHER RELATED RESULTS

Enhancement of GR transcriptional activity by TPA is not mediated through induction of cJun, JunB, JunD and cFos.

Previous studies reported that TPA augmented dexamethasone-induced transcriptional activation of the MMTV LTR in several T cell lines. The mechanism was ascribed to cooperation of TPA induced-Jun proteins with GR. This hypothesis was supported by transfection of c-jun, jun-B, or junD expression vectors, which can synergize with GR to activate GRE (Maroder et al., 1993). On the other hand, it was also reported that c-jun and jun-B inhibit GR-dependent GRE activity in Hela cells (Shemshedini et al.,1991). To test whether the TPA effect can be attributed to interaction of GR with Jun proteins in Hela cells, we transfected increasing amounts of c-jun, jun-B, junD, or c-Fos expression vectors in Hela cells. Consistently, TPA treatment stimulated GR transcriptional activity 2- to 3- fold (figure 10, lane 4 to lane 2). However, none of the AP-1 members stimulated GR activity in the presence of dexamethasone (figure 10, compare lanes 6, 8, 10, 12, 14, 16, 18, 20, 22, 24, 26, 28 to lane 2). In fact, transient transfection of 3 μg of c-Jun and JunB repressed AP-1 activity around 50% (figure 10, compare lane 10 and lane 16 to lane 2). We conclude that overexpression of cJun, JunB, JunD and cFos does not mimic the TPA effect, implying that enhancement of GR transcriptional activity by TPA is not mediated through induction of cJun, JunB, JunD or cFos.

Figure 10. Overexpression of increasing amounts of cJun, JunB, JunD, and cFos does not mimic the effect of TPA on GR transcriptional activity.

Hela cells were transfected with increasing amounts (0.1 μg/100 mm-dish, 1 μg/100 mm-dish, or 3 μg/100 mm-dish) of RSV-cJun, RSV-JunB, RSV-JunD, or RSV-cFos. Reporter vector pGRE5-TATA-CAT (2 μg/100 mm-dish) was cotransfected. 24 h posttransfection, cells were incubated with dexamethasone (25 nM) in the presence or absence of TPA (10 ng/ml) as indicated for another 20 h. Then cells were harvested and CAT assay was performed as described previously.

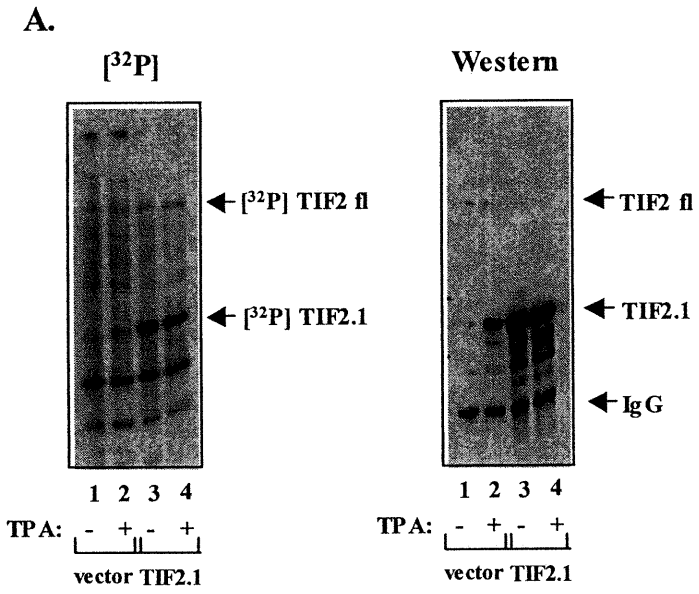

Hela
Reporter: GRE5-TATA-CAT
CAT activity (arbitrary units)
80
70
60
50
40
30
20
10
0
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24 25 26 27 28
DEX: - + - + - + - + - + - + - + - + - + - + - + - + - + - + - +
TPA: - - + + -
0.1µg 1µg 3µg 0.1µg 1µg 3µg 0.1µg 1µg 3µg 0.1µg 1µg 3µg
0
RSV-cJun
RSV-JunB
RSV-JunD
RSV-cFos

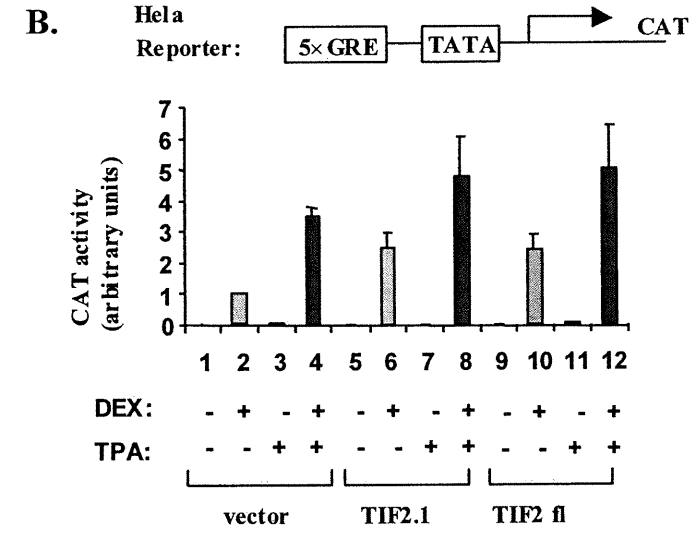
Phosphorylation of TIF-2 is not the limiting phenomenon in the potentiation of GR-dependent transcription by TPA.

To further analyze the mechanism of the TPA effect on GR transcriptional activation, we tested whether TPA can upregulate phosphorylation of a GR-interacting coactivator. The best characterized nuclear receptor coactivators are members of the p160 family. SRC-1, GRIP1, and AIB1 have all been identified as ERK2 substrates *in vitro* (Rowan et al., 2000; Lopez et al., 2001; Font de Mora and Brown 2000). Mutations of GRIPI S736 to alanine substantially reduce the ability of GRIP1 to enhance transcription of the EGF activated PR and of ER costimulated by estrogen and EGF (Lopez et al., 2001). So we performed *in vivo* labeling experiments to investigate whether TPA can modulate TIF-2 phosphorylation *in vivo*. Hela cells express TIF2 to low levels. To achieve a better signal, we transiently transfected Hela cells with a highly expressed TIF2 truncated mutant, TIF2.1 (aa 624-1287). TIF-2.1 was phosphorylated *in vivo*. (Fig. 10A, lane 3). TPA treatment did not affect levels of phosphorylation (lane 4 versus lane 3). Western blot confirmed equal loading of the samples. Note that a band of high molecular weight (~ 160 kDa) was also detected by the antibody, likely representing TIF2 fl (full length TIF2). Phosphorylation of this band was not altered by TPA (figure 10A lane 4 versus lane 3). To further analyze the role of TIF2, we overexpressed the active TIF2 truncated mutant TIF2.1 and TIF2 full length expression vector in Hela cells by transient transfection. If TIF2 is the target of TPA, when we overexpress TIF2, we would expect the TPA effect on GR transcription to be further enhanced. Our results indicate that both TIF2.1 and TIF2 full length overexpression enhance dexamethasone induced GR transcriptional activity (figure 10B, compare lane 6 and lane 10 to lane 2). However, the TPA effect on GR-transcription was not further increased (figure 10B, compare fold induction between lane 4 versus lane2, lane 8 versus lane 6 and lane 12 versus lane 10). These results indicate that phosphorylation of TIF-2 is not the limiting factor that mediates the TPA effect.

Figure 11. Phosphorylation of TIF-2 is not the limiting factor in the potentiation of GR-dependent transcription by TPA.

(A) Hela cells (1.5×10^6/100-mm dish) were serum starved for 24 h, then incubated with [^{32}P] phosphoric acid (1 mCi/dish) for 5h. 15 min before harvest, cells were treated with vehicle, or TPA (10 ng/ml). TIF-2 was immunoprecipitated with a monoclonal TIF-2 antibody (Asc 3Ti 3Fi), immunorecipitations was eletrophoresed on an 8% SDS-PAGE gel and transferred to a PVDF membrane. Following autoradiography, the membrane was subjected to Western blotting with the monoclonal anti-TIF-2 antibody. (B) Hela cells were transfected with pSG5, pSG5-TIF2.1(aa 624-1287 of TIF2) or pSG5-TIF2 full length (6 μg/100 mm-dish) and pGRE5-TATA-CAT (2 μg/100 mm-dish). 24 h posttransfection, cells were incubated with dexamethasone (25 nM) in the presence or absence of TPA (10 ng/ml) for 20 h. CAT assay was performed as described previously.


A.
[32P]
Western
[32P] TIF2 fl
[32P] TIF2.1
TIF2 fl
TIF2.1
IgG
1 2 3 4
TPA: - + - +
vector TIF2.1
1 2 3 4
TPA: - + - +
vector TIF2.1

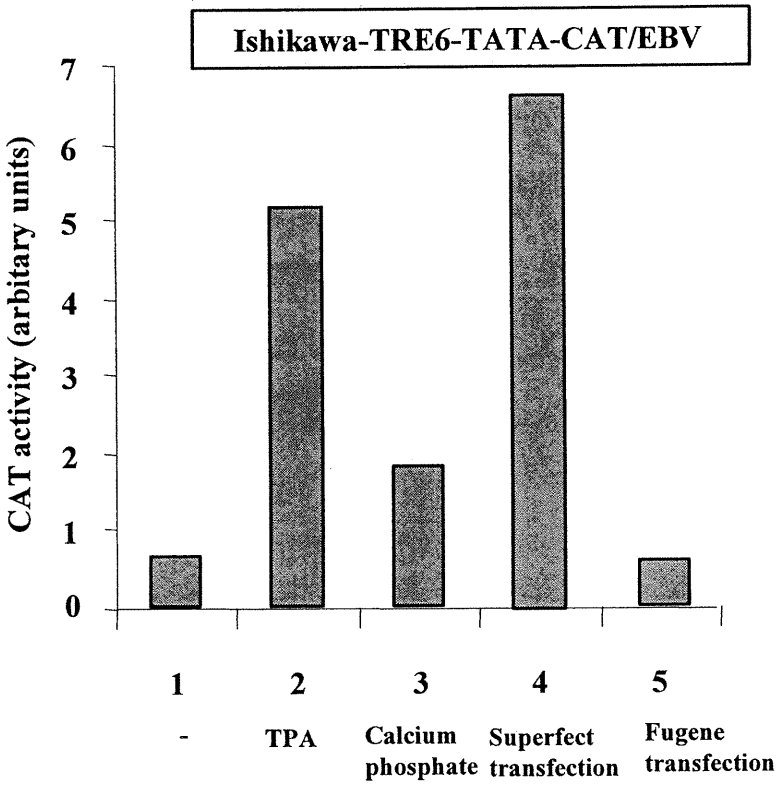

B.
Hela
Reporter:
5× GRE
TATA
CAT
CAT activity
(arbitrary units)
0 1 2 3 4 5 6 7
1 2 3 4 5 6 7 8 9 10 11 12
DEX: - + - + - + - + - + - +
TPA: - - + + - - + + - - + +
vector
TIF2.1
TIF2 fl

Modulation of AP-1 activity by transient transfection

In our efforts to identify the divergence between our studies and published reports regarding the effects of activation of MAP kinase on GR transcriptional activity, we found that most of the published results were based on transient transfection, while we used stably transfected cell lines that propagate the GRE reporter vector. So we decided to test whether the effect of TPA can be attenuated by transient transfection, our results is shown in figure 1D. Theses results suggest that transient transfection itself may modulate signaling of the cells, then we performed an experiment to check whether AP-1 activity can be regulated by transient transfection. Using the stably transfected Ishikawa-6×TRE-TATA-CAT/EBV, we performed mock transfections with the carrier DNA Bluescribe M13+ via different tranfection methods. Our results indicate that while AP-1 activity can be induced in response to TPA up to 6-7 fold (figure 12, compare lane 2 to lane 1), calcium transfection induced AP-1 activity 2.5-fold (figure 12, compare lane 3 to lane 1) and Superfect induced AP-1 activity up to 8-9 fold (figure 12, compare lane 4 to lane 1). In contrast, Fugene transfection did not activate AP-1 activity (figure 12, compare lane 5 to lane 1). This result indicates that transient transfection can modulate AP-1 activity depending on the transfection method, suggesting that the Fugene transfection method might be a more reliable transfection reagent, since it did not modulate cell signaling such as the AP-1 pathway under our conditions.

Figure 12. Transient transfection increases AP-1 transcriptional activity in Ishikawa cells.

