Université de Montréal

LE RÔLE DES CAVÉOLES ET DES RADEAUX LIPIDIQUES DANS L'ENDOCYTOSE

par

Phuong Uyen Le

Département de pathologie et biologie cellulaire

Faculté de médecine

Thèse présentée à la Faculté des études supérieures

en vue de l'obtention du grade de

Philosophiae Doctor (Ph.D.)

en biologie cellulaire du cancer

Octobre 2003

Université de Montréal

Faculté des études supérieures

Cette thèse intitulée :

LE RÔLE DES CAVÉOLES ET DES RADEAUX LIPIDIQUES DANS L'ENDOCYTOSE

Présentée par:

Phuong Uyen Le

Est évaluée par un jury composé des personnes suivantes:

Représentant du Doyen . . . . . .

Docteur Jacques Paiement. . . . . Président-rapporteur

Docteur Ivan Robert Nabi. . . . . Directeur de recherche

Docteure Audrey Claing . . . . . Membre du jury

Docteur John R. Silvius. . . . . Examinateur externe

Thèse acceptée le: . . . . . .

À toutes les personnes
que j'aime

SOMMAIRE

La phosphoglucose isomérase est équivalente à la neuroleukine, au facteur de maturation et au facteur autocrine de motilité (AMF) et elle agit comme cytokine au près des cellules neuronales, des lymphocytes et des cellules tumorales. La liaison de l'AMF à son récepteur, AMF-R, induit la motilité et l'invasion des cellules tumorales. L'AMF-R est un récepteur à sept domaines transmembranaires qui est concentré au niveau des cavéoles à la membrane plasmique et à l'intérieur de la cellule, au niveau d'un compartiment spécialisé du réticulum endoplasmique lisse, les tubules AMF-R. La localisation de l'AMF-R au niveau des cavéoles et du réticulum endoplasmique lisse suggère qu'il puisse être recyclé entre ces deux compartiments. Nous avons donc entrepris l'étude de l'endocytose de l'AMF/AMF-R.

Dans les cellules NIH-3T3 le complexe AMF-R et son ligand AMF sont endocytés via deux voies distinctes. La première voie est effectuée par les vésicules de clathrine vers des endosomes multivésiculaires, puis ce complexe est ensuite recyclé vers les fibrilles de fibronectine. La dynamine est une GTPase qui induit la fission des cavéoles et des vésicules de clathrine de la membrane plasmique et l'expression d'une forme dominante négative de la dynamine (K44A) bloque cette voie d'endocytose. Cette voie est également inhibée lors d'une acidification cytoplasmique et par l'expression cellulaire d'une forme mutante négative de la clathrine et elle correspond donc à une voie clathrine-dépendante. La deuxième voie d'endocytose est médiée par une voie

indépendante des vésicules de clathrine vers le REL. Cette voie d'endocytose est inhibée lors d'un traitement des cellules avec la méthyl-β-cyclodextrine et par l'expression cellulaire de la dynamine K44A. Cette voie d'endocytose correspond donc à une voie dépendante des cavéoles/radeaux lipidiques.

Les cavéoles sont des invaginations lisses de la membrane plasmique qui sont enrobées par la protéine cavéoline. De plus, elles font parties d'un sous-domaine des radeaux lipidiques, riche en cholestérol et en sphingolipide. Afin d'identifier le rôle précis des cavéoles dans l'endocytose de l'AMF, nous avons étudié l'internalisation de l'AMF/AMF-R dans les cellules NIH-3T3 transformées avec les oncogènes H-ras ou v-abl qui montrent une forte diminution de l'expression des cavéoles et de la cavéoline. Malgré la forte réduction de la cavéoline et des cavéoles dans ces cellules, l'endocytose de l'AMF/AMF-R vers le réticulum endoplasmique est augmentée en comparaison avec les cellules non transformées et cette endocytose est inhibée par la méthyl-ß-cyclodextrine. La surexpression de la dynamine K44A dans les cellules NIH-3T3 transformées induit la formation des invaginations plasmalemmales lisses qui sont morphologiquement identiques aux cavéoles. Une forte diminution de l'expression de la cavéoline n'empêche pas la formation et l'endocytose des cavéoles. Ces études indiquent donc que les radeaux lipidiques, des domaines enrichis en cholestérol, pourraient aussi s'invaginer pour former une vésicule endocytaire morphologiquement équivalente aux cavéoles.

La réintroduction de la cavéoline-1 dans les cellules NIH-3T3 transformées induit également la formation des cavéoles et réduit le taux d'endocytose de l'AMF vers le réticulum endoplasmique au niveau des cellules NIH-3T3 non-transformées. Ces résultats démontrent que la présence de la cavéoline n'est pas requise pour l'internalisation des cavéoles, mais elle est nécessaire pour la stabilisation de ces organites et dans la régulation de leur capacité endocytaire. Ensuite, pour vérifier si la cavéoline régule un autre ligand des cavéoles, nous avons étudié l'endocytose de la toxine de choléra dans les cellules NIH-3T3. Nous avons montré que la surexpression de la cavéoline réduit aussi l'endocytose de la toxine de choléra vers l'appareil de Golgi.

Il a été montré que l'endocytose du virus SV40 et de la toxine de choléra passe par un compartiment intermédiaire, appelé cavéosome. À partir des cavéosomes, SV40 est transporté au niveau du réticulum endoplasmique et la toxine de choléra est internalisée vers l'appareil de Golgi. Un traitement des cellules avec le nocodazole, un agent qui dépolymérise les microtubules, inhibe l'endocytose de SV40 au niveau des cavéosomes. Les cellules NIH-3T3 traitées avec le nocodazole montrent une inhibition de l'endocytose de la toxine de choléra vers l'appareil de Golgi, alors que l'endocytose de l'AMF vers le réticulum endoplasmique n'est pas affectée. Un traitement avec la brefeldin A ou une endocytose à 20°C bloque l'endocytose de la toxine de choléra vers l'appareil de Golgi. Toutefois, l'endocytose de l'AMF vers le réticulum endoplasmique n'est pas bloquée en présence de la brefeldin A ou à 20°C. De

plus, une cointernalisation de l'AMF avec la toxine de choléra montre qu'après seulement cinq minutes l'AMF est déjà internalisé vers le réticulum endoplasmique, alors que la majorité de la toxine de choléra est encore en surface. L'AMF est donc directement endocyté vers le réticulum endoplasmique sans avoir besoin de traverser les cavéosomes et l'appareil de Golgi.

Globalement, ce travail démontre que l'AMF est endocyté par deux voies distinctes : 1) via les vésicules de clathrine vers les corps multivésiculaires pour être ensuite recyclé vers les filaments de fibronectine; 2) via les cavéoles/radeaux lipidiques vers le réticulum endoplasmique. Cette dernière voie d'endocytose est la première démonstration de l'endocytose médiée par un récepteur qui est effectuée par les cavéoles vers le réticulum endoplasmique. Ces travaux nous ont également permis de caractériser le rôle de la cavéoline-1 dans la régulation des capacités endocytaires des cavéoles et des radeaux lipidiques.

Mots-cléfs : Facteur autocrine de motilité, réticulum endoplasmique, toxine de choléra, cavéoline, dynamine, fibronectine, motilité cellulaire, transformation cellulaire

SUMMARY

Phosphoglucose isomerase is equivalent to neuroleukin, maturation factor and autocrine motility factor (AMF) that function as cytokines in neuronal cells, lymphocytes and tumour cells. The binding of AMF to its receptor, AMF-R, induces cell motility and invasion of cancer cells. AMF-R is a protein with seven transmembrane domains localized to cell surface caveolae and to smooth ER tubules. The colocalization of AMF-R to both caveolae and the smooth ER suggest that AMF-R must recycle between these two cellular compartments. We therefore undertook the study of AMF/AMF-R endocytosis.

We show in NIH-3T3 fibroblasts that the AMF/AMF-R complex is internalised by two distinct pathways. First, AMF is internalized via a clathrin-dependent pathway to multivesicular bodies (MVBs) that mediate its recycling to fibronectin fibrils. This endocytic pathway is inhibited by cytoplasm acidification and expression of the clathrin hub as well as the dominant negative mutant of dynamin (K44A). Dynamin GTPase activity is responsible for the fission of both clathrin-coated pits and caveolae from the plasma membrane. AMF/AMF-R complex is also internalised by a clathrin-independent pathway to smooth ER tubules that is blocked in the presence of ß-cyclodextrin and by the expression of the mutant dynamin K44A. This pathway therefore corresponds to the caveolae/raft-dependent pathway.

Caveolae are smooth plasmalemmal vesicles associated with caveolin that form a subdomain of cholesterol, sphingomyelin-rich glycolipid rafts. We studied AMF/AMF-R internalisation in NIH-3T3 cells transformed with the oncogene H-ras or v-abl that present a strong reduction of caveolin and caveolae expression, respectively. AMF/AMF-R internalization studies by EM shows that in spite of the reduction of caveolin and caveolae, the AMF-R complex is still internalised to the ER and this pathway is sensitive to methyl-ß-cyclodextrin. Indeed, quantification showed the increased endocytosis of AMF to ER in transformed cells. Overexpression of dynamin K44A in transformed NIH-3T3 cells induced the formation of smooth plasmalemmal vesicles morphologically identical to caveolae. Reduced caveolin expression does not prevent glycolipid rafts to form endocytic vesicles morphologically equivalent to caveolae.

Expression of caveolin-1 in the ras and abl transformed NIH-3T3 cells induced the de novo formation of caveolae and reduced AMF endocytosis to the ER to the level of NIH-3T3 cells. These results indicated that caveolin-1 expression is not necessary for caveolae endocytosis but rather stabilizes caveolae expression at the plasma membrane and regulates their endocytic function. In order to determine whether caveolin-1 regulates the endocytosis of ligands other than AMF, we also investigated cholera toxin internalization in NIH-3T3 cells. Overexpression of caveolin-1 in these cells also reduced cholera toxin endocytosis to the Golgi apparatus.

SV40 virus and cholera toxin are endocytosed via caveolae to a caveolin-positive endosomal compartment, the caveosome. From this compartment, SV40 is transported to the ER while Cholera toxin is internalized to the Golgi apparatus. Nocodazole, a microtubule depolymerization agent has been shown to cause an accumulation of SV40 in the caveosome. Nocodazole treatment of NIH-3T3 cells blocked cholera toxin endocytosis to the Golgi apparatus but did not affect AMF endocytosis to the ER. Furthermore, while brefeldin A and a 20°C block inhibited cholera toxin endocytosis to the Golgi apparatus, AMF endocytosis to the ER was not affected. Cholera toxin and AMF cointernalization studies show that after only five minutes incubation AMF is already internalized to the ER, while the majority of cholera toxin remained at the cell surface. Therefore, our results indicate that from caveolae, AMF is directly internalized from caveolae to the ER.

In summary, these studies show that AMF is internalized by two distinct pathways: 1) clathrin-dependent endocytosis to MVBs and then recycling to fibronectin fibrils; and 2) caveolae/raft-dependent endocytosis to the ER. Caveolae-mediated endocytosis of AMF to the ER is the first demonstration of caveolae-mediated receptor endocytosis to the ER. Study of AMF endocytosis has further enabled the identification of a regulatory effect of caveolin-1 on caveolae/raft-dependent endocytosis.

Keywords: Autocrine motility factor, endoplasmic reticulum, cholera toxin, caveolin, dynamin, fibronectin, cell motility, cell transformation.

REMERCIEMENTS

Je tiens à adresser mes remerciements les plus sincères aux personnes suivantes:

Ivan Robert Nabi, mon directeur de recherche, pour m'avoir accueillie dans son laboratoire et pour m'avoir guidée tout au long de mes études graduées. Sous son adroite supervision, j'ai acquis une solide formation scientifique. Sa passion pour la science, son dynamisme et sa grande générosité ont contribué à ma réussite. Je tiens également à le remercier pour la patience, dont il a fait preuve à mon égard durant ma rédaction de thèse.

Ginette Guay, pour toute l'aide technique qu'elle m'a apportée, pour le montage des figures et pour la lecture et la correction de ma thèse. Merci Ginette pour tout ce que tu as fait pour moi.

Jacques Paiement et Nicole Leclerc, qui à plusieurs reprises, ont eu la gentillesse de me fournir des lettres de recommandations.

Jean Léveillé, un excellent photographe, pour son infinie gentillesse à mon égard. Grâce à lui, les photos que j'ai utilisées pour mes publications sont d'une qualité nettement supérieure.

Marielle Chèvrefils, pour tous les petits services qu'elle m'a rendus.

Anne Guénette, Diane Gingras et Christine Lavoie, pour leur constant support et encouragement.

Aux membres du laboratoire, passés et présents, plus particulièrement à Naciba Benlimame pour m'avoir si bien montré les techniques de laboratoire.

Ma famille, pour leur affection.

Van, pour ses encouragements, son support et son réconfort dans les moments difficiles et surtout pour son amour.

En terminant, je remercie les membres du jury qui ont pris le temps de faire la lecture de cette thèse.

TABLE DES MATIÈRES

LISTE DES TABLEAUX

Page

Thèse

Article 1

LISTE DES FIGURES

Thèse

Article 1

Article 2

Article 3

Article 4

LISTE DES ABRÉVIATIONS

AMF	Facteur autocrine de motilité
AMFb	Facteur autocrine de motilité biotinylé
AMF-R	Récepteur pour le facteur autocrine de motilité
AP	Protéine adapteur
ATP	Adénosine triphosphate
ARN	Acide ribonucléique
CNF1	Cytotoxic necrotizing factor 1
COP	Coat protein
CSD	Scaffolding domain
CTX	Toxine de choléra
DynK44A	Mutant dominant négatif de la dynamine
EGF	Facteur de croissance de l'épiderme
EGFR	Récepteur du facteur de croissance de l'épiderme
eNOS	Synthase de l'oxyde nitrique
FRET	Fluorescence resonance energy transfer
GDP	Guanosine diphosphate
GFP	Green fluorescent protein
GPI	Glycosylphosphatidylinositol-anchored proteins
GTP	Guanosine triphosphate
LDLR	Récepteur des lipoprotéines à faible densité
mβCD	Méthyle-β-cyclodextrine
MDCK	Madin Darby Canin Kidney Cells

MSV-MDCK	Cellules MDCK transformées avec le virus de sarcome de Moloney
MTOC	Centre d'organisation des microtubules
MVBs	Corps multivésiculaires
NIH-ras	Cellules NIH-3T3 transformées avec l'oncogène H-ras
NIH-abl	Cellules NIH-3T3 transformées avec l'oncogène v-abl
PDGF	Facteur de croissance dérivé des plaquettes sanguines
PGI	Phosphoglucose isomérase
PIP2	L'inositol phosphatidyl biphosphate
RE	Réticulum endoplasmique
REL	Réticulum endoplasmique lisse
REG	Réticulum endoplasmique rugeux
SV40	Simian virus 40
TFGβ	Transforming growth factor-β
TFR	Récepteur de transferrine
VEGF	Facteur de croissance vasculaire endothéliale

I. INTRODUCTION

1. ENDOCYTOSE

L'endocytose est un processus cellulaire qui sert à transporter des substances du milieu externe vers l'intérieur de la cellule. Ainsi, elle permet à la cellule d'internaliser des nutriments, des hormones et d'autres molécules à la membrane plasmique vers les compartiments intracellulaires. Elle joue un rôle essentiel dans le contrôle de la quantité de récepteurs et de leurs ligands à la surface cellulaire ainsi que dans le milieu extracellulaire, soit en les séquestrant dans des compartiments intracellulaires ou en les dégradant. De plus, l'endocytose joue un rôle important dans l'activation et la propagation des voies de signalisation. Plus récemment, il a été montré que différents pathogènes utilisent l'endocytose pour rentrer dans la cellule. L'endocytose est donc un processus très important à la vie cellulaire.

Le mécanisme d'internalisation des molécules est subdivisé en deux grandes catégories : 1) la phagocytose est un processus par lequel la cellule engloutie des particules ayant plus de 0,5 micromètre de diamètre en les entourant d'un pseudopode; 2) l'endocytose est un processus par lequel la cellule internalise de petites molécules à l'aide des invaginations de la membrane plasmique (Mellman, 1996). L'endocytose peut être effectuée soit par : 1) les vésicules enrobées de clathrine ; 2) les vésicules sans clathrine ; 3)

les cavéoles (Figure1). Dans la prochaine section, nous définirons plus en détail chacune de ces voies d'internalisation.