Ishikawa stably tansfected with the pTRE×6-TATA-CAT/EBV reporter vector were either non-transfected, or mock transfected with carrier DNA via calcium phosphate, or Superfect, or Fugene transfection reagent for 20 hours, then treated with vehicle or TPA (10 ng/ml). CAT assay was performed as previously described.


Ishikawa-TRE6-TATA-CAT/EBV
CAT activity (arbitary units)
0
1
2
3
4
5
6
7
1
2
3
4
5
-
TPA
Calcium phosphate
Superfect transfection
Fugene transfection

Chapter IV

Discussion and Perspectives

I. Mechanisms of regulation of AP-1 by steroid hormones

1. AP-1 is not sensitive to estrogen in Ishikawa cells

Estrogens have long been recognized as being important for stimulating the growth of a large portion of tumors of the female reproductive system, but the mechanism by which estradiol acts as a mitogen is still not clear. Rapid activation of the mitogen-activated protein kinase (MAPK) signaling pathway by estrogen is one of the recently identified mechanism (Migliaccio et al., 1996; Filardo et al., 2000; Manthey et al., 2001; Wade et al., 2001). Activation of MAPK by estrogen is likely to mediate regulation of transcription factors such as AP-1. Umayahara et al. (1994) reported that the AP-1 site is essential to mediate estrogen stimulation in the insulin-like growth factor-I promoter. Kushner's group reported that estrogens and tamoxifen stimulated transcription of promoters regulated by AP-1 sites including the human collagenase gene promoter (-73 to +63) and constructs in which an AP-1 site is fused to the herpes thymidine kinase promoter. Tamoxifen agonism at AP-1 sites is cell type specific, occurring in cell lines of uterine, but not of breast origin. It thus parallels tamoxifen agonism *in vivo*. All these data suggest that regulation of the AP-1 pathway by estrogen and antiestrogens could be a major route by which ER affects target tissue growth and differentiation *in vivo* (Webb et al., 1995).

Since antiestrogens have a well characterized agonistic effect in uterus tissues, we studied the regulation of the AP-1 pathway by estrogen and antiestogens in a well-differentiated human uterine endometrial adenocarcinoma cell line Ishikawa. In the literature, It was reported that Ishikawa cells express both ERα and ERβ (Bhat and

Pezzuto, 2001). These cells are estrogen-responsive, since at the transcriptional level, we observed the induction of natural estrogen-responsive genes such as PR by estradiol pretreatment. We used both transient transfection methods and stably transfected cell lines, which have been demonstrated to be closer to physiological conditions. The partial antiestrogen tamoxifen has been widely used for the treatment of breast cancer patients and for the prevention of breast cancer in women at high-risk. However, long-term tamoxifen treatment increases the risk of endometrial cancer, as introduced previously. Moreover, elucidation of the molecular mechanisms of tamoxifen tissue-specific agonistic activity could provide the basis for novel drug design. Based on the literature, we tested two potential mechanisms for the agonistic activity of partial antiestogens like tamoxifen. The first is whether an estrogen-response element is sufficient to mediate agonism of partial antiestrogens. The second potential mechanism is whether partial antiestrogens can regulate AP-1 activity to mediate their agonistic effects.

Our results indicate that the presence of EREs in promoters is sufficient to mediate cell-specific agonism of antiestrogens. Activation of AP-1 by estradiol was observed in a promoter context-dependent manner in transient transfection experiments of Ishikawa cells, but not in stable cell lines carrying the same reporter gene. We also performed cDNA arrays to identify the target genes of estradiol and partial antiestrogens. Consistently, our results of cDNA arrays show that the AP-1 family members c-jun and c-fos mRNA are not regulated by estrogen (data not shown). We also attempted to test whether estrogen can activate MAP kinase in Ishikawa cells, and we failed to detect activation of ERK by estrogen using phospho-

ERK antibody and the IP-kinase assay (Meloche et al., unpublished results). So we conclude that AP-1 is insensitive to estrogen in Ishikawa cells, and that activation of MAP kinase and AP-1 via estrogen may be cell type specific.

The mechanisms of the activation of MAPK by estrogen are still controversial. Previously, Migliaccio et al (1996) showed that estradiol triggers rapid and transient activation of erk-1 and erk-2 in MCF-7 cells, but not in Cos-7 cells which lack ER, while stimulation of erk-2 by estradiol can be observed in Cos-7 cells transfected with an ERα expression vector. These results indicate that ER is required to confer estradiol responsiveness of erk-2. Recently, Filardo et al (2000) provided evidence that estrogen-induced Erk-1/-2 activation occurs independently of known estrogen receptors, but requires the expression of the G protein-coupled receptor homolog, GPR30. They observed that 17β-estradiol activates Erk-1/-2 not only in MCF-7 cells (ERα+ and ERβ+), but also in SKBR3 breast cancer cells, which fail to express either receptor (ERα- and ERβ-). MDA-MB-231 breast cancer cells (ERα-, ERβ+) are GPR30 deficient and insensitive to Erk-1/-2 activation by 17β-estradiol. But overexpression of GPR30 protein resulted in conversion to an estrogen-responsive phenotype. More recently, Wade et al (2001) showed that nuclear ERs are necessary for estrogen's effects at the membrane. These studies suggest that the existance of several possible reasons for lack of responsiveness of AP-1 to estradiol in Ishikawa cells, such as the absence of GPR30.

Other connections between ER and AP-1 have been described. It has been proposed that there is a direct protein-protein interaction between ER and c-Jun, a component of the AP-1 complex, mediating induction of AP-1 transcriptional activity

through recruitment of ER-associated cofactors (Martínez-Lacaci and Dickson, 1996). It is unknown, however, whether other members of the Jun family can substitute for c-Jun for interaction with ER. Another explanation for AP-1 insensitivity to estrogen in Ishikawa cells could be due to expression of a different AP-1 family member rather than c-Jun.

In addition of AP-1 signlaing pathway, growth factor may also medite the effect of estrogen. It has been shown that TGFα mRNA and protein levels can be induced by physiological concentrations of 17β-estradiol. This induction can be blocked with antiestrogens, suggesting that this effect is mediated through the ER. In addition, it has been shown that EGF receptor mRNA and protein levels are modulated by estrogen and can be blocked by antiestrogens (Martínez-Lacaci and Dickson, 1996). Further, estrogen induces IGF-1 in the uterus and it is believed to be responsible for the uterotropic response observed in stromal and epithelial cells (Macgregor and Jordan, 1998). Recently, vascular endothelial growth factor (VEGF) has been found to be induced by estrogen in primary human endometrial epithelial cells, stromal cells and Ishikawa cells. This effect appears to require the ER DNA-binding domain and a variant ERE located at the VEGF promoter (Mueller et al., 2000). Collectively, these results indicate that estrogen can exert its mitogenic effects through multiple mechanisms.

2. AP-1 is sensitive to glucocorticoids in Hela, but not Ishikawa cells

As introduced previously, it is widely accepted the GR's transcriptional repressive effect of GR (this ability to directly inhibit a transcription factor activity, in the absence of a GRE was termed transrepression) on AP-1 and other transcription factors (such as NF-κB) underlies the anti-inflammatory and immunosuppressive activity of glucocorticoids. However, the molecular mechanisms of GR-dependent repression of AP-1 is still not determined, although intensive research has been conducted all over the world for more than ten years. Several hypotheses have been proposed and none of them can satisfactorily explain the exact molecular mechanism. Obviously, understanding GR transrepression mechanisms may lead to development of novel selective drugs. Our results (as shown in chapter 2, figure 8) indicate that AP-1 is repressed by glucocorticoids in Hela cells, but not in Ishikawa cells.

Since GR transrepression was shown to involve a physical interaction between GR and AP-1, we investigated whether the differential effect between Hela and Ishikawa cells is due to a different expression ratio of GR versus AP-1. Overexpression of GR in Ishikawa cells should thus restore GR transrepression. Our results show that overexpression of GR in Ishikawa cells cannot reestablish this repressive effect (chapter 2, figure 10).

In a recent review, De Bosscher et al (2000) proposed that activated GR might disrupt essential contacts between p65 (NF-κB is a heterodimer of p65 and p50) and factors of the basal transcription machinery, but the precise target was not identified. Very recently, Ito and colleagues found that glucocorticoids can repress histone acetylation by a combination of direct inhibition of p65-associated CBP HAT activity

and by recruiting HDAC2 (Ito et al., 2001). This hypothesis raises several questions: Can glucocorticoids affect histone acetylation when tethered to AP-1? If yes, is this mechanism cell-type specific? The cell-type specific responses we observed highlight the need to search for novel factors participating in this crosstalk.

3. Regulation of AP-1 by high concentrations of partial antiestrogens

We observed that AP-1 was activated by high concentrations (micromolar) of tamoxifen in stably transfected Ishikawa cells. In contrast, we did not observe AP-1 regulation by estrogen. We speculate that the mechanisms of regulation of AP-1 by estrogen and partial antiestrogens in Ishikawa cells are independent. It is well accepted that many effects of tamoxifen are elicited via estrogen receptor-independent routes. Search for a potential mechanism of AP-1 activation by tamoxifen has been focused on activation of PKC, PKA, and increase of cytosolic free Ca^{2+} levels by tamoxifen.

Protein kinase C is a family of serine/threonine protein kinases that is activated in the presence of phospholipid and Ca^{2+} ions. PKC is present in the cytosol of unstimulated cells in an inactive form. When diacylglycerol (DAG) or phorbol ester tumor promoters such as TPA bind to PKC, the enzyme becomes activated and translocates to the membrane. To date, at least 12 subspecies of PKC have been identified, and these are divided into three groups based on their structures: conventional (α-, βI-, βII-, and γ-PKC), novel (δ-, ε-, η-, and θ-PKC), and atypical PKC (ζ- and λ-PKC). Conventional PKCs have binding sites for DAG and phorbol ester (Shirai et al., 1998).

Lavie and collegues reported that tamoxifen induced PKC-epsilon translocation in MCF-7 human breast cancer cells, which was time-dependent (as early as 5 min post-treatment) and dose-dependent (5.0-20 microM). Tamoxifen did not influence translocation of alpha, beta, gamma, delta or zeta PKC isoforms. Structure-activity relationship studies demonstrated chemical requirements for PKC-epsilon translocation, compounds without the basic amino side chain or minus a phenyl group were not active. *In vitro* cell growth assays showed a correlation between agent-induced PKC-epsilon translocation and inhibition of cell growth (Lavie et al., 1998). When we attempted to investigate the mechanisms of AP-1 activation by high concentrations of tamoxifen in Ishikawa cells, we also tested whether tamoxifen can exert its effect via activation of PKC. Consistently, we did not observe α-, β-, and γ-PKC translocation induced by tamoxifen. Other commercial available PKC isozyme-specific antibodies we tested were not sensitive enough to detect a tamoxifen effect (data not shown). Future studies may adopt more sensitive techniques, such as recently reported subspecies of PKC-GFP fusion protein system (Shirai et al., 1998) which has been shown to be able to monitor the translocation of subspecies of PKC in living cells, rather than produce PKC isozyme-specific antibodies by ourselves.

Involvement of the protein kinase A system in response and resistance to tamoxifen treatment was also reported (Miller et al., 1997; Lee et al., 2000). In addition, Jan et al reported that tamoxifen evoked a rise in cytosolic free Ca^{2+} levels in a concentration dependent fasion between 1 and 50 microM with an EC50 of 10 microM in Madin Darby canine kidney (MDCK) cells. Tamoxifen (5 microM) also increased Ca^{2+} in neutrophils, bladder cancer cells, and prostate cancer cells from

humans and glioma cells from rats. The Ca^{2+} increase was accompanied by cytotoxicity (Jan et al., 2000).

Our results (Chapter 2, figure 7) show that AP-1 activity can be induced by high concentrations of tamoxifen, but not raloxifen (data not shown). Since the non-steroidal skeleton of tamoxifen and raloxifen are similar (refer to Chapter I, figure 2), this suggests that the non-steroidal skeleton of these drugs are not important to mediate tamoxifen activation of AP-1. RU39,411 has the same side chains as tamoxifen, however we did not detect activation of AP-1 by RU39,411 either, suggesting that the side chains of tamoxifen is not sufficient to mediate AP-1 activation. Taken together, these results suggest that the effect of tamoxifen on AP-1 is unique to tamoxifen. Further investigation of the potential mechanisms of the effect of tamoxifen on AP-1 may provide a novel drug target to facilitate rational drug design for novel antiestrogens.