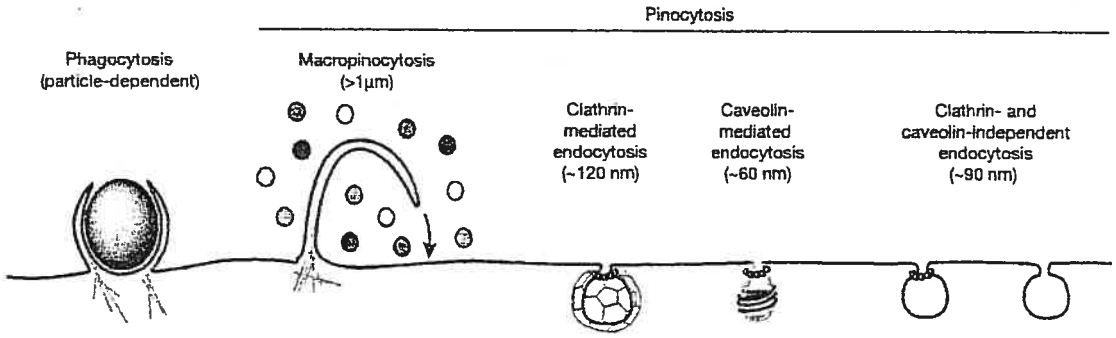


Figure 1. Les multiples voies d'internalisation dans les cellules de mammifères. Les différentes voies d'endocytose se distinguent par la taille des vésicules endocytaires, la nature des cargos (les ligands, les récepteurs et les lipides) et par les mécanismes de formation des vésicules endocytaires. Les vésicules de clathrine sont caractérisées par un recouvrement de clathrine du côté cytoplasmique qui leurs donnent une apparence de membrane épaissie. Alors que les différentes classes de vésicules dépourvues de clathrine ont une morphologie de vésicules plasmalemmales lisses. Les cavéoles et les radeaux lipidiques se distinguent des vésicules non-enrobées de clathrine par l'implication de la dynamine dans leur détachement de la membrane plasmique. Les cavéoles diffèrent des radeaux lipidiques dû à un enrichissement de la protéine cavéoline du côté cytoplasmique (Adapté de Conner et Schmid, 2003).

1.1 L'endocytose effectuée par les vésicules de clathrine

L'endocytose médiée par les vésicules enrobées de clathrine est la voie d'internalisation classique des cellules de mammifère. Elle est reconnue pour son rôle dans l'endocytose médiée par l'intermédiaire d'un récepteur et dans l'internalisation de la phase fluide. De nombreux récepteurs sont internalisés par cette voie. Le récepteur de transferrine (TFR), le récepteur des lipoprotéines à faible densité (LDLR) et le récepteur du facteur de croissance de l'épiderme (EGFR) sont les récepteurs les plus utilisés pour l'étude de cette voie d'endocytose (Mellman, 1996; Schmid, 1997; Brodsky et al., 2001).

L'endocytose effectuée par les vésicules enrobées de clathrine dans les cellules de mammifères débute avec la formation d'un puit de clathrine qui va s'invaginer pour former une vésicule de clathrine et cette vésicule va finalement se détacher de la membrane plasmique pour devenir une vésicule endocytaire. Dans le cytoplasme, la clathrine et les protéines adaptatrices vont se dissocier des vésicules de clathrine et ces vésicules vont ensuite fusionner avec les endosomes précoces. Puis, les récepteurs endocytés sont recyclés vers la membrane plasmique, soit à partir des endosomes précoces ou des compartiments plus tardifs. Les molécules destinées à la dégradation sont quant à elles envoyées vers les lysosomes. De cette façon, l'endocytose médiée par les vésicules de clathrine aide au contrôle du nombre de récepteurs à la surface (Sorkin, 2000; Brodsky et al., 2001).

1.1.1 La formation des vésicules de clathrine

La formation d'une vésicule de clathrine est effectuée en plusieurs étapes et débute avec : 1) le recrutement des protéines adaptatrices (AP2), des molécules cargo, de la clathrine et des protéines accessoires à la membrane plasmique; 2) la constriction et ensuite le détachement des vésicules de clathrine; 3) la dissociation de la clathrine des vésicules endocytaires.

1.1.1.1 Le recrutement de la clathrine et des protéines résidentes des puits de clathrine

La clathrine est une des principales composantes impliquées dans la formation du "manteau" de clathrine. Dans le cytoplasme, la clathrine se présente sous la forme de triskelion. Le triskelion est composé de trois chaînes lourdes identiques et de trois chaînes légères. Chacun de ces triskelions est retenu par un domaine centrale appelé "hub". La clathrine a la propriété de s'autoassembler et la formation d'une "cage" est effectuée par une oligomérisation des triskelions (Kirchhausen, 2000). Des essais *in vitro* ont montré que l'assemblage de la clathrine seul pouvait générer une force qui provoque une courbature de la membrane (Mahaffey et al., 1989; Smythe et al., 1992). Toutefois, *in vivo*, d'autres protéines sont nécessaires pour la formation des vésicules de clathrine.

En plus de la clathrine, les protéines adaptatrices et AP180 sont également impliquées dans l'assemblage de la clathrine et dans l'endocytose. La protéine adaptatrice 1 (AP1) est composée des sous-unités β1, γ, μ1A et σ1, alors que AP2 est composée des sous-unités α, β2, μ2, et σ2 (Kirchhausen, 1999). AP1 est localisé au niveau du réseau trans-golgien et AP2 est spécifique à la membrane plasmique (Pearse and Robinson, 1990). Les protéines adaptatrices, AP3 et AP4, similaires à AP1 et AP2 ont été identifiées. AP3 est impliquée dans le transport spécialisé du réseau trans-Golgien et AP4 est faiblement exprimée et elle a été localisée dans la partie trans-Golgienne (Kirchhausen 1999 ; Dell' Angelica et al., 1999 ; Hirst et al., 1999). La fonction de AP4 n'est pas encore claire, mais il a des évidences qui montrent que AP4 peut être impliquée dans le ciblage des protéines vers les lysosomes (Aguilar et al., 2001) ou vers le côté basolatéral des cellules MDCK (Simmen et al., 2002).

AP1 et AP2 induisent l'assemblage de la clathrine à travers leurs sous-unités β1 et β2 (Gallusser and Kirchhausen, 1993). Les sous-unités μ1 et μ2 sont impliquées dans la liaison avec les molécules cargo. Il a été montré que la sous-unité μ se liait particulièrement aux récepteurs qui présentent un motif YXXφ dans leur domaine cytoplasmique (Ohno et al., 1995). D'autres sous-unités peuvent également lier spécifiquement différentes protéines tel que β2-adaptine et β2-arrestine. Les protéines adaptatrices sont donc impliquées dans la reconnaissance et la sélection de molécules cargo.

La clathrine peut aussi interagir directement avec AP180 qui se lie aussi avec AP2. AP180 et AP2 interagissent également avec l'inositol phosphatidyl bisphosphate (PIP2) (Brodsky et al., 2001). PIP2 est présent en grande concentration à la membrane plasmique et il serait impliqué dans le recrutement de AP2 et AP180 à la membrane (Cremona and De Camilli, 2001). Les complexes AP2 et AP180, à leur tour, vont recruter les triskelions de clathrine (Takei and Haucke, 2001). AP180 aurait pour fonction de stimuler l'assemblage de la clathrine et de réguler la grandeur des vésicules de clathrine (Zhang et al., 1998; McMahon, 1999; Mao et al., 2001).

1.1.1.2 Les mécanismes de recrutement des protéines cargo dans les vésicules de clathrine

Les vésicules de clathrine recrutent trois différentes classes de récepteurs par un mécanisme qui est régulé par des peptides de signalisation (sorting signals). La première classe de récepteurs (TfR et LDLR) sont ceux qui se concentrent dans les vésicules de clathrine de façon constitutive. La seconde classe de récepteurs comprend ceux qui sont recrutés aux niveaux des vésicules de clathrine suite à une stimulation du récepteur et cette stimulation est effectuée par la liaison du ligand au récepteur. Parmi ces récepteurs, il y a les récepteurs de type tyrosine kinase et les récepteurs couplés aux protéines G tels que les récepteurs β2-adrénergique et les EGFRs.

Les récepteurs qui sont recrutés dans les puits de clathrine de façon constitutive sont généralement des récepteurs qui contiennent un peptide signal à base de tyrosine de type YXXΦ du côté cytoplasmique. Il a été montré que les molécules AP2 interagissent directement avec ce peptide (Pearse, 1988; Ohno et al., 1995). Dans un système *in vitro,* les récepteurs contenant le peptide signal à base de tyrosine augmentent l'interaction entre AP2 et la synaptotagmine et cette liaison servirait à augmenter la liaison de AP2 à la membrane des cellules neuronales (Farsad et al., 2001). Récemment, une autre étude a montré que c'est la phosphorylation de la sous-unité μ de la molécule AP2 qui augmente son affinité pour les peptides ayant un motif à base de tysosine (Fingerhut et al., 2001). Ces résultats impliquent AP2 dans la reconnaissance des récepteurs contenant une séquence peptidique à base de tyrosine et qui a pour effet de faciliter le recrutement de ces récepteurs dans les vésicules de clathrine. Il a également été rapporté qu'une surexpression de TfR dans les cellules de poulets est corrélée avec une augmentation du nombre de vésicules de clathrine (Miller et al., 1991). Toutefois, aucun effet sur le nombre de vésicules de clathrine a été observé lors de la surexpression de TfR dans d'autres lignées cellulaires (Warren et al., 1997; Brown et al., 1999).

Chez les mammifères, une stimulation du récepteur par le ligand n'induit pas la formation des vésicules de clathrine (Santini et al., 1998). Toutefois, la liaison du ligand induit une stimulation de la reconnaissance du récepteur par les molécules AP2. Pour les récepteurs de type tyrosine kinase, une

phosphorylation de leur domaine cytoplasmique induit un changement de conformation exposant ainsi le site de liaison avec AP2 (Chen et al., 1989). Pour EGFR, il a été montré que suite à la liaison de EGF à son récepteur, une phosphorylation de la clathrine se produit et la clathrine est recrutée à la membrane plasmique. La phosphorylation de la clathrine est effectuée par une SRC kinase. Une inhibition de cette kinase empêche la phosphorylation de la clathrine et cause un retardement de l'internalisation de EGFR (Wilde et al., 1999). L'ubiquitination pourrait également être un signal pour la séquestration des récepteurs dans les vésicules de clathrine. L'ubiquitination est associée avec l'internalisation de plusieurs récepteurs (Alves dos Santos et al., 2001; van Kerkhof et al., 2002; Cook et al., 2003; Haglund et al., 2003; Mosesson et al., 2003).

Par contre, le recrutement des récepteurs couplés aux protéines G, tel que le récepteur β-2 adrénergique, dans les vésicules de clathrine est initié par la liaison du ligand au récepteur et cette activation induit la phosphorylation du récepteur et le recrutement des β-arrestines. β-arrestine se lie avec le récepteur activé ainsi qu'avec la clathrine et AP2, ces liaisons permettent le recrutement des récepteurs couplés aux protéines G dans les vésicules de clathrine (Goodman et al., 1997; Goodman et al., 1998; Laporte et al., 1999). Il a été montré que β-arrestine 1 recrute la tyrosine kinase c-SRC à la membrane plasmique afin de phosphoryler des protéines intermédiaires tel que la

dynamine, une protéine impliquée dans le détachement des vésicules de clathrine de la membrane plasmique (Miller et al., 2000 ; Kim et al., 2003).

1.1.1.3 Le recrutement des protéines accessoires

Les protéines accessoires sont des protéines qui se lient soit à la clathrine, AP2 ou PIP2 et elles sont recrutées de façon transitoire à la membrane plasmique. Elles participent à la sélection de cargo ainsi qu'à la formation des puits de clathrine. Les protéines Eps15 et epsin sont des protéines candidates pour la sélection de cargo et dans l'assemblage des vésicules de clathrine. Eps15 et epsin contiennent un motif qui s'associe avec l'ubiquitine et ces protéines pourraient être impliquées dans le recrutement des molécules cargo ubiquitinées (Polo et al., 2002). En plus d'être impliquée dans la sélection des molécules cargo, Eps15 est aussi impliquée dans l'assemblage de la clathrine et au réarrangement des clathrines en réseau (lattice) par son association avec les protéines AP180 et AP2 (Benmerah et al., 1998; Morgan et al., 2003).

La structure de epsin-1 est très similaire à AP180, son côté N-terminal se lie avec les lipides inositols et sa queue cytoplasmique contient des sites de liaison avec la clathrine, AP-2 et Eps15 (Kalthoff et al., 2002). Il a été montré *in vitro* que l'epsin-1 recombinant induit l'assemblage de la clathrine en forme de cage (Kalthoff et al., 2002) et *in vivo*, epsin-1 est essentiel pour l'endocytose

(Chen et al., 1998). Ainsi, en association avec AP180, epsin-1 contribue au recrutement rapide et efficace de la clathrine et de AP2 aux niveaux des puits de clathrine (Kalthoff et al., 2002). De plus, des études *in vitro* sur une monocouche lipidique ont montré que par son association avec les lipides inositols, epsin-1 est capable de modifier la courbature de la membrane plasmique et ainsi, elle facilite la formation des vésicules de clathrine (Ford et al., 2002).

1.1.1.4 La constriction et le détachement des vésicules de clathrine

La dynamine, l'endophilin, l'amphiphysin sont également des protéines accessoires. Toutefois, ces protéines vont aider ou contribuer à la constriction et à la fission des vésicules de clathrine.

La dynamine est une grosse protéine qui possède une activité GTPasique. Elle est constituée de plusieurs domaines, dont un domaine GTPase du côté N-terminal qui lui permet de se lier au GTP et d'hydrolyser le GTP en GDP ; un domaine PH (pleckstrin homology) dans la région centrale qui lui permet de se lier spécifiquement au PtdIns(4, 5)P_2 ; un domaine GED (GTPase effector domain) du côté C-terminal qui est impliqué dans l'oligomérisation et l'auto-assemblage de la dynamine. Ce domaine est suivi par un domaine PRD qui, par son association avec les domaines SH3, permet à la dynamine de se lier avec d'autres protéines tel que amphysine, endophiline et actine (Hinshaw, 2000; McNiven et al., 2000).

Il a été montré que la dynamine est recrutée au niveau du col des vésicules de clathrine où elle participe à la constriction et au détachement de ces vésicules de la membrane plasmique. Une mutation dans le domaine GTPasique de la dynamine (K44A) résulte en une défaillance dans sa capacité de lier et d'hydrolyser le GTP. Donc, les cellules qui expriment la dynamine mutante K44A montrent une accumulation des vésicules de clathrine et des cavéoles à la membrane plasmique. De plus, une inhibition de l'endocytose effectuée par les vésicules de clathrine, des cavéoles et des radeaux lipidiques a été observée lors de l'expression de la dynamine K44A (Hinshaw, 2000; McNiven et al., 2000).

De plus, des essais *in vitro* ont montré qu'une interaction de la dynamine avec amphiphysin et avec PIPs pouvait induire une déformation des lipides de la membrane plasmique. Cette interaction avec les lipides pourrait contribuer à la déformation de la membrane durant la fission des vésicules de clathrine (Takei et al., 1999). Endophilin-1 est une enzyme cytosolique qui convertie l'acide lysophosphatidique en acide phosphatidique. Cette réaction enzymatique pourrait générer un changement dans la courbature de la membrane plasmique. Par son interaction avec la dynamine, l'endophilin facilite l'invagination des vésicules de clathrine et contribue ainsi au détachement des vésicules de clathrine (Schmidt et al., 1999; Huttner and Schmidt, 2000; Farsad et al., 2001).

1.1.1.5 La dissociation de la clathrine

Suivant le détachement des vésicules de clathrine de la membrane plasmique (encore entièrement recouvertes de clathrine), la clathrine et subséquemment, les molécules adaptatrices vont se dissocier de la vésicule. Ces étapes sont effectuées avec l'aide de plusieurs molécules tel que : la synaptojanin, auxilin et hsc70.

La synaptojanin-1 est une polyphosphoinositide phosphatase et dans un système in vitro, la synaptojanin-1 déphosphoryle plusieurs PIPs. Elle clive spécifiquement les 3-, 4- et 5-phospates des PI(3)P, PI(4)P, PI(3, 5)P_2 et PI(3, 4, 5)P_3 (Mcpherson et al. 1996). Une perte de la synaptojanin-1 chez la souris est léthale et cause une accumulation des vésicules de clathrine (Cremona et al., 1999; Harris et al., 2000; Kim et al., 2002). Une mutation de l'homologue de la synaptojanin-1, unc-26, chez le vers montre une déficience dans le recrutement, la fission des vésicules de clathrine et une dissociation de la clathrine (Harris et al., 2000). Ces résultats montrent un rôle pour la synaptojanin-1 dans la dissociation du manteau de clathrine.