II. Mechanisms of the regulation of glucocorticoid receptor activity by AP-1 activators

1. Conflicting literature reports

While glucocorticoids can repress AP-1 activity, the effect of activators of AP-1 on GR transcriptional activity are controversial (Rogatsky et al 1998; Moyer et al., 1993; Maroder et al., 1993; Vacca et al., 1989), as shown in table 1. Rogatsky et al demonstrated that selective activation of either ERK or JNK *in vivo* inhibits GR-

mediated transcriptional activation. Moyer et al observed that TPA cotreatment enhances GR transcriptional activity. To address this issue, we studied the effects of MAP kinase activators on endogenous GR transcriptional activity using stably transfected vectors. We believe that our cellular model system is more physiological than the transient transfection system used in several of the published studies for several reasons. (1) We use Hela and Ishikawa cells, which express endogenous physiological level of GR. (2) The reporter vector we used is composed of five GREs upstream of an adenoviral minimal promoter TATA box. Complex promoters such as MMTV contain other cis-regulatory elements which may mediate transcriptional effects. (3) Transient transfection itself exerts certain effects on cell signaling (chapter 3, figure 12). Our results indicate that MAPK activators can positively modulate GR transcriptional activity. To analyze the mechanisms of this transcriptional regulation, we proposed four potential mechanisms: 1) upregulation of GR expression by TPA. 2) upregulation of GR phosphorylation by TPA. 3) upregulation of GR coactivator phosphorylation by TPA. 4) Protein-protein interactions between GR and TPA induced Jun or Fos leading to enhanced GR transcriptional activity. The working model is shown in figure 1.

Table 1. Summary of the conflicting reports in the literature regarding mitogen-activated protein kinases' effect on glucocorticoid receptor transcriptional activity

Reports	Effect	Mechanism	Experimental procedures			
			Cell line	Reporter vector	GR species	Methods of transfection and activtion of MAPK
Rogatsky et al., 1998	Inhibition	GR S246(P) by JNK GR cofactor (P) by ERK	U-2 OS Hela	ΔGTCO-CAT (2 GREs)	Rat Human	Transient, Selectively activate ERK or JNK
Moyer et al., 1993	Enhancement	GR(P) excluded GR cofactor (P) ?	T47-D	MMTV-luc	Rat	Stable, TPA treatment
Maroder et al., 1993	Enhancement	GR(P) excluded Jun cooperate with GR	T-cell lines	MMTV-CAT ΔAP1-2×GRE-tk-CAT	Human	Transient, TPA treatment
Vacca et al., 1989	Inhibition	Activation of PKC	NIH-3T3	MMTV-CAT	Mouse	Transient, TPA treatment; Stably overexpress Ha-ras

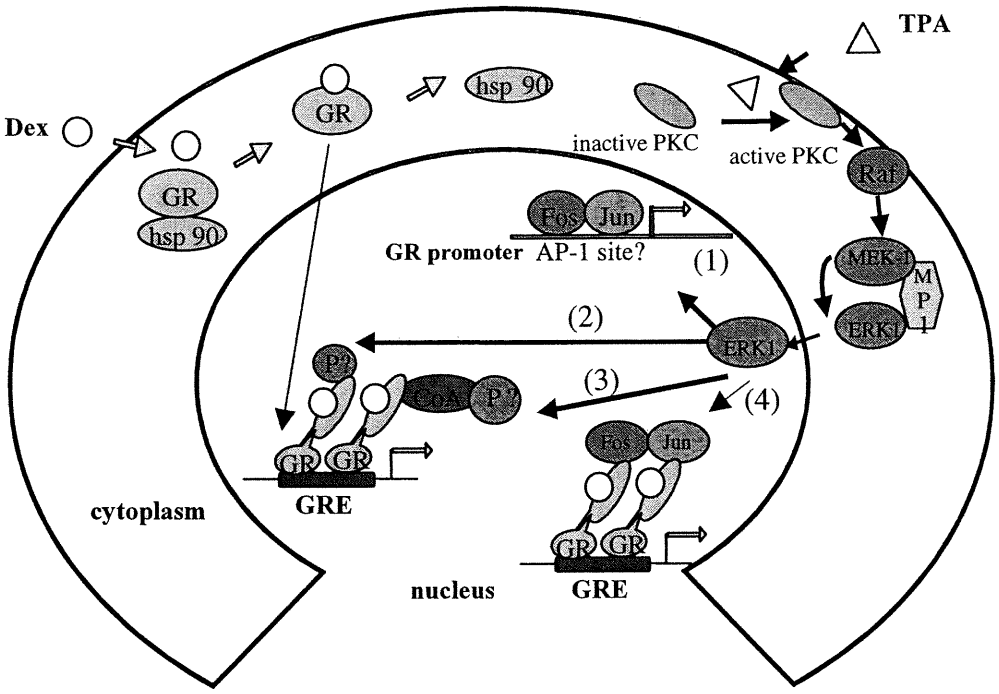


Figure 1. Working model to study the mechanism of TPA effect on GR transcription.

To analyze the stimulatory effect of TPA on GR transcription, we investigated four potential mechanisms: 1) upregulation of GR expression by TPA. 2) upregulation of GR phosphorylation by TPA. 3) upregulation of GR coactivator phosphorylation by TPA. 4) Protein-protein interactions between GR and TPA induced Jun or Fos leading to enhanced GR transcriptional activity.

2. Can MAP kinase activators regulate GR expression?

Characterization of the human glucocorticoid receptor promoter revealed a wide variety of prospective promoter elements (Zong et al., 1990). However, none of these elements have yet been shown to be functionally operative; all identifications were made using at least 75% identity to the consensus sequence for these elements. The human GR promoter contains a putative AP-1 site (TGACACA), which differs by one nucleotide (underlined) from the consensus AP-1 site (TGAC/GTCA). To test whether this putative AP-1 site is truly functional, we performed Northern blot analysis to monitor GR mRNA levels in response to TPA. Our results of Northern blot show that GR mRNA is not regulated by TPA. So we conclude that the AP-1 site in the human GR promoter is not functional, at least in Hela cells.

3. Do MAP kinase activators regulate phosphorylation of GR or GR coactivators?

(1) Analysis of human glucocorticoid receptor phosphorylation

The phosphorylation sites of human GR have not been characterized yet. What has been shown in figure 13 (chapter 1) is several potential phosphorylation sites, deduced from the mouse GR phosphorylation site. Krstic et al reported that mitogen-activated protein kinases (MAPKs) phosphorylate rat GR threonine 171 and serine 246, Serine 246 is conserved in humans, the corresponding site being serine 226. Our results indicate that the MAP kinase ERK2 can phosphorylate human GR Ser226 *in*

vitro. However, GR phosphorylation levels were not increased detectably *in vivo* by TPA stimulation, and human GR mutants S226E and S226A did not alter GRE-mediated transcriptional activity in COS-7 cells and in Jurkat cells. In addition, a GR τ1 deletion mutant can still mediate TPA effect on GR transcriptional activity, suggesting that phosphorylation of the τ1 region is not essential to mediate TPA effect. All these results indicate that TPA effect on GR transcriptional effect is not mediated through regulation of GR phosphorylation, especially Ser 226.

The divergence between our results of human GR phosphorylation and rat GR phosphorylation published by krstic et al, may be attributed to species differences between human and rat. Species-specific differences between human and rat glucocorticoid receptor signaling at the level of GSK-3 phosphorylation has been evidenced (Rogatsky et al., 1998).

(2) The role of glucocorticoid receptor coactivator phosphorylation

Coactivators play important roles in the regulation of nuclear receptor transcription. According to their function and mechanism, coactivators can be divided into two general classes: members of the SWI/SNF family and members of the histone acetyltransferase family. Both classes have the capacity to modify the chromatin environment facilitating transcription indirectly by alleviating the repressive effects of histone-DNA contacts. They can also potentially influence the activity of the basal transcriptional machinery directly through protein-protein contacts (Collingwood, Urnov, and Wolffe, 1999). To date only a few coactivators have been analyzed for GR in more detail. These include the p160 coactivators, the BRG-1 (SWI/SNF) complex,

the P/CAF (ADA/SAGA) complex, CBP/p300, and components of the DRIP/TRAP complex DRIP 150 and DRIP205.

a. p160 family coactivators

To assess whether MAP kinases can modulate GR transcription through regulation of coactivator phosphorylation, we first examined whether MAP kinase activators can modulate the phosphorylation of the p160 family coactivators. As described in the introduction (chapter 1), ERK phosphorylation sites have been identified in SRC-1, GRIP-1, AIB1 and CBP. These results suggest that coactivator phosphorylation may regulate steroid receptor transcriptional activity. Unfortunately, we did not detect modulation of TIF-2 phosphorylation levels in response to TPA *in vivo*. It is still possible that TPA-induced phosphorylation may not be detectable due to high levels of phosphorylation of TIF2. Further phosphopeptide maps may be more sensitive to detect regulation at a single phosphorylation site. However, overexpression of TIF-2 did not further enhance the effect of TPA on GR transcription, implying that TIF-2 is not the target of TPA that mediates GR transcriptional enhancement. In addition, our observation that the effect of TPA is specific to GR in Ishikawa cells, suggests that p160 family members, which are general nuclear receptor coactivators, are not mediating the GR-specific effects of TPA. We proposed that GR-specific coactivators might be the targets of MAP kinase activators.

b. BRG-1 and hbrm complex

Several lines of evidence support that chromatin remodeling factors, such as hbrm and BRG-1 (human homologues of yeast SWI/SNF), can potentiate the activity

of GR through the N-terminal τ1 in mammalian cells. Could hbrm or BRG-1 be a potential target of MAP kinase? Alignment and analysis of protein sequences of BRG-1 and hbrm indicate that there is a number of potential ERK MAP kinase phosphorylation sites at the N-terminal domain of BRG-1 and hbrm, and that these potential phosphorylation residues are highly conserved between BRG-1 and hbrm, as shown in figure 2. Additionally, one perfect ERK consensus sequences are identified in BRG-1, two perfect ERK consensus sequence is identified in hbrm.

Recently, Brown's group demonstrated that transcriptional activation by the estrogen receptor (ER) also requires functional BRG-1 and that the coactivation of estrogen signaling by either SRC-1 or CBP is BRG-1 dependent. In response to estrogen, ER recruits BRG-1, thereby targeting BRG-1 to the promoters of estrogen-responsive genes in a manner that occurs simultaneously to histone acetylation. These results indicate that BRG-1 is not specific to GR, and BRG-1 might not be responsible for the effects of MAP kinase activators on GR transcription in Ishikawa cells.

c. DRIP 150

Coactivators that associate specifically with GR remain largely undefined. DRIP 150 has been shown to specifically enhance GR AF-1-mediated transactivation (Hittelman et al.,1999). Analysis of the sequences of DRIP150 revealed two ERK MAP kinase consensus sequences: $PPS_{986}P$ and $PGS_{1112}P$. It will be of interest to test whether these two consensus sequences are indeed ERK phosphorylation sites *in vivo* and further to determine whether DRIP150 is the real target of MAP kinase activators that mediate TPA effect on GR transcription.

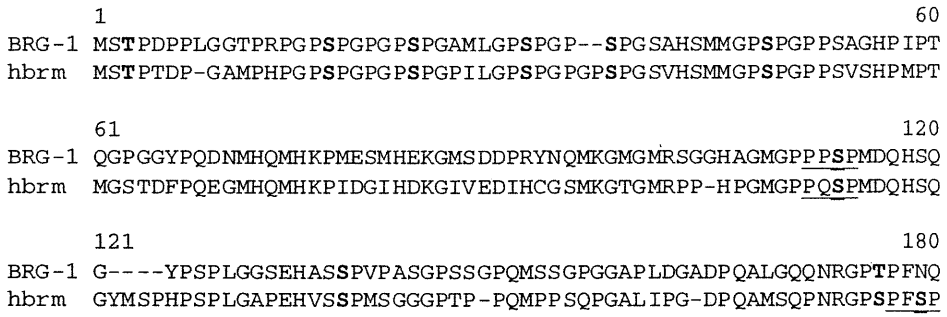


Figure 2. Analysis of potential ERK MAP kinase phosphorylation sites in two human homologs of SWI/SNF complex.

Sequences are from PubMed, alignment was done by CLUSTALW. Potential ERK phosphorylation sites are in bold character, perfect ERK consensus sequences are underlined (almost all potential phosphorylation sites are at N-terminal, BRG-1 is a protein of 1647 aa, hbrm is a protein of 1586 aa.).

4. The effects of AP-1 components on GR transcription

Maroder et al reported that enhancement of GR transcription by TPA is mediated through TPA-induced Jun proteins cooperating with GR (Maroder er al., 1993). To test this potential mechanism, we overexpressed c-Jun, JunB, JunD, and c-Fos in Hela cells. Our results show that overexpression of c-Jun, JunB, JunD, and c-Fos does not mimic the effects of TPA on GR transcription (Chapter III, figure 10).