La synaptojanin-2 est exprimée de façon ubiquitaire et son domaine catalytique est fortement homologue à la synaptojanin-1. Une délétion de la synaptojanin-2 avec un siRNA (small-interfering RNA) bloque l'endocytose de EGF et montre une réduction du nombre d'invagination et de vésicules de

clathrine à la membrane plasmique. Ces résultats suggèrent que la synaptojanin-2 soit aussi impliquée dans la formation des vésicules de clathrine et non seulement dans la dissociation de la clathrine (Rusk et al., 2003).

En plus de la synaptojanin, auxilin (ou GAK (G-associated kinase), l'homologue de auxilin dans les cellules non-neuronales), est aussi impliquée dans la dissociation de la clathrine. Une délétion d'un homologue de l'auxilin chez la levure montre une déficience dans la dissociation du manteau de clathrine (Pishvaee et al., 2000). Auxilin induit la dissociation de la clathrine en recrutant hsc70 au niveau du manteau de clathrine, en stimulant l'activité ATPase de hsc70 (Greener et al., 2000; Pishvaee et al., 2000; Morgan et al., 2001). Il a été montré qu'une mutation dans le gène codant pour l'auxilin chez les vers et dans la levure inhibe l'endocytose et provoque une accumulation des vésicules où la clathrine est incapable de se dissocier (Umeda et al., 2000; Zhao et al., 2001).

1.1.2 Les compartiments endosomiales de la voie d'endocytose classique

Suite à la dissociation de la clathrine et des molécules adaptatrices, les vésicules de clathrine fusionnent avec les endosomes précoces et les molécules cargo sont triées, puis envoyées vers les compartiments suivants. Les endosomes précoces ont une forme vésiculotubulaire et un pH de 6.2-6.5. À partir des endosomes précoces, les protéines cargo sont directement recyclées

vers la membrane plasmique, ou triées vers le compartiment de recyclage, ou envoyées vers les endosomes tardifs. Le compartiment de recyclage est juxtaposé au réseau trans-golgien et il est responsable du retour des molécules cargo vers la membrane plasmique.

Les endosomes tardifs (également appelés corps multivésiculaires) ont une forme plus constante et sphérique. Ils sont caractérisés par la présence de l'acide lysobisphosphatidique et de vacuoles ou des membranes internes. De plus, ils ont un pH plus acide (5.5) que celui des endosomes précoces. Par le processus de maturation, les endosomes tardifs peuvent devenir des lysosomes ou transférer leur contenu vers les lysosomes via les vésicules de transport. Les lysosomes ont un contenu plus dense et ont la caractéristique de posséder des hydrolases et des protéases (Mellman, 1996; Mukherjee et al., 1997; Sorkin, 2000).

Les petites GTPases de la famille des rabs sont reconnues pour leur rôle dans la régulation du transport membranaire. Rab5 est nécessaire pour la formation des endosomes précoces, rab7 est impliqué dans le transport entre les endosomes tardifs et les lysosomes, rab4 et 11 sont impliqués dans la voie de recyclage et rab9 contrôle le transport entre les endosomes tardifs et le réseau trans-golgien (Mellman, 1996; Mukherjee et al., 1997; Sorkin, 2000). Par conséquent, une mutation des rabs pourrait être un outil efficace pour bloquer le transport au niveau d'un compartiment endocytaire spécifique.

1.1.3 Quelques exemples des inhibiteurs qui sont utilisés pour bloquer l'endocytose des vésicules de clathrine.

Traditionnellement, l'endocytose effectuée par les vésicules de clathrine peut être bloquée soit par une acidification cytoplasmique, soit par une déplétion de potassium, soit par un traitement avec la chlorpromazine. L'acidification cytoplasmique et la déplétion de potassium ont pour effet d'empêcher l'assemblage de la clathrine (Carpentier et al., 1989; Heuser, 1989; Altankov and Grinnell, 1993). La chlorpromazine est une drogue qui bloque l'assemblage de la clathrine à la membrane plasmique et la dissociation de la clathrine aux niveaux des endosomes (Wang et al., 1993). Cependant, l'utilisation des dominants négatifs des protéines impliquées dans la formation des vésicules de clathrine est une approche plus spécifique pour l'analyse de l'endocytose. L'emploi de la forme dominante négative de la dynamine (K44A), où l'hydrolyse de la GTP en GDP est bloquée, empêche la fission des vésicules de clathrine de la membrane plasmique (Henley et al., 1999; Hinshaw, 2000; McNiven et al., 2000). L'expression des mutants de l'Eps15 (Carbone et al., 1997; Benmerah et al., 1998; Benmerah et al., 1999) ou de la clathrine hub (Liu et al., 1998; Altschuler et al., 1999) induit aussi une inhibition de l'endocytose des vésicules de clathrine.

1.2 L'endocytose indépendante des vésicules de clathrine

L'endocytose indépendante des vésicules de clathrine peut être effectuée soit par les cavéoles, les radeaux lipidiques ou par les vésicules non-enrobées de clathrine. Ces voies d'endocytose ont été observées lors de l'inhibition de l'endocytose par les vésicules de clathrine et les mécanismes qui sont impliqués dans ces voies d'endocytose sont encore peu connus.

1.2.1 L'endocytose via les vésicules non-enrobées de clathrine

L'endocytose via les vésicules non-enrobées de clathrine ou la pinocytose constitutive se distingue de l'endocytose dépendante des vésicules de clathrine, des cavéoles ou des radeaux lipidiques par son indépendance de l'activité GTPasique de la dynamine et par son insensibilité aux agents qui extraient le cholestérol tels que la filipine, la nystatine ou la méthyle-β-cyclodextrine (mβCD) (Damke et al., 1995; Llorente et al., 1998; Contamin et al., 2000; Sabharanjak et al., 2002). Cette voie d'endocytose est caractérisée par l'internalisation du ricin. À la surface des cellules, la ricin se lie aux glycoprotéines et aux glycolipides qui contiennent la galactose du côté terminal et par conséquent elle peut être endocytée par différents mécanismes d'internalisation (Sandvig and van Deurs, 1996).

En effet, lors d'une acidification cytoplasmique dans les cellules Hep2 afin d'inhiber l'endocytose médiée par les vésicules de clathrine, l'internalisation de la transferrine est totalement bloquée alors que des lectines tels que la ricin et la concanavalin A continuent d'être endocytées. Suite à leur internalisation, la ricin et la concanavalin A sont transportées vers les même endosomes impliqués dans le transport des molécules provenant de l'endocytose médiée par les vésicules de clathrine; elles sont par la suite ciblées vers l'appareil de Golgi et par un transport rétrograde vers le réticulum endoplasmique (Moya et al., 1985; van Deurs et al., 1986; Sandvig et al., 1987; Hansen et al., 1993). De plus, lorsque l'endocytose via les vésicules de clathrine ou les cavéoles est bloquée avec l'expression d'un mutant de la dynamine, les cellules sont quand même infectées par la ricin (Simpson et al., 1998). Un blocage de l'internalisation des vésicules de clathrine, des cavéoles et des radeaux lipidiques induit par une déplétion du cholestérol (Rothberg et al., 1990a; Rodal et al., 1999; Subtil et al., 1999) n'affecte nullement l'internalisation de la ricin.

De façon similaire à la ricin, l'utilisation des inhibiteurs spécifiques pour bloquer l'endocytose médiée par les vésicules de clathrine ou par les cavéoles, il a aussi été rapporté que le récepteur muscarinique M2, TGF-ß (transforming growth factor- ß), la toxine bactérienne CNF1 (cytotoxic necrotizing factor 1) et certains récepteurs conjugués avec la GPI (glycosylphosphatidylinositol-anchored proteins) sont endocytés par les vésicules non enrobées de clathrine (Pals-Rylaarsdam et al., 1997; Skretting et al., 1999; Vilhardt et al., 1999;

Contamin et al., 2000; Zwaagstra et al., 2001; Sabharanjak et al., 2002) (Voir table 1).

Table 1: Les molécules et les récepteurs qui sont endocytées par les voies clathrine-indépendantes

Molécules	Références
Vésicules non-enrobées de clathrine	
Récepteur Muscarinique M2	(Pals-Rylaarsdam et al., 1997; Roseberry and Hosey, 2001)
CNF1	(Contamin et al., 2000)
Concanavalin A	(Hansen et al., 1993)
Dopamine	(Vickery and von Zastrow, 1999)
Protéines ancrées au GPI	(Sabharanjak et al., 2002)
Récepteur de la toxine de Diphteria conjugué au GPI	(Skretting et al., 1999)
Récepteur UPA conjugué au GPI	(Vilhardt et al., 1999)
Ricin	(Moya et al., 1985; van Deurs et al., 1986; Sandvig et al., 1987; Llorente et al., 2000)
TGF-ß	(Zwaagstra et al., 2001)
Radeaux lipidiques	
CD59	(Nichols et al., 2001)
GPI-GFP	(Nichols et al., 2001)
Interleukine-2	(Lamaze et al., 2001)
Lactosyl ceramide	(Puri et al., 2001)
Toxine de Shiga	(Nichols, 2001 #2038)
Toxine de Tétanus	(Montesano et al., 1982; Herreros et al., 2001; Munro et al., 2001)

Cavéoles	
Acide folique	(Rothberg et al., 1990b; Anderson et al., 1992; Rijnboutt et al., 1996)
Albumine	(Schnitzer et al., 1994; Minshall et al., 2000)
Alkaline phosphatase	(Parton et al., 1994)
AMF	(Benlimame et al., 1998; Le et al., 2000; Le et al., 2002)
Echovirus 1	(Marjomaki et al., 2002)
Endothéline	(Chun et al., 1994)
Hormones de croissance	(Lobie et al., 1999)
Simian virus 40 (SV40)	(Kartenbeck et al., 1989; Stang et al., 1997; Pelkmans et al., 2001; Pelkmans et al., 2002)
Toxine de Choléra	(Montesano et al., 1982; Parton, 1994; Orlandi and Fishman, 1998; Le et al., 2003)
Virus respiratoire syncytial	(Werling et al., 1999)

Cette table a été compilé à partir de Nichols et Lippincott-Schwartz, 2001; Pelkmans et Helenius, 2002.

1.2.2 Les radeaux lipidiques

La membrane plasmique est formée par une double couche de phospholipides qui peuvent diffuser latéralement. La membrane plasmique peut aussi exister en état de "liquid ordered" où l'assemblage des lipides est plus rigide et leurs mouvements limités. Ce type de domaine est causé par une concentration de cholestérol et des phospholipides et il est également appelé radeau lipidique (Brown and London, 1997 ; Simons and Toomre, 2000).

Les radeaux lipidiques consistent en un assemblage dynamique de cholestérol et de sphingolipides du côté extracellulaire de la membrane plasmique. Les protéines sont sélectivement incluses ou exclues des radeaux lipidiques et ceci pourrait être modulé par un stimulus intra- ou extracellulaire. Cet assemblage de lipides forme des plateformes qui concentrent des molécules de signalisation et les radeaux lipidiques sont impliqués dans la transduction de signaux (Simons and Ikonen, 1997; Kurzchalia and Parton, 1999; Mukherjee and Maxfield, 2000; Simons and Toomre, 2000; Brown, 2002).

Les radeaux lipidiques peuvent réguler la transduction des signaux soit en activant ou en inactivant les protéines de signalisation. Ainsi, en étant un domaine qui concentre plusieurs protéines de signalisation, les radeaux lipidiques favorisent le rapprochement et l'interaction entre ces protéines. Elles

peuvent également inactiver la transduction des signaux en ségréguant les molécules de signalisation et ainsi empêcher leur interaction et leur activation.

Durant les dernières années, il a aussi été rapporté que les radeaux lipidiques sont le site d'internalisation de différents pathogènes et bactéries (Rosenberger et al., 2000; Shin and Abraham, 2001). Depuis, d'autres études ont montré que les radeaux lipidiques sont aussi impliqués dans l'endocytose (voir table 1).

1.2.2.1 Les dimensions des radeaux lipidiques

Les radeaux lipidiques sont impliqués dans plusieurs processus cellulaires et pourtant, leur existence *in vivo* est incertaine et controversée. Les évidences qui montrent leur existence sont surtout basées sur la capacité de les isoler avec l'utilisation de différents détergents (Simons and Ikonen, 1997; Kurzchalia and Parton, 1999; Mukherjee and Maxfield, 2000; Simons and Toomre, 2000; Brown, 2002). Pour cette raison, plusieurs groupes de chercheurs ont tenté de visualiser et de mesurer ces domaines dans les cellules vivantes.

Les radeaux lipidiques sont difficiles à séparer des autres membranes. Il est donc difficile de mesurer directement la largeur de ces microdomaines. L'utilisation de différentes méthodes indirectes permet d'évaluer la largeur des

radeaux lipidiques. Cependant, une variabilité dans la largeur des radeaux lipidiques a été observée selon la technique utilisée. Les protéines liés aux GPI sont enrichies dans les radeaux lipidiques et sont souvent utilisées pour marquer les radeaux lipidiques (Brown and Rose, 1992; Schnitzer et al., 1995b). Une analyse de la diffusion latérale des protéines GPI et des gangliosides suggèrent que les radeaux lipidiques ont un diamètre de 200-300 nm (Jacobson et al., 1995; Sheets et al., 1997; Simson et al., 1998).

D'autres études ont montrées par FRET (fluorescence resonance energy transfer) que les récepteurs GPI-folate se trouvent dans des domaines qui sont plus petits que 70 nm (Varma and Mayor, 1998). Un autre groupe de chercheurs a aussi essayé de détecter les radeaux lipidiques par la technique de FRET en utilisant la toxine de choléra (CTX) et les protéines GPI comme marqueurs des radeaux lipidiques. Toutefois, la concentration des protéines GPI et de CTX ou l'existence de larges domaines lipidiques stables n'ont pas été observés. Ils ont donc suggéré que les radeaux lipidiques sont des domaines instables de la membrane plasmique et qu'ils existent seulement de façon transitoire (Kenworthy and Edidin, 1998; Kenworthy et al., 2000). Cette hypothèse concorde avec le fait que le regroupement des protéines GPI ou de d'autres composantes des radeaux lipidiques sont seulement visible lorsqu'ils sont entrecroisés (cross-linked) avec un anticorps secondaire (Mayor et al., 1994; Parton, 1994; Fujimoto, 1996). Donc, il a été suggéré qu'*in vivo*, les radeaux lipidiques sont des structures dynamiques qui sont stabilisées par l'aggrégation

des détergents ou des anticorps secondaires (Harder and Simons, 1997; Simons and Ikonen, 1997; Jacobson and Dietrich, 1999).

1.2.2.2 L'isolation et la purification des radeaux lipidiques

Dû à la composition lipidique des radeaux, il est possible de les isoler avec des méthodes biochimiques. En effet, la purification et la caractérisation des radeaux lipidiques ont permis l'identification des molécules qui sont enrichies dans ces microdomaines. Les radeaux lipidiques peuvent être isolés biochimiquement dû à leur résistance à la solubilisation par les détergents non-ioniques à 4°C, tel que le Triton X-100. Ensuite, ils sont purifiés par flotaison dans un gradient de sucrose de 5-30% où les radeaux lipidiques se retrouvent dans les fractions légères (Brown and Rose, 1992; Lisanti et al., 1995; Lisanti et al., 1999). Dernièrement, d'autres détergents tels que le NP-40, l'octylglucoside, CHAPS, le Lubrol et le Brij98 ont été utililés pour isoler les radeaux lipidiques (Ilangumaran et al., 1999; Roper et al., 2000; Drevot et al., 2002). Les radeaux lipidiques peuvent même être isolés sans l'utilisation de détergents (Song et al., 1996a). Cependant, selon la méthode d'isolation et du détergent utilisé, les lipides et les protéines qui sont enrichies dans les radeaux lipidiques peuvent varier d'une technique à l'autre.

La composition lipidique des cavéoles est très similaire aux radeaux lipidiques. Les cavéoles sont des microdomaines enrichies en cholestérol, en

phospholipides ainsi qu'en sphingolipides tels que la sphingomyéline, la céramide et les gangliosides (Parton, 1994; Liu and Anderson, 1995; Fujimoto, 1996). Même si les cavéoles partagent les propriétés biochimiques des radeaux lipidiques, les cavéoles sont définies comme un sous-domaine des radeaux lipidiques ayant des fonctions spécifiques qui leurs sont conférées par la présence de la cavéoline (Simons and Toomre, 2000). Toutefois, nos travaux suggèrent que l'endocytose effectuée par les radeaux lipidiques et les cavéoles sont des mécanismes similaires qui sont régulés par la dynamine, le cholestérol et la cavéoline (Nabi et Le, 2003). Nous allons donc utiliser le terme cavéoles/radeaux lipidiques pour identifier cette voie d'endocytose (voir la section III).