5. Mapping of GR domains responsible for TPA effect

Furthermore, when we attempted to map the GR domains responsible to TPA effect, we found that the GR tau1 or AB region is not necessary to mediate the effect of TPA, as we could still obtain TPA stimulatory effect on GR transcription, whereas the GR ligand binding domain is required to mediate the effect of TPA, because the effect of TPA is not detectable with the GR ligand binding domain deletion mutants (Chapter III, figure 8). Most importantly, the GR DNA binding domain is crucial to mediate the effect of TPA (Chapter III, figure 9). This suggests that the DNA-binding domain of GR might be a direct or indirect target of TPA, or that the GR DNA-binding domain interacting proteins are the target of TPA.

Sequence analysis of GR DBD and ER DBD shows that one serine residue (Ser 425) is present in the first zinc finger of GR but not in that of ER. Thus a possible mechanism of the effect of TPA may be the phosphorylation of Ser 425 in the GR DNA binding domain in vivo, although it is unlikely that GR is the direct target of

MAP kinase, as the ser 425 surrounding sequences do not resemble to the MAP kinase consensus sequences.

In addition, it is possible that TPA may regulate the phosphorylation of GR-specific cofactors interacting with the DNA binding domain of GR, although such cofactors have not been characterized to date.

6. GR transrepression and GR transactivation are independent cellular events

Finally we found that the mechanism of repression of AP-1 activity by glucocorticoid receptor and the mechanism of upregulation of GR mediated transcription by activators of AP-1 are independent cellular events, as both events are observed in Hela cells (chapter 2, figure 9 and chapter 3, figure 1), but not in Ishikawa cells (chapter 2, figure 9 and chapter 3, figure 4).

III. Conclusions and contributions to knowledge

• To investigate the tissue-specific agonism of antiestrogens like tamoxifen, we compared transcriptional activation of reporter constructs containing classical estrogen response elements (EREs) as well as AP-1 response elements in the breast carcinoma cell line MCF7 and in the endometrial carcinoma cell line Ishikawa. Our results indicate that the presence of EREs in promoters is sufficient to mediate cell-specific agonism of antiestrogens. Activation of AP-1 by estradiol was observed in a promoter context-dependent manner in transient transfection experiments of Ishikawa cells, but not in stable cell lines carrying the same reporter gene. AP-1 was activated by high concentrations (micromolar) of TAM in stably transfected Ishikawa., suggesting that tamoxifen may elicit its pharmacological effects via other estrogen receptor-independent routes.

• Using stably transfected Hela cell lines that propagate AP-1 responsive reporter vectors, we have observed that physiological concentrations of glucocorticoids can repress AP-1 activity in Hela cells, but not in Ishikawa cells that propagate the same AP-1 reporter vector. Our results also show that overexpression of GR in Ishikawa cells can not establish this repressive effect. This cell-type specific responses we observed highlight the need to search for tissue-specific factors participating in this crosstalk.

To correlate the mechanisms of GR transrepression on AP-1 and enhancement of GR transactivation by AP-1 activators like TPA, we think that the mechanisms of GR transrepression and the mechanisms of GR transactivation by AP-1 activators are independent, as both cellular events are observed in Hela cells, but not in Ishikawa cells.

• Contrary to the reported inhibition of GR transcriptional activity in Hela cells by activation of the JNK and the ERK pathways, we have observed a strong potentiation of GR dependent transcription by activation of the MAP/SAP kinase pathway. We have investigated the effect of MAP kinase inducers TPA and EGF on GR-dependent transcription in HeLa cells and in Ishikawa cells, two epithelial cell lines that express glucocorticoid receptors. This effect was more marked when using stably integrated or episomal vectors than with transiently transfected reporter vectors, possibly because transient transfection of culture cells itself stimulates MAP kinase signaling. In addition, our results indicate that GR expression is not regulated by TPA *in vivo*. Although phosphorylation of the GR AB region by ERK could be observed *in vitro*, overall phosphorylation levels of GR were not altered by TPA treatment *in vivo*. Furthermore, point mutants of GR phosphorylation sites and deletion of the τ1 or AB region did not abolish the effect of TPA.

Finally, while the integrity of the ligand binding domain was necessary to observe enhancement by TPA, characterization of chimeric ER-GR proteins indicates that the GR DNA binding domain cannot be replaced by that of ER without losing

TPA effect. These results suggest that the effect of TPA on GR-dependent transcription may be mediated by cofactors of GR that interact with the DNA binding domain, or that the DNA binding domain may allosterically regulate the activity of GR cofactors recruited via the ligand binding domain.

Our results confirm that there is crosstalk between nuclear receptors and AP-1 at different levels, and indicate that these effects are cell-specific. Our work and that of others shows that control of gene expression by steroid hormones is far more complex than was apparent at the time when the genes for steroid hormone receptors were isolated, contributing to the pleiotropic effects of steroid hormones in physiological and pathological processes. Molecular mechanisms and levels of crosstalk between these two pathways still remains an area of exciting future investigation.

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APPENDIX

Specific activation of MAP/SAP kinase signaling and AP1-dependent transcription by Cremophor EL and ricinoleic acid.

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Running title: Modulation of MAP/SAP kinases by Cremophor EL and ricinoleic acid

Cremophor EL is used clinically as a solvent for hydrophobic drugs, but can trigger hypersensitivity reactions and cytotoxicity. Here we report that Cremophor EL can induce apoptosis and activate intracellular signaling pathways leading to Ras activation, MAP/SAP kinase phosphorylation and increased AP1-dependent transcription. These effects could be reproduced using ricinoleic acid, the major component of castor oil, from which Cremophor EL is derived. Ricinoleic acid was a stronger inducer of MAP/SAP kinase phosphorylation and AP1 activity than arachidonic acid and linoleic acid, but unlike arachidonic acid did not activate protein kinase C. Our results demonstrate that a hydroxylated mono-unsaturated fatty acid can act as a signaling agent of MAP/SAP kinase pathways. These studies expand the repertoire of fatty acids modulating intracellular signaling and show that different fatty acids can control activity of MAP/SAP kinases by distinct mechanisms.

Cremophor EL is a castor oil derivative with pharmacological applications as an excipient for hydrophobic drugs such as Taxol (paclitaxel), cyclosporin A, miconazole, teniposide, diazepam and some vitamin preparations. In early clinical trials, paclitaxel administration was associated with hypersensitivity reactions, varying from dyspnea, rashes, and bronchospasms to severe hypotension (52). These effects are now attributed to Cremophor EL (41), which also induces histamine release and hypotension in dogs within 10 min of administration (31), and activates complement *in vitro* (46). Angioedema, rhinitis, asthma and rashes have also been reported after oral administration of castor oil, and contact dermatitis from castor oil in lipsticks and creams occurs frequently (47). Nephrotoxicity has also been associated with Cremophor EL administration (48). *In vitro* experiments have indicated that plasma levels of Cremophor EL achieved during paclitaxel therapy inhibit the activity of the P-glycoprotein in multidrug resistant human T-cell leukemia cells cellular models, suggesting that Cremophor EL may be contributing to the activity of anti-cancer drugs (6, 51). Finally, Cremophor EL has cytostatic and/or cytotoxic effects through unknown mechanisms (6, 8, 39), but was also reported to be associated with an increase in the incidence of spontaneous multiple myeloma in long-term immunosuppressed aging mice (37).

Here, we have investigated the effect of Cremophor EL administration on cell survival and on the transcriptional activity of the AP1 complex, which is involved in the immediate early cellular response to signaling molecules and stresses. We conclude that Cremophor EL can induce cellular death and AP1 activity. In addition, immediate effects of Cremophor EL administration are activation of Ras and induction of MAP kinase phosphorylation. We have also investigated the effect of oleic, linoleic and ricinoleic acids, the main unsaturated fatty acid constituents of Cremophor EL, on the same parameters. Polyunsaturated fatty acids such as linoleic acid and arachidonic acid have been previously shown to modulate MAP/SAP kinase function (3, 19, 20, 21, 22, 38), but the effect of the hydroxylated mono-unsaturated fatty acid ricinoleic acid on these signaling pathways had not been investigated. Interestingly, we find that ricinoleic acid mimics Cremophor EL effects and is more efficient than linoleic acid in all these assays, suggesting that the hydroxyl group on carbon 12 contributes more to activity as a signaling molecule than the double

bond present at the same position in linoleic acid. Our results also show that different types of fatty acids can modulate intracellular signal transduction by distinct mechanisms, since we observe that contrary to linoleic acid, ricinoleic acid does not activate the classical isoforms of protein kinase C.

MATERIALS AND METHODS

Materials. Castor oil, Cremophor EL, 12-O-tetradecanoylphorbol 13-acetate (TPA), D-sorbitol, oleic acid, linoleic acid, ricinoleic acid, HRP-conjugate anti-mouse and anti-rabbit IgG were purchased from Sigma Aldrich. MAP kinase inhibitors PD 98059 and SB 203580 as well as the Pan-Ras antibody were from Calbiochem. TNF-α and the PKC inhibitor GF 109203X were purchased from Boehringer Mannheim. EGF was from Upstate Biotechnology. Cell culture media, fetal bovine serum (FBS), geneticin (G418 sulfate) were purchased from Life Technologies.

Cell culture. HepG2 cells were routinely maintained in α-minimal essential medium (αMEM) supplemented with 10% fetal bovine serum (FBS), sodium pyruvate (1%), glutamine (1%) and penicillin-streptomycin (1%). HeLa cells were maintained in Dulbecco's modified essential medium (DMEM) supplemented with FBS (5%), sodium pyruvate (1%), glutamine (1%) and penicillin-streptomycin (1%). The stably transfected clones HeLa/TRE6-CAT#5 and #14 (4) were maintained in the same medium supplemented with G418 (0.3 mg/ml). To assay for AP1-dependent transcription, HeLa-TRE6-CAT cells (2.5 million per 10 cm plate) were incubated at 37°C in the presence of indicated concentrations of drugs (see figure legends). Concentrations of Cremophor EL used (0.7, 1.4, 2.8, or 4.2 µl/ml) correspond to those found in dilutions of taxol from a 7.03 10^{-3}M stock solution in 1:1 V/V Cremophor EL/ethanol to final concentrations of 5, 10, 20 and 30 µM, respectively, in cell culture media. Cells were harvested after indicated periods of time and assayed for CAT activity as previously described (4)

Cytotoxicity and apoptosis assays. Exponentially growing cells were seeded in 96-well plates (10^3 cells/well) and treated 16 h later with varying concentrations of Cremophor EL or fatty acids. After 72 h, cell culture media were replaced by fresh media containing 10 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid buffer, pH 7.4, and supplemented with 50 µl of 2.5 mg/ml solution of 3-(4,5-dimethylthiazo-2-yl-2,5-diphenyltetrazolium bromide (MTT) in PBS, pH 7.4. After 3 h, the precipitate of reduced MTT was dissolved in 200 µl DMSO, followed by addition of 25 µl glycine buffer (glycine 0.1M, NaCl 0.1M, pH 10.5). Absorbance at 570 nm was

determined (microplate reader BIORAD, model 450). The IC50 was calculated as the concentration of drug causing a 50% reduction in absorbance compared to control cells.

For apoptosis assays, cells were seeded in T75 cm^2 plates (1 million/plate), and treated with Cremophor EL or fatty acids for 72 h. Cells were then collected by scraping, washed twice in PBS and distributed in 96 well-plates (1 million cells/well). Fixation was performed in ethanol 70% at 4°C for 30 min with continuous shaking. After washing with PBS, cells were permeabilized with a 1% solution of TritonX-100 in 0.1% sodium citrate on ice for 2 min. Cells were then washed twice in PBS and labeled with 50 µl/well TUNEL reaction mix (*In situ* death detection kit, Boehringer Mannheim) at 37°C in the dark for 1 h. Cells were then washed 3 times with 1% BSA in PBS and resuspended in 500 µl PBS for analysis by flow cytometry.

Reverse-transcription PCR. Total RNAs were prepared using the RNAgent Total RNA Isolation System from HeLa cells after incubation with TPA (100 ng/ml), Cremophor EL (14.2 µl/ml), ricinoleic acid (200 µM) or vehicle (ethanol) for 6 h, and reverse-transcribed using Moloney Murine Leukemia Virus reverse transcriptase (MMLV RT, Pharmacia Biotech) and a random hexamer following conditions specified by the manufacturer. Aliquots of the resulting cDNAs were amplified by PCR using VentR DNA polymerase (New England Biolabs) and collagenase or β-actin primers:

collagenase forward: 5'-CTGTTCAGGGACAGAATGTGCT-3'

collagenase reverse: 5'-TTGGACTCACACCATGTGTT-3'

β -actin forward: 5'-CGGAATTCGCTGTGCTATCCCTGTACGC-3'

β-actin reverse: 5'-CGGGATCCGCCAATGGTGATGACCTGGC-3'

40 cycles (94°C, 1 min; 62°C, 1 min; 72°C, 1 min) and 25 cycles (94°C, 1 min; 63°C, 1 min; 72°C, 1 min) were performed for amplification of collagenase and β-actin cDNAs, respectively, followed by a final elongation step (72°C, 15 min). The amplified products were resolved on a 1.8% agarose gel. Identity of the amplified fragments was confirmed by sequencing.

Western blot analyses. For monitoring of MAP/SAP kinase phosphorylation levels, cells ($7x10^6$ per 10 cm plate) were washed in PBS and incubated in DMEM without FBS for 20 h. After

treatment, cells were lysed in sample buffer (Tris-HCl pH 6.8, 62.5 mM, SDS 2%, glycerol 10%, DTT 50 mM, bromophenol blue 0.1%, NaF 50 mM and Na_3VO_4 1 mM). Cell lysates were sonicated for 15 s, boiled for 5 min and centrifuged prior to loading on a 10% polyacrylamide-SDS gel. After electrotransfer, nitrocellulose membranes were blocked in TBST (TBS containing 0.1% Tween-20) and 5% milk and incubated overnight with anti-p44/p42, anti-Phospho-p44/p42, anti-JNK, anti-Phospho-JNK, anti-p38, or anti-Phospho-p38 (New-England BioLabs). Secondary antibodies were horseradish peroxidase-conjugated anti-rabbit or anti-mouse IgG, as appropriate. Bands were revealed using an ECL kit (NEN Life Science Products). For p21-ras detection, protein extracts were loaded onto a 12.5% polyacrylamide-SDS gel after column purification. Nitrocellulose blots were blocked in TBST-5% milk, and incubated sequentially with the Pan-Ras antibody (Calbiochem) and with horseradish peroxidase-conjugated anti-mouse IgG(Sigma). The classical isoforms of protein kinase C were detected using the Pan-PKC antibody (Upstate Biotechnology).

PKC activity assays. For translocation assays, cytosolic and membrane fractions were prepared from cells after treatment with Cremophor EL or with different fatty acids as follows. Cells ($4x10^6$ cells per 10 cm plate) were washed in ice cold PBS, harvested in translocation buffer (TrisHCl pH7.5 20 mM, EDTA 2mM, EGTA 0.5 mM, sucrose 0.33M, PMSF 0.2 mM, leupeptine 10µg/ml, aprotinine 10 µg/ml), sonicated for 15 s and centrifuged (4°C, 45 min, 15000g). Supernatants (cytosolic fraction) were collected and frozen in dry ice, and pellets were resuspended in translocation buffer supplemented with triton X-100 (0.1%) and incubated overnight at 4°C with constant shaking. Debris were eliminated by centrifugation (4°C, 45 min, 15000g), yielding the particulate fraction.

For protein kinase C activity assays, cells maintained in DMEM without serum for 20 h and treated with Cremophor EL or ricinoleic acid for 10 min were washed in ice cold PBS, harvested in extraction buffer (TrisHCl pH7.4 25 mM, EDTA 0.5mM, EGTA 0.5 mM, β-mercaptoethanol 10 mM, PMSF 0.2 mM, leupeptine 10µg/ml, aprotinine 10 µg/ml), homogenized with a Dounce homogenizer and centrifuged (4°C, 15 min, 14000g). Membrane pellets were washed in extraction

buffer and homogenized in extraction buffer supplemented with Triton X-100 (0.05%). After incubation at 4°C for 1 h with rotation followed by centrifugation to remove insoluble debris, protein concentration was estimated by Bradford assay and Protein kinase C activity was determined using SigmaTECT Protein Kinase C assay (Sigma Aldrich).

Purification of GST-RBD. Expression of the ras binding domain (RBD) of raf fused to GST was induced in E coli cells transformed with the pGST-RBD expression vector (obtained from Dr F. McKenzie, State University of New York) by induction with Isopropylthio-β-D-galactoside (IPTG, 0.5 mM) for 2h30 at 37°C. Cells were pelleted, resuspended in lysis buffer (TrisHCl pH7.4 20 mM, NaCl 100mM, EDTA 1 mM, DTT 1mM and protease inhibitors) and lysed by sonication. The cleared bacterial lysate was incubated with glutathione-sepharose 4B beads for 1 h at 4°C. After three washes with NENT buffer (TrisHCl pH7.4 20 mM, EDTA 1 mM, NaCl 100 mM, NP-40 0.5%), GST-RBD was eluted twice with elution buffer (TrisHCl pH7.4 100 mM, NaCl 120 mM) and protein determination was performed by Bradford assay. Purity (over 90%) was determined by SDS-PAGE analysis of protein extracts.

Ras activity assays. For determination of ras activity, cells were grown to confluence in 15 cm plates in Dulbecco's modified Eagle Medium containing 5% FBS, and switched to DMEM without FBS for 24 h. After treatment with EGF, Cremophor EL or ricinoleic acid for 1-15 min, cells were washed with ice cold PBS and harvested in lysis buffer (Tris-HCl pH 7.5, 25 mM, EGTA 5mM, NaCl 15 mM, MgCl2 5mM, TritonX-100 1%, N-Octyl-glucoside 1% and protease inhibitors). After agitation for 10 min at 4°C, cell extracts were centrifuged and protein concentration was determined by Bradford assays. Protein extracts (1.5 mg) were incubated at 4°C for 2h with gluthatione-sepharose beads preloaded with GST-RBD fusion protein (30μg). The beads were then washed three times with extraction buffer and column-associated proteins were analyzed by western blot assay using the Pan-Ras antibody (see above).

RESULTS

Cremophor EL and ricinoleic acid induce apoptotic cell death of HeLa cells. Preliminary experiments indicated that Cremophor EL (CreEL) was cytotoxic to HeLa cells in culture (not shown). In order to investigate the effect of CreEL on cellular growth, MTT conversion was measured after 72h of incubation of HeLa cells with increasing concentrations of solvent. Inhibition of cell growth was observed in a dose-dependent manner, with an IC50 (the concentration required to inhibit cell growth by 50%) of 1µl/ml (Fig. 1A). To determine whether growth inhibition resulted from induction of apoptotic death, TUNEL assays were performed. Whereas less than 0.3% cells were apoptotic in the absence of treatment, 5.8% and 88.3% cells were apoptotic after incubation for 72h with 0.5 and 1µl/ml CreEL, respectively (Fig. 2B).

CreEL is obtained by polyethoxylation of castor oil, whose main components are triglycerides containing ricinoleic, linoleic and oleic acids (Fig. 1C). We compared the capacity of the three fatty acids to induce cellular death. In MTT assays, ricinoleic acid inhibited cell growth with an IC50 of approximately 210 µM (Fig 1B). Inhibition was also observed after incubation with linoleic acid albeit at higher concentrations (IC50 360 µM), and more than 50% cells survived at the highest concentration of oleic acid used (500 µM). Ricinoleic acid like CreEL caused cellular death via induction of apoptosis: 37.5 and 72.5% cells were apopototic after incubation with 150 and 300µM ricinoleic acid for 72h, respectively (Fig. 2A). Consistent with results of the survival assay, linoleic acid (data not shown) and oleic acid (Fig. 2C) were less efficient at inducing apoptosis than ricinoleic acid.

Cremophor EL and ricinoleic acid induce AP1 transcriptional activity in HeLa and Ishikawa cells. Activation of the AP1 transcription factor is often associated with induction of apoptosis (25, 27). To investigate whether CreEL can modulate AP1 activity, we used two different recombinant cell lines (HeLA/TRE6-CAT clones 5 and 14) derived from HeLa cells by chromosomal integration of an AP1-sensitive reporter vector. When cells were incubated with CreEL (2.8 µl/ml) for 20 h, CAT expression was increased alone by 3.5- to 5-fold in clones 14

and 5, respectively, compared to control cells treated with ethanol (Fig. 3A, compare lanes 3 to 1 and 6 to 4). Stimulation of these cells with TPA (10 ng/ml) yielded comparable levels of stimulation in both cases (Fig. 3A, lanes 2 and 5). Stimulation of CAT activity by CreEL was dose-dependent. Whereas control levels of activity were observed at 0.7 µl/ml (Fig. 3B, lane 4), levels of CAT activity were comparable to those induced by EGF at 1.4 µl/ml (3-fold stimulation, Fig. 3B compare lane 5 to lane 3) and reached more than 10-fold stimulation at 4.2 µl/ml (Fig. 3B, lane 7). Similarly, CreEL induced a dose-dependent increase in CAT activity in epithelial uterine Ishikawa cells stably propagating the TRE6-TATA-CAT reporter vector (Fig. 3C, compare lanes 3-6 to lane 1), demonstrating that this effect is not restricted to HeLa cells. Finally, we also assessed the effect of CreEL on transcription of the collagenase gene, which contains a TRE motif in its promoter sequences. CreEL induced transcription of the collagenase gene, albeit to a lower extent (6.5-fold, Fig. 3D lane 3) than TPA (~18-fold, Fig. 3D lane 2). In conclusion, CreEL activates AP1 transcriptional activity both on synthetic and natural promoters.

To assess the effects of oleic acid, linoleic acid or ricinoleic acid on AP1 activity, CAT activity was quantitated from extracts of HeLa/TRE6-CAT cells treated with increasing concentrations of these unsaturated fatty acids. No significant induction of CAT activity was observed with concentrations of oleic (OA) or linoleic acid (LA) as high as 250 µM (Fig. 3E, compare lanes 3 and 4 to lane 1). However, increasing stimulation of CAT expression was observed with concentrations of ricinoleic acid (RiA) ranging from 100 to 250 µM. Induction of CAT activity in the presence of 200 µM ricinoleic acid was comparable (9-fold) to that obtained with the optimal concentration (10 ng/ml) of TPA (Fig. 3E lanes 2 and 7). In addition, ricinoleic acid (200 µM) and TPA (100 ng/ml) induced expression of the collagenase gene to similar levels (Fig. 3D, lanes 2 and 4). Therefore, ricinoleic acid mimics the effects of Cremophor EL on induction both of AP1 activity and of apoptotic death.

Cremophor EL and ricinoleic acid activate MAP/SAP kinases ERK and p38 in HeLa and HepG2 cells. MAP/SAP kinases are activated both by mitogenic and apoptotic

signals (27), and are involved in the transmission of numerous types of signals from the membrane to the nucleus. Upon activation by phosphorylation, kinases belonging to the ERK, JNK and p38 families translocate to the nucleus and phosphorylate numerous transcription factors including AP1. Here, we examined whether CreEL modulates MAP/SAP kinase phosphorylation. Phosphorylation of MAP kinases ERK and p38, but not JNK could be detected 7.5 min after incubation of HeLa cells with CreEL (Fig. 4A, compare lane 6 to lane 5). Phosphorylation levels of ERK family kinases at 15 min were comparable to those observed with 0.4 M sorbitol, 50 ng/ml EGF or 100 ng/ml TPA (Fig. 4A, compare lane 7 to lanes 1, 3 and 4) and returned to basal levels at 30 min (Fig. 4A, lane 8). Induction of p38 phosphorylation was more prolonged and yielded levels of phospho-p38 comparable to those obtained using 10 ng/ml TNF-α or 100 ng/ml TPA (Fig. 4A, compare lanes 6-8 to lanes 2 and 4). No variations were observed at any time point in the levels of phospho-JNK (Fig. 4A, bottom panel) or in the levels of unphosphorylated ERK or p38 kinases (Fig. 4A, second and fourth panels). Similar results were obtained using hepatocarcinoma HepG2 cells, although weak induction of JNK phosphorylation was detected in these cells (not shown, but see also Fig.4E lanes 5-7). Induction of ERK phosphorylation was inhibited by preincubation with the MEK1 inhibitor PD98059 (Fig. 4B, compare lanes 4 to 2). Similarly, no phosphorylation of p38 could be observed if cells were pretreated with the selective p38 inhibitor SB203580 (Fig. 4B, compare lanes 8 to 6).