1.2.3 Les cavéoles

Dans les années 1950, Palade (Palade, 1953) et Yamada (Yamada, 1955) ont été les premiers à identifier les cavéoles selon leur morphologie par microscopie électronique. Palade les a appelé vésicules plasmalemmales (Palade, 1953) et Yamada les a nommé caveolae intracellularis pour "little cave" (Yamada, 1955). Environ 40 ans plus tard, la cavéoline fut identifiée comme un marqueur des cavéoles (Rothberg et al., 1992). Depuis, plusieurs fonctions ont été attribuées aux cavéoles tel que la transcytose dans les cellules endothéliales, la signalisation cellulaire, l'endocytose, le métabolisme des lipides, la régulation de la synthèse de l'oxyde nitrique et même l'internalisation

des pathogènes (Anderson, 1998; Rosenberger et al., 2000; Mineo and Anderson, 2001; Shin and Abraham, 2001).

1.2.3.1 Définition et morphologie

Par microscopie électronique, les cavéoles se distinguent des vésicules de clathrine par leur morphologie et leur taille. Elles se présentent comme de petites invaginations lisses de la membrane plasmique ayant un diamètre de 50-100 nm (Anderson, 1998; Mineo and Anderson, 2001). Leur composition, apparence et fonction varient selon le type cellulaire. Par exemple, dans les cellules endothéliales, les cavéoles peuvent avoir la forme d'une invagination ou d'une vésicule détachée de la membrane plasmique ou elles peuvent fusionner pour former un tubule ou un canal (Simionescu et al., 1975); dans les cellules adipocytes, lorsqu'elles sont détachées de la membrane plasmique, elles ont la forme de rosette (Scherer et al., 1994) et elles sont sous la forme de "grappe" dans les cellules musculaires squelettiques (Parton et al., 1997).

L'abondance des cavéoles varie selon le type cellulaire, toutefois elles sont morphologiquement identifiables dans la plupart des cellules et tissus. Les cavéoles sont présententes dans la plupart des cellules et elles sont surtout abondantes dans les cellules endothéliales et adipeuses. Toutefois, elles sont absentes dans les globules rouges, les plaquettes sanguines, les lymphocytes

(Fra et al., 1994), les neuroblastomes (Gorodinsky and Harris, 1995) et les cellules CaCo2 (Mirre et al., 1996).

1.2.3.2 La biogenèse des cavéoles

Les cavéoles sont des petites invaginations plasmalemmales lisses et la présence du cholestérol est essentiel pour leur formation. Une séquestration ou une déplétion du cholestérol membranaire par la filipine, la nystatine ou la mβCD a pour effet d'aplatir les cavéoles et d'inhiber leur internalisation (Rothberg et al., 1990a; Schnitzer et al., 1994; Smart et al., 1994; Anderson et al., 1996; Hailstones et al., 1998). La cavéoline lie le cholestérol (Murata et al., 1995) et le cholestérol est nécessaire pour stabiliser les oligomères de la cavéoline (Monier et al., 1995). Une oxydation du cholestérol induit le transfert de la cavéoline présente dans les cavéoles vers le réticulum endoplasmique (RE) et les compartiments de l'appareil de Golgi. La perte de la cavéoline à la membrane plasmique n'induit pas de diminution des cavéoles et n'affecte pas la morphologie des cavéoles (Smart et al., 1994).

Toutefois, d'autres études proposent que c'est la cavéoline qui est importante pour la formation des cavéoles. L'oligomérisation de la cavéoline autour des cavéoles servirait à former un "manteau" et à induire l'invagination des cavéoles (Lisanti et al., 1993). En accord avec cette hypothèse, une réduction ou une absence de la cavéoline est directement proportionnelle avec

la réduction ou l'absence des cavéoles à la surface des cellules, mais une transfection de la cavéoline-1 dans les cellules induit de nouveau la formation des cavéoles (Fra et al., 1995; Engelman et al., 1997; Fujimoto et al., 2000).

Les souris nulles de la cavéoline-1 montrent une grande perte des cavéoles à la membrane plasmique, cependant quelques invaginations similaires aux cavéoles peuvent être détectées (Drab et al., 2001; Razani et al., 2001a; Zhao et al., 2002). De même, dans les souris knock-out de la cavéoline-3 une perte des cavéoles a également été observée dans les cellules musculaires, alors que les autres types cellulaires qui expriment toujours les cavéoline-1 et 2 présentent des cavéoles à la membrane plasmique (Galbiati et al., 2001). Une double délétion de la cavéoline-1 et 3 montre une perte des cavéoles dans les cellules non-musculaires et musculaires (Park et al., 2002). Alors qu'une perte de la cavéoline-2 chez la souris montre aucun changement dans l'expression des cavéoles à la membrane plasmique (Razani et al., 2002a).

Lorsque la cavéoline-2 est exprimée dans les cellules qui n'expriment pas la cavéoline-1, la cavéoline-2 est incapable d'induire la formation des cavéoles. En fait, lorsque la cavéoline-2 est seulement exprimée dans les cellules, elle reste au niveau du Golgi et elle requiert la présence de la cavéoline-1 pour être transloquée à la membrane plasmique (Scheiffele et al., 1998; Mora et al., 1999; Parolini et al., 1999). La présence de la cavéoline-1 ou 3 semble être essentielle

à la formation des cavéoles. Il est possible que la cavéoline-1 et 3 aident au maintien du cholestérol dans les cavéoles. L'expression de la cavéoline aurait pour effet d'augmenter la quantité de cholestérol associé aux cavéoles et de stabiliser la formation des cavéoles.

1.2.3.3 Le mécanisme de l'endocytose des cavéoles

En plus de la cavéoline, les cavéoles contiennent aussi la dynamine (Henley et al., 1998; Oh et al., 1998), une GTPase qui est impliquée dans la fission des vésicules de clathrine (Hinshaw, 2000; McNiven et al., 2000). Cette molécule a été localisée au niveau du cou des invaginations cavéolaires (Henley et al., 1998; Oh et al., 1998) et similairement aux vésicules de clathrine, elle est également impliquée dans le détachement des cavéoles de la membrane plasmique pour former une vésicule libre (Henley et al., 1998; Oh et al., 1998). Les cavéoles des cellules endothéliales contiennent les machineries nécessaires pour que les cavéoles puissent se détacher de la membrane plasmique et être internalisées (Schnitzer et al., 1995a). En accord avec ces études, il a été montré dans un système in vitro que les cavéoles pouvaient se détacher de la membrane plasmique et que ce processus nécessitait la présence d'ATP, GTP ainsi que des facteurs cytosoliques (Gilbert et al., 1999). Ces études indiquent que les cavéoles possèdent les composantes requises pour se détacher de la membrane plasmique et éventuellement transporter son cargo vers l'intérieur de la cellule.

1.2.3.4 L'internalisation des cavéoles

Les cavéoles sont abondantes dans les cellules endothéliales et la première fonction qui fut attribuée aux cavéoles est la transcytose. La transcytose est un processus par lequel les protéines sanguines sont transportées à travers les cellules des capillaires (Simionescu et al., 1975). Avec des études de "time lapse" en microscopie électronique, il a été montré que l'albumine conjuguée à l'or colloïdale était transportée à travers les cellules endothéliales via les cavéoles (Ghitescu and Bendayan, 1992; Schnitzer and Oh, 1994; Predescu et al., 1997). Puis, durant les dernières années, plusieurs études ont montré que les cavéoles sont aussi impliquées dans l'endocytose de multiples molécules et récepteurs (voir Table 1).

CTX fut le premier marqueur à être utilisé pour l'étude des cavéoles dans l'endocytose des cellules non-endothéliales. CTX est concentré dans les cavéoles et ces vésicules médient l'internalisation de cette toxine vers les compartiments intracellulaires (Montesano et al., 1982; Parton, 1994). Le traitement des cellules avec des agents qui lient le cholestérol tel que la filipin et la nystatin bloque l'endocytose de CTX (Orlandi and Fishman, 1998; Gilbert et al., 1999). Toutefois, de récentes études montrent que CTX pourrait être concentré dans les cavéoles à la surface des cellules, mais elle n'est pas nécessairement endocytée par les cavéoles (Shogomori and Futerman, 2001a;

Torgersen et al., 2001). Les mécanismes d'internalisation de CTX sont complexes et seront discutés plus en détail dans le chapitre 4.