We then tested whether oleic, linoleic or ricinoleic acid induced phosphorylation of MAP/SAP kinases. As shown in Fig.4C, ricinoleic acid (400 μM) also strongly stimulated phosphorylation of MAP kinases of the ERK and p38, but not JNK families (lane 7). Levels of phosphorylated MAP kinases were much weaker when using linoleic acid (Fig.4C lane 5) and undetectable using the same concentration of oleic acid (Fig.4C lane 4). Stimulation of ERK and p38 kinase phosphorylation by ricinoleic acid could be detected at concentrations as low as 25 μM (Fig. 4D, compare lane 4 to lane1). Stimulation of phosphorylation of ERK, p38 and to a lower degree JNK MAP kinases by ricinoleic acid (200 μM) was also observed in HepG2 cells (Fig. 4E lane 10). Note that in both cell lines, ricinoleic acid was more active than the C20:4 fatty acid arachidonic

acid (AA), which has a well characterized role as second messenger in animal cells following stimulation by a variety of signaling molecules (Fig.4E lane 11 and data not shown).

Cremophor EL and ricinoleic acid activate the small G protein Ras, but not protein kinase C in HeLa cells. Recruitment of protein kinase C (PKC) to the membrane is an early response to a variety of signaling molecules including some activators of MAP/SAP kinases such as TPA (23). Indeed, global PKC activity was induced 4.75-fold in HeLa cells after a 10 min incubation with TPA (Fig. 5A, lane 2). On the other hand, neither Cremophor EL (4.2 μl/ml) or ricinoleic acid (400 μM) induced PKC activity (Fig. 5A, lanes 3-4). However, this assay does not discriminate between the several isoforms of protein kinase C. HeLa cells have been reported to express PKC α, β and ε (36). Western analysis of cytosolic and particulate fractions of HeLa cells using an antibody that recognizes PKC isoforms α, β, γ demonstrated recruitment to the membrane of different PKC isoforms after treatment with TPA (Fig. 6B lanes 2, 7) or with arachidonic (Fig. 5B lanes 5, 10) or linoleic acid (Fig. 5B lanes 3, 8). However, neither Cremophor EL (data not shown) nor ricinoleic acid (Fig. 5B lanes 4-5 and 6-7) induced translocation of the classical PKC isoforms. Further, preincubation of HeLa cells with GF109203X (GF), a highly selective inhibitor of PKCα, βI, βII, γ, δ, and ε isozymes, did not diminish levels of phospho-ERK and phospho-p38 induced by ricinoleic acid (Fig. 5C, compare lanes 8 and 9). On the other hand, stimulation by TPA was drastically reduced (Fig. 5C, compare lanes 4 and 5). Similarly, stimulation of MAP/SAP kinase phosphorylation by Cremophor EL was not affected by GF109203X (Fig. 5C, compare lanes 6 and 7). Together, these results suggest that ricinoleic acid does not activate classical PKC isoforms in HeLa cells, and that other pathways are involved in MAP/SAP kinase activation by this fatty acid.

The small G protein Ras is activated by numerous signaling molecules that interact with membrane receptors and/or kinases, and activates in turn the first components of the MAP/SAP kinase cascade, the MAP kinase kinase kinases (MAP3Ks). Incubation of HeLa cells with Cremophor EL (not shown) or with ricinoleic acid (50 and 200 μM) led to a marked increase in

intracellular concentrations of activated Ras (Fig. 6A, lanes 3 and 4). The kinetics of Ras activation by EGF (50 ng/ml) or ricinoleic acid (200 μM) were similar, with peaks of activity around 2.5 min (Fig. 6B). Therefore, activation of Ras is an immediate response to ricinoleic acid stimulation.

DISCUSSION

Cytotoxicity of Cremophor EL, an excipient for hydrophobic drugs such as taxol and cyclosporin A, has been previously reported (6, 13) albeit mechanisms were not characterized. Observations described in this paper demonstrate that Cremophor EL can induce cellular death by apoptosis at concentrations achieved following clinical administration of paclitaxel in a 1:1 (V/V) ethanol/Cremophor EL formulation (41, 51). Indeed, patients treated with a dose level of 175 mg/m^2 paclitaxel achieved plasma concentrations of Cremophor EL of 1 μl/ml or more during 9-10 hours (40). We also demonstrate that Cremophor EL stimulates Ras activity, MAP/SAP kinase phosphorylation, and AP1-mediated transcription. Activation of Ras and MAP/SAP kinase phosphorylation is extremely rapid and may represent the inductive signal for apoptosis. Stimulation of AP1 transcriptional activity is more delayed and may either induce apoptosis or coincide with its induction. Activation of MAP/SAP kinase and of AP1 activity has been associated with induction of cell proliferation and differentiation as well as with apoptosis (25, 27). Therefore, activation of these pathways by Cremophor EL might trigger apoptosis or modulate proliferation and/or differentiation depending on the cellular context. Activation of MAP/SAP kinase pathways by Cremophor EL is also a potential mechanism for the previously reported hypersensitivity reactions to Cremophor EL (10, 52) and to ricinoleic acid in castor oil (47). We can further speculate that this signaling pathway may contribute to the 6- to 10-fold increase in the incidence of spontaneous multiple myeloma observed in immunosuppressed aging mice treated with Cremophor EL (37). Interestingly, taxol was reported to induce activation of Ras, JNK, and AP1 activity in a variety of cell lines in the absence of Cremophor EL (1, 30, 50), raising the possibility that Cremophor EL and taxol may have combined effects on MAP/SAP kinase signaling. It has been previously reported that Cremophor EL synergized with taxol to induce cytotoxicity of drug-resistant breast tumor cells (13). This observation could be explained by modulation of intracellular signaling in the presence of Cremophor EL as well as by inhibition of glycoprotein P (6, 51).

All effects observed with Cremophor EL could be mimicked by the hydroxylated unsaturated C18 fatty acid ricinoleic acid, the major fatty acid in castor oil. This suggests that ricinoleic acid is mainly responsible for the observed effects of Cremophor EL. It should be noted however that ricinoleic acid is present in triglycerides within castor oil, and is modified at both the carboxyl and hydroxyl locations by polyoxyethylene polymerization during preparation of Cremophor EL. It is difficult to evaluate how the different modifications in the resulting polydisperse products may affect the signaling properties of ricinoleic acid or those of the other fatty acid components of Cremophor EL. Finally, we cannot exclude contribution from other less abundant components of Cremophor EL.

Linoleic and arachidonic acid are the main fatty acid component of animal cell membranes and play an important role as second messenger released from membrane phospholipids by the action of cytosolic phospholipase A2 (2, 9, 24). They can also act as exogenous signaling molecules when released in the circulation either from adipocytes or from dietary sources, either by free diffusion through the plasma membrane or by interaction with membrane or soluble receptors (14, 15, 28). Arachidonic acid and its metabolites are critical to a variety of biological processes such as cell spreading (7), chemotaxis (45), and inflammation (16). It is also thought that induction of cellular growth by some peptide growth factors such as epidermal growth factor and fibroblast growth factor requires arachidonic acid release and metabolism (11, 17, 42). In addition, arachidonic acid and linoleic acid or their metabolites have been found to stimulate cell growth, differentiation or apoptosis in a variety of cell types (12, 18, 34, 35, 44). Activation of ERK, JNK or p38 activities by exogenously-added arachidonic acid has been reported in several cell lines (3, 19, 20, 38). These effects are cell-specific, as JNK activity is stimulated in Jurkat cells, but not in neutrophils, whereas the converse was observed for p38 phosphorylation (20), while all three MAP/SAP kinases were induced in monocytes (3). Linoleic acid was shown to activate ERK in smooth muscle cells (21) and JNK in mesangial cells (22). We also observed a cellular specificity in the induction of MAP/SAP kinases by ricinoleic acid, since phosphorylation of JNK was detected in HepG2 but not in HeLa cells. Interestingly, ricinoleic acid was a stronger inducer of

MAP/SAP kinase phosphorylation, AP1 activation and apoptotic death than linoleic or arachidonic acid. On the other hand, ricinoleic acid did not detectably induce translocation of the classical isoforms of protein kinase C, while arachidonic acid and linoleic acid both stimulated translocation of protein kinase Cβ. These results suggest that the higher levels of activity observed with ricinoleic acid in the other assays (MAP/SAP kinase phosphorylation, AP1 activity, apoptotic death) do not simply result from increased solubility of this hydroxylated fatty compared to arachidonic and linoleic acids. Induction of protein kinase C observed here by the latter fatty acids is consistent with previous observations (32, 33). Specific isozymes of protein kinase C are translocated to the cellular membrane in a cell-specific manner in response to arachidonic acid (23). For instance, incubation of rat liver epithelial WB cells with arachidonic acid resulted in the translocation of PKC α, δ, and ε but not ζ to a particulate fraction (19). Arachidonic acid induced translocation of PKC α, βI and βII but not δ or ζ in human neutrophils (20), and of PKC β, δ,ζ and λ in monocytes (3). Activation of ERK by arachidonic acid was inhibited by pretreatment with GF109203X, an inhibitor of classical PKC isozymes (49), whereas activation of p38 or JNK was only attenuated or unaffected by this inhibitor (3, 20). On the other hand, consistent with the absence of translocation of classical PKC isoforms consecutive to treatment with ricinoleic acid, the PKC inhibitor GF109203X did not affect stimulation of either ERK or p38 by ricinoleic acid. Note that stimulation by TPA was completely inhibited under the same conditions. These results suggest that activation of MAP/SAP kinases by ricinoleic acid is mediated by other pathways. Accordingly, we observed that an immediate response to ricinoleic acid is Ras activation. This effect is similar to a previously reported activation of p21ras by arachidonic acid and linoleic acid in renal proximal tubular cells (26), a possible mechanism being inhibition of the catalytic activity of Ras GTPase-activating proteins (43).

Accumulating evidence suggests that arachidonic acid may not be itself the active species in the activation of PKC or MAP/SAP kinases, but that metabolism to eicosanoids by 15-lipoxygenase is required. Indeed, nordihydroguaiaretic acid (NDGA), a potent inhibitor of the lipoxygenase system, blocked the arachidonic acid-induced activation of ERK in vascular smooth muscle cells

(38). In addition, inhibition of lipoxygenase blocked AA-initiated F-actin formation and cell spreading (7). However, induction of p38 by arachidonic acid was not blocked by NDGA in neutrophils (20). Contrary to the polyunsaturated fatty acids arachidonic acid and linoleic acid, ricinoleic acid may not be a substrate for lipoxygenases. Lipoxygenation involves abstraction of a proton from a doubly allylic methylene group (29), which is absent in ricinoleic acid. While linoleic acid is produced by insertion of a double bond between carbons 12 and 13 of oleic acid by oleate desaturases present in all higher plants, ricinoleic acid (D-12-hydroxyoctadec-cis-9-enoic acid) is synthesized by oleate desaturases only in a few plant species (5). Comparison of activities of oleic and ricinoleic acid suggest that the hydroxyl group at position 12 is crucial to the activity of ricinoleic acid in the induction of Ras, MAP/SAP kinase and AP1 activities and of apoptosis. The product of linoleic acid lipoxygenation is a hydroxylated doubly unsaturated fatty acid, 13-HODE (29). However, the position of the hydroxyl group in 13-HODE differs from that of the hydroxyl group in ricinoleic acid (C12), and 13-HODE contains two conjugated double bonds at positions 9 and 11 instead of the single one at position 9 in ricinoleic acid. This suggests that the two fatty acids may not interact with the same enzymes. In this light, the differences observed in the signaling pathways activated by arachidonic or linoleic acid on one hand and ricinoleic acid on the other hand are not unexpected. Further studies will be required to identify upstream effectors of Ras involved in ricinoleic acid action, and to investigate whether intracellular metabolism of ricinoleic acid is needed for activation of the Ras-MAP/SAP kinase-AP1 signaling pathway.

ACKNOWLEDGMENTS

We are grateful to pGST-RBD expression vector Dr F. McKenzie (State University of New York) for the GST-RBD expression vector. This work was supported by a grant from the Canadian Breast Cancer Initiative (#008491) to M.A. A-J. and S.M. W.G. is recipient of a graduate scholarship from the Fonds pour la Formation de Chercheurs et l'Aide à la Recherche. M.A. A-J. and S.M. are Chercheur-Boursiers du Fonds de la Recherche en Santé du Québec.

Figure Legends.

FIG. 1 Cremophor EL and ricinoleic acid treatments inhibit HeLa cell growth. A. Percentages of surviving cells were measured using MTT assays after incubation of HeLa cells with increasing concentrations of Cremophor EL for 72 hr. B. Percentages of surviving cells were measured using MTT assays after incubation of HeLa cells with increasing concentrations of oleic, linoleic and ricinoleic acid for 72 hr. C. Chemical structure of oleic, linoleic and ricinoleic acids.

FIG. 2 Cremophor EL and ricinoleic acid treatments induce apoptosis of HeLa cells. TUNEL assays were performed after incubation of HeLa cells with ricinoleic acid (150 or 300 μM, panel A), Cremophor EL (0.5 or 1 ml/ml, panel B) or oleic acid (150 or 300 μM, panel C).

FIG. 3: Cremophor EL and ricinoleic acid stimulate AP1 activity in HeLa and Ishikawa cells. A. Cells from two different clones obtained by stable transfection of HeLa cells with the TRE6-TATA-CAT reporter construct were treated with ethanol (EtOH, lanes 1,4), TPA (10 ng/ml, lanes 2,5) or Cremophor EL (2.8 μl/ml lanes 3,6) for 20 hours. CAT activity was assayed from extracts standardized for protein concentration. B. Cells from HeLa/TRE6-TATA-CAT clone #5 were treated for 20 hr with EtOH (lane 1), TPA (10 ng/ml, lane 2), EGF (50 ng/ml, lane 3) or with increasing volumes of Cremophor EL (CreEL 0.3, 0.7, 1.4 or 2.8 μl/ml in lanes 4 to 6). CAT activity was assayed as in A. C. Ishikawa/TRE6-TATA-CAT cells were treated with EtOH (lane 1), TPA (10 ng/ml, lane 2) or Cremophor EL (CreEL 0.3, 0.7, 1.4 or 2.8 μl/ml in lanes 3 to 6) for 18 hours. CAT activity was assayed from whole cell extracts standardized for protein concentration. D. HeLa cells were treated with ethanol (EtOH, lane 1), TPA (100 ng/ml, lane 2), Cremophor EL (CreEL 4.2μl/ml) and ricinoleic acid (RiA 200μM, lane 4) for 6 hours. mRNAs prepared from each plate were reverse-transcribed and fragments from the collagenase and actin cDNAs were amplified by polymerase chain reaction and run on 1.5% agarose gels. E. Cells from HeLa/TRE6-TATA-CAT clone #5 were treated with ethanol (EtOH, lane 1), TPA (10 ng/ml, lane 2), oleic acid (OA 250μM lane 3), linoleic acid (LA 250μM lane 4) or ricinoleic acid (RiA 100,

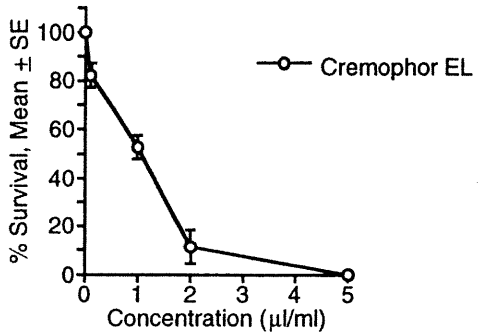
150, 200, 225 and 250μM, lanes 5-9) for 20 hours. CAT activity was assayed from whole cell extracts standardized for protein concentration.

FIG. 4. Cremophor EL induces phosphorylation of MAP/SAP kinases ERK and p38 in HeLa and HepG2 cells. A. HeLa cells were treated with ethanol (EtOH, lane 5) or with MAP/SAP kinase activators sorbitol (0.4 M., lane 1), TNF-α (10 ng/ml, lane 2), EGF (50ng/ml, lane 3), TPA (10 ng/ml, lane 4) for 30 minutes. Treatments with Cremophor EL (3 μl/ml) were performed for 7.5 min (lane 6), 15 min (lane 7) and 30 min (lane 8). Equal amounts of protein extracts (equivalent to approximately 0.3 million cells) were subjected to electrophoresis through a 10% polyacrylamide-SDS gel and western blotting. Blots were incubated with, from top to bottom panels: anti-p44/p42, anti-phospho-p44/p42, anti-phospho-SAPK/JNK, anti-p38, anti-phospho-p38, and appropriate secondary antibodies (see Materials and Methods). B. HeLa cells were treated with the MEKK1 inhibitor PD 098059 (50 μM, lanes 3,4) or the p38 inhibitor SB 203580 (10 μM, lane 7,8) for 30 min prior to a 15 min incubation with ethanol (lanes 1 and 5) or Cremophor EL (CreEL 2.8 μl /ml, lanes 2, 4 and 6, 8). C. HeLa cells were treated with ethanol (EtOH, lane 1), sorbitol (0.4M, lane 2), EGF (50 ng/ml, lane 3), TPA (10 ng/ml, lane 4), oleic acid (OA, 400 μM, lane 5), linoleic acid (LA, 400 μM, lane 6), ricinoleic acid (RiA, 400 μM, lane 7) or Cremophor EL (CreEL, 2.8 μl/ml, lane 8) for 15 min. Levels of phospho-p44/42, phospho-JNK and phospho-p38 were assayed by western blotting as described above. D. HeLa cells were treated with ethanol (EtOH, lane 1), sorbitol (0.4M, lane 2) or increasing concentrations of ricinoleic acid (lanes 3-7, as indicated) for 15 min. Levels of phospho-p44/42, phospho-JNK and phospho-p38 were assayed by western blotting. E. HepG2 cells were treated with ethanol (EtOH, lane 1), Sorbitol (0.4 M, lane 2), TPA (100 ng/ml, lane 3), EGF (50 ng/ml, lane 4), or with Cremophor EL (0.7 μl/ml, 2.8 μl/ml and 4.2 μl/ml in lanes 5-7) oleic acid (OA, 200 μM, lane 8), linoleic acid (LA, 200 μM, lane 9), ricinoleic acid (RiA, 200 μM, lane 10) or arachidonic acid (AA, 200 μM, lane 11) for 15 min. Levels of phospho-p44/42, phospho-JNK and phospho-p38 were assayed by western blotting.

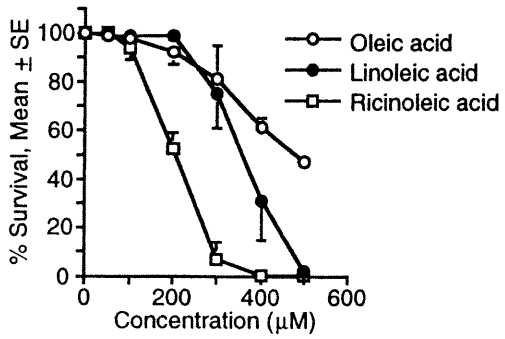
FIG. 5: Activation of MAP kinases by Cremophor EL and ricinoleic acid is not mediated by protein kinase C in HeLa cells. A. HeLa cells were treated with ethanol (EtOH, lane 1), TPA (100 ng/ml, lane 2) Cremophor EL (CreEL, 4.2 µl/ml) or ricinoleic acid (RiA, 400 µM, lane 4) for 10 min. Particulate fractions were isolated from whole cell extracts and levels of protein kinase C activity were measured (see Materials and Methods). B. HeLa cells were treated with ethanol (EtOH lanes 1,6), with 100 ng/ml TPA (lanes 2,7), or with 400 µM linoleic acid (LA, lanes 3,8), ricinoleic acid (RiA, lanes 4,9), or arachidonic acid (AA, lanes 5,10) for 3 or 15 min as indicated. Soluble and particulate fractions were isolated from whole cell extracts and analyzed for levels of protein kinase C isoenzymes by immunoblotting using a Pan-PKC antibody (see Materials and Methods). C. HeLa cells were pretreated with EtOH (lanes 1, 2, 4, 6, 8) or with the PKC inhibitor GF109203X (GF, 5 µM, lanes 3, 5, 7, 9) for 30 min prior to incubation with Sorbitol (0.4M, lane 2), TPA (100 ng/ml, lane 4 and 5) Cremophor EL (CreEL, 2.8 µl/ml, lane 6 and 7), ricinoleic acid (RiA, 400 µM, lane 8 and 9) for 15 min. Whole cell extracts were analyzed for levels of phospho-ERK and phospho-p38 by immunoblotting as described in Fig. 4.

FIG. 6. Ricinoleic acid activates p21-ras. A. HeLa cells were treated with Ethanol (EtOH, lane 1), EGF (50 ng/ml, lane 2) or ricinoleic acid (RiA 50 or 200 _M in lanes 3 and 4, as indicated) for 2.5 min. Equal amounts of protein extracts were incubated with Sepharose beads pre-coupled with GST-RBD fusion protein, which binds Ras-GTP. Proteins retained on the beads were analyzed by 12.5% polyacrylamide-SDS gel and western blotting using a Pan-Ras antibody. B. HeLa cells were treated with EGF (50 ng/ml) or ricinoleic acid (200 µM) for increasing amounts of time, and levels of Ras-GTP in the resulting whole cell extracts were measured as described above. Stimulated levels of Ras activity are shown since basal activity was subtracted.

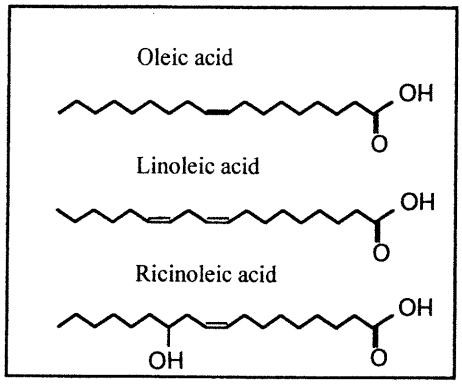
A


% Survival, Mean ± SE
100
80
60
40
20
0
0 1 2 3 4 5
Concentration (µl/ml)
Cremophor EL

B


% Survival, Mean ± SE
100
80
60
40
20
0
0 200 400 600
Concentration (µM)
Oleic acid
Linoleic acid
Ricinoleic acid

C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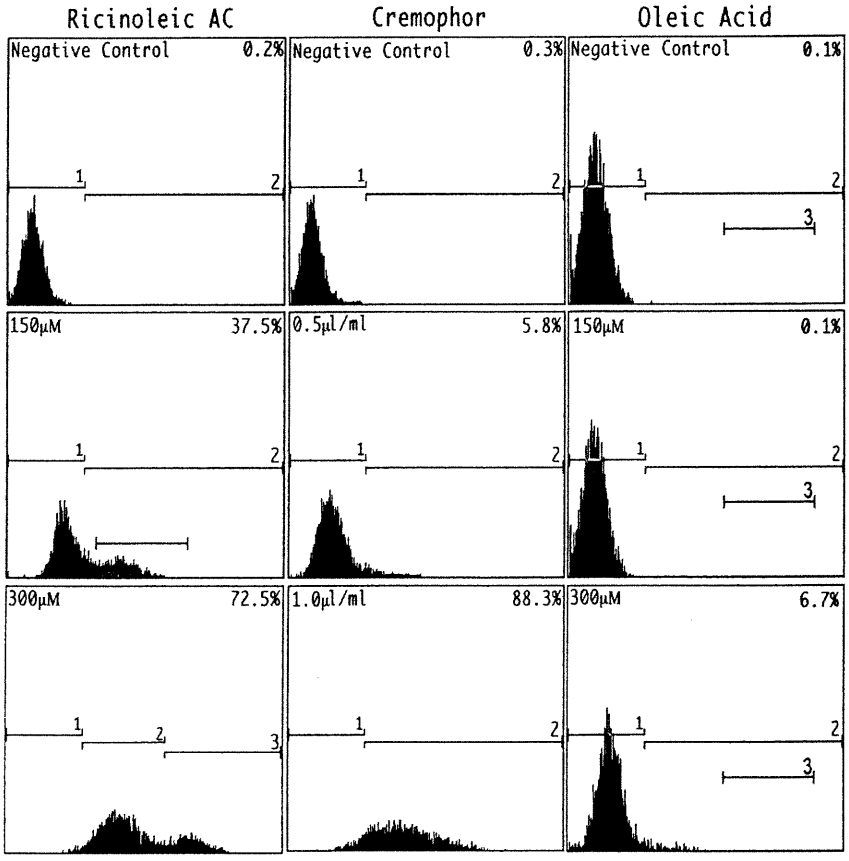
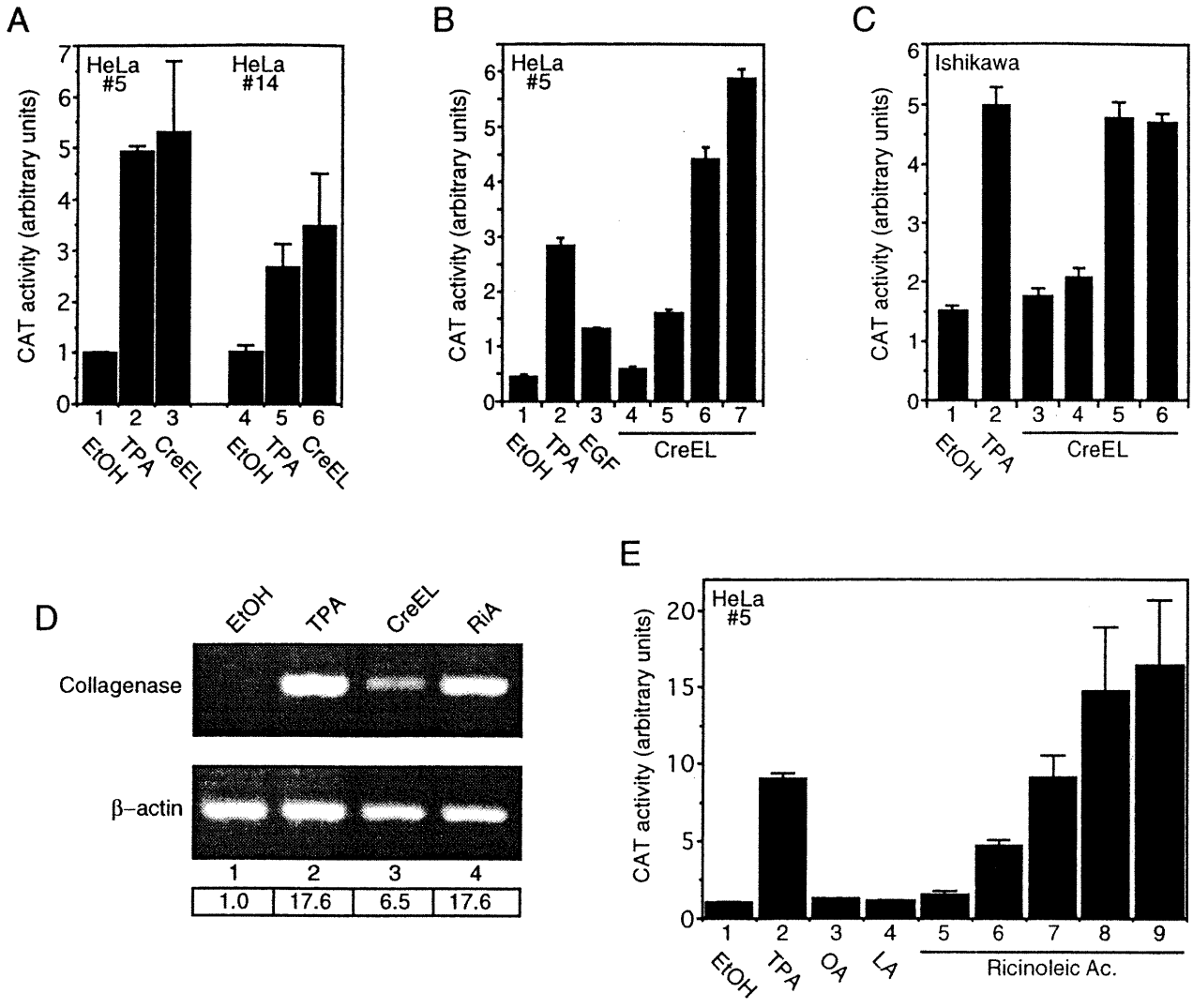
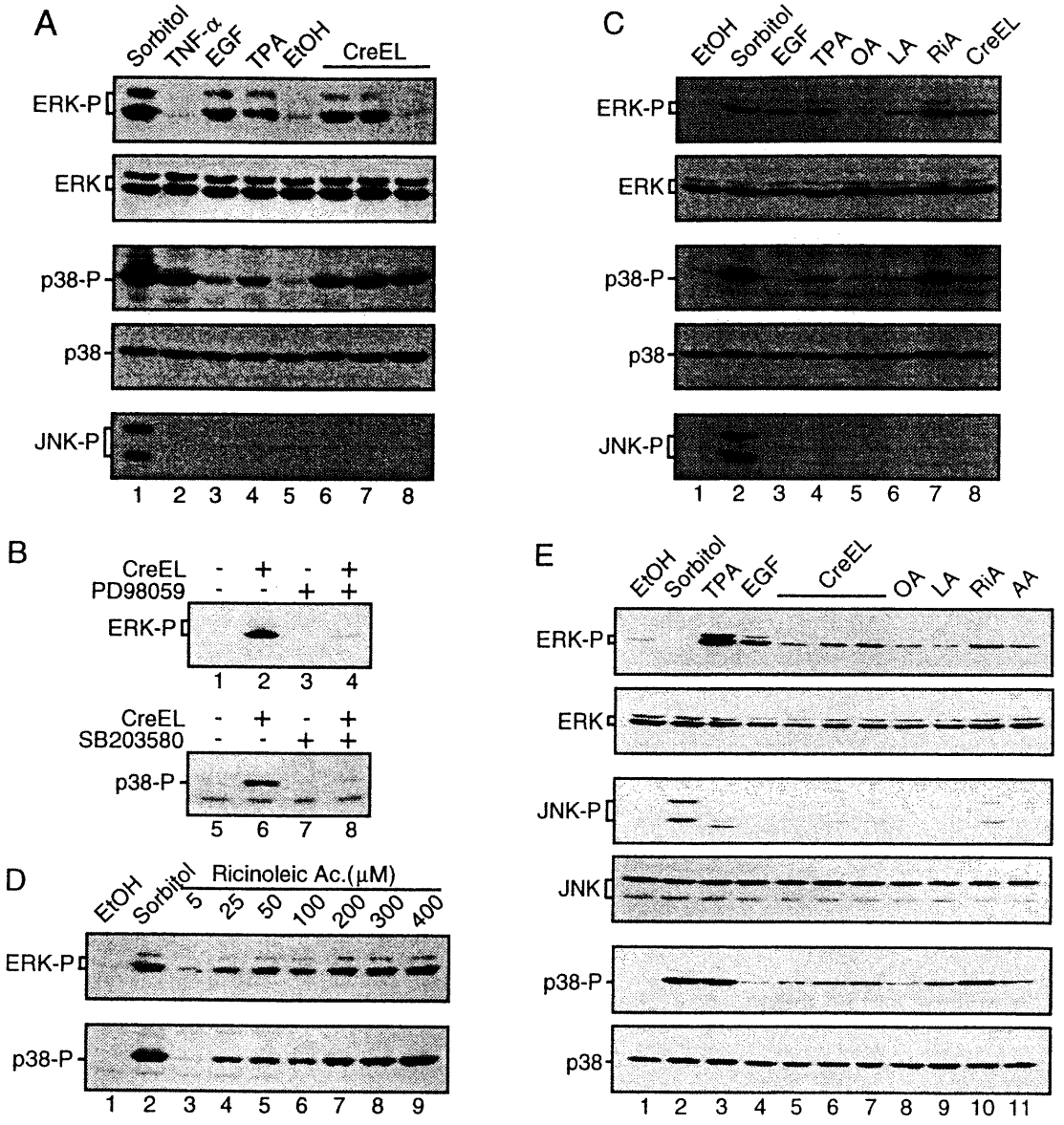
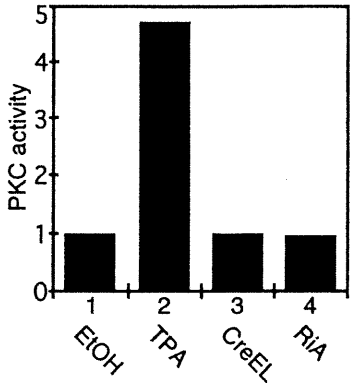
OH
O
Linoleic acid
OH
O
Ricinoleic acid
OH
OH
O


Ricinoleic AC
Cremophor
Oleic Acid
Negative Control
0.2%
Negative Control
0.3%
Negative Control
0.1%
150µM
37.5%
0.5µl/ml
5.8%
150µM
0.1%
300µM
72.5%
1.0µl/ml
88.3%
300µM
6.7%

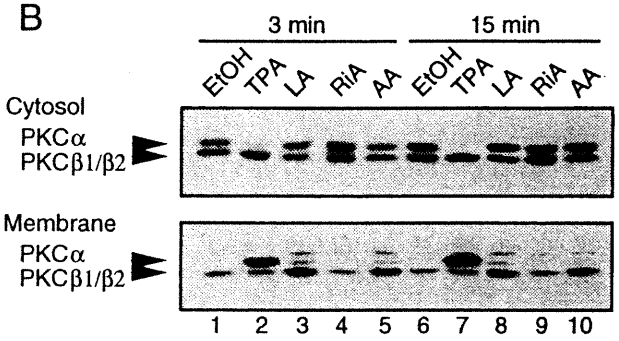

A
HeLa #5
HeLa #14
CAT activity (arbitrary units)
EtOH
TPA
CreEL
B
HeLa #5
EGF
C
Ishikawa
D
EtOH
TPA
CreEL
RiA
Collagenase
β-actin
1.0
17.6
6.5
17.6
E
HeLa #5
OA
LA
Ricinoleic Ac.


A
Sorbitol
TNF-α
EGF
TPA
EtOH
CreEL
ERK-P
ERK
p38-P
p38
JNK-P
1 2 3 4 5 6 7 8
C
EtOH
Sorbitol
EGF
TPA
OA
LA
RiA
CreEL
ERK-P
ERK
p38-P
p38
JNK-P
1 2 3 4 5 6 7 8
B
CreEL - + - +
PD98059 - - + +
ERK-P
1 2 3 4
CreEL - + - +
SB203580 - - + +
p38-P
5 6 7 8
D
EtOH
Sorbitol
Ricinoleic Ac.(μM)
5
25
50
100
200
300
400
ERK-P
p38-P
1 2 3 4 5 6 7 8 9
E
EtOH
Sorbitol
TPA
EGF
CreEL
OA
LA
RiA
AA
ERK-P
ERK
JNK-P
JNK
p38-P
p38
1 2 3 4 5 6 7 8 9 10 11

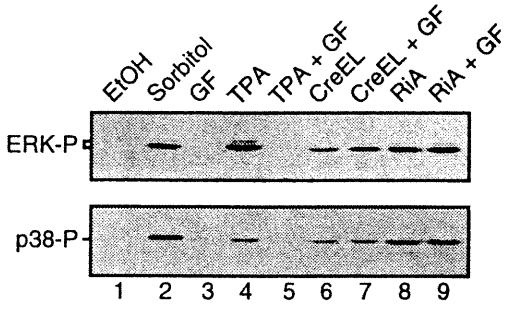
A

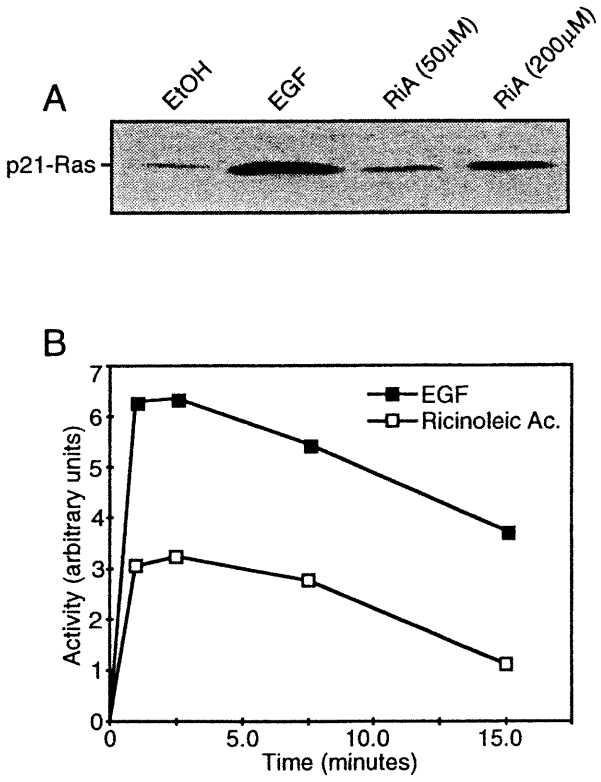

PKC activity
0
1
2
3
4
5
1
2
3
4
EtOH
TPA
CreEL
RiA

B


3 min
15 min
EtOH
TPA
LA
RiA
AA
EtOH
TPA
LA
RiA
AA
Cytosol
PKCα
PKCβ1/β2
Membrane
PKCα
PKCβ1/β2
1 2 3 4 5 6 7 8 9 10

C


EtOH
Sorbitol
GF
TPA
TPA + GF
CreEL
CreEL + GF
RiA
RiA + GF
ERK-P
p38-P
1 2 3 4 5 6 7 8 9


A
EtOH
EGF
RiA (50μM)
RiA (200μM)
p21-Ras
B
EGF
Ricinoleic Ac.
Activity (arbitrary units)
0
1
2
3
4
5
6
7
0
5.0
10.0
15.0
Time (minutes)

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