L'internalisation de la phosphatase alkaline montre également le rôle des cavéoles dans l'endocytose. Il a été démontré que la phosphatase alkaline est recrutée dans les cavéoles suite à une liaison croisée "cross linking" avec un anticorps et que les cavéoles médient son internalisation. De plus, cette internalisation est accélérée par un traitement avec un inhibiteur des phosphatases cellulaires, l'acide okadaic. Des études de microscopie électronique ont montré que sous l'action de l'acide okadaic les cavéoles pouvaient se détacher de la membrane plasmique et être internalisées dans la cellule. Une destruction des filaments d'actine avec la cytochalasine D bloque l'internalisation de la phosphatase alkaline (Parton et al., 1994). Le rôle des cavéoles dans l'endocytose fut également mis en évidence avec la démonstration de l'internalisation du simian virus 40 (SV40) vers le réticulum endoplasmique (Kartenbeck et al., 1989; Anderson et al., 1996; Stang et al., 1997; Chen and Norkin, 1999).

Plus récemment, avec l'utilisation de la cavéoline-1 marquée avec la GFP (green fluorescent protein) et des techniques de vidéo microscopie, il a été démontré dans les cellules vivantes que les cavéoles contenant la cavéoline ainsi que SV40 ou CTX se détachent de la membrane plasmique pour transporter son cargo vers l'intérieur de la cellule (Pelkmans et al., 2001;

Nichols, 2002; Pelkmans et al., 2002). Plus spécifiquement, la liaison de SV40 avec les cavéoles induit l'activation des tyrosines kinases et ensuite, un remodelage du cytosquelette d'actine ainsi que le recrutement de la dynamine aux niveaux des cavéoles (Pelkmans et al., 2002).

Ces études ont également identifié l'existence d'un compartiment intermédiaire dans l'internalisation de SV40 vers le réticulum endoplasmique et de CTX vers l'appareil de Golgi. Ce compartiment endocytaire appelé cavéosome se distingue des endosomes et des lysosomes classiques par la présence de la cavéoline et l'absence de marqueurs tels que la transferrine, EEA1, rab5 et rab11 (Pelkmans et al., 2001; Mundy et al., 2002; Nichols, 2002; Pelkmans et al., 2002). Les cavéosomes ont un pH neutre (Pelkmans et al., 2001) et sont localisé près du centre d'organisation des microtubules (MTOC) et dans la même région que les endosomes de recyclage positifs pour le récepteur de transferrine. Ainsi, par microscopie à immunofluorescence, il est difficile de faire la distinction entre les deux compartiments (Mundy et al., 2002).

Même si ces études mettent en évidence le rôle des cavéoles dans l'endocytose, ce ne sont pas toutes les cavéoles à la membrane plasmique qui peuvent être internalisées. Des analyses par vidéo microscopie et par photo-bleaching (FRAP) dans les cellules vivantes transfectées avec la cavéoline-1-GFP indique que les cavéoles sont des structures plutôt stationnaires, soutenues par l'actine cortical sous la membrane plasmique et seulement une

minorité d'entres elles sont dynamiques et se déplacent vers l'intérieur de la cellule. Ainsi, les cavéoles ne sont pas impliquées dans l'endocytose de façon constitutive, mais il semblerait qu'elles soient uniquement internalisées suite à une signalisation cellulaire (Mundy et al., 2002; Thomsen et al., 2002; van Deurs et al., 2003).

2. LA CAVÉOLINE

Un patron de cercle concentrique a été observé à la surface cytosolique des cavéoles par des techniques de microscopie à balayage à haute résolution et par cryodécapage (Peters et al., 1985; Rothberg et al., 1992). Il a été démontré par immunocytochimie que la cavéoline décore la surface cytosolique des cavéoles (Rothberg et al., 1992; Monier et al., 1995). Depuis cette découverte, la cavéoline est considérée comme un marqueur des cavéoles et comme la clathrine, elle est la protéine "manteau" qui entoure les cavéoles.

2.1 Définition

La cavéoline est une protéine intégrale de la membrane de 21 kDa, elle a d'abord été identifiée comme étant une protéine phosphorylée sur son résidu de tyrosine dans les cellules transformées par le virus de Rous sarcoma (Glenney, 1989). Il existe trois gènes de la cavéoline dans les cellules de mammifère, les cavéolines 1, 2 et 3 et ceux-ci code pour cinq différentes isoformes de la

cavéoline (Smart et al., 1999). Il existe deux isoformes de la cavéoline-1: la cavéoline 1α et 1β. La présence des deux isoformes de la cavéoline-1 a été observée dans les cavéoles fortement invaginées, alors que seul l'isoforme β était détecté dans les cavéoles moins invaginées (Fujimoto et al., 2000).

Les cavéolines-1 et 2 sont souvent exprimées ensemble dans la plupart des cellules de mammifère et la cavéoline-3 est surtout exprimée dans les cellules musculaires, les astrocytes et les chondrocytes (Song et al., 1996b; Tang et al., 1996; Scherer and Lisanti, 1997; Ikezu et al., 1998; Okamoto et al., 1998; Nishiyama et al., 1999; Schwab et al., 1999). En plus de sa localisation à la membrane plasmique, la cavéoline est aussi présente dans le réseaux trans-Golgien (Kurzchalia et al., 1992; Dupree et al., 1993) et dans un compartiment endocytaire appelé cavéosome (Pelkmans et al., 2001).

Toutes les cavéolines possèdent un domaine centrale hydrophobique d'environ trente-trois acides aminés et ce domaine leur permet d'adopter une conformation d'épingle à cheveux dans la membrane laissant ainsi, le côté N- et C-terminal face au cytoplasme (Monier et al., 1995). La cavéoline peut former des dimères ou des oligomères *in vitro* et *in vivo* (Monier et al., 1995). De plus, elle se lie directement avec le cholestérol (Murata et al., 1995) ainsi qu'aux multiples molécules de signalisation cellulaire (Liu et al., 2002).

2.2 La cavéoline et la signalisation cellulaire

La purification biochimique des cavéoles a montré une concentration de plusieurs molécules de signalisation incluant les protéines Gα, H-ras, la famille des tyrosine kinases Src, la synthase de l'oxyde nitrique (eNOS) (Razani et al., 2002b). La localisation de toutes ces protéines dans les cavéoles suggère un rôle pour les cavéoles et la cavéoline dans la signalisation cellulaire. La cavéoline possède un domaine appelé "scaffolding domain" (CSD) qui lui permet d'interagir directement avec d'autres molécules de signalisation qui sont aussi localisées dans les cavéoles (Okamoto et al., 1998).

La sous-unité Gα des protéines G est enrichie au niveau des cavéoles et elle interagit avec la cavéoline via son domaine CSD. La localisation de la sous-unité Gα dans les cavéoles serait dans le but de garder la sous-unité Gα sous une forme inactive (Li et al., 1995 ; Li et al., 1996). De plus, il a été montré que la cavéoline se lie préférentiellement avec la forme inactive de la tyrosine kinase c-Src et la liaison de la cavéoline avec c-Src aurait pour effet de supprimer l'auto-activation de celle-ci (Li et al., 1996). Un autre exemple du rôle de la cavéoline dans la signalisation cellulaire est son interaction avec eNOS. Cette enzyme est localisée dans les cavéoles et lorsqu'elle interagit avec la cavéoline, sa fonction est inhibée. Ainsi, l'interaction de la cavéoline avec eNOS a pour effet de maintenir cette enzyme inactive (Garcia-Cardena et al., 1997).

Il a également été montré que les récepteurs couplés aux protéines G sont concentrés dans les cavéoles. Le récepteur β-adrénergique est localisé au niveau des cavéoles (Schwencke et al., 1999 ; Ostrom et al., 2000 ; Rybin et al., 2000). Ces récepteurs sont concentrés dans les cavéoles lorsqu'ils sont en état de repos et suite à une stimulation, ils sont recrutés vers les vésicules de clathrine pour être internalisés (Ostrom et al., 2001). La liaison ou l'association de certaines protéines avec la cavéoline se produit dans le but de maintenir les molécules de signalisation inactives. La cavéoline agirait donc comme un régulateur négatif pour inhiber l'activité basale de plusieurs protéines de signalisation cellulaire et suite à une stimulation, l'effet inhibitrice de la cavéoline serait relâché et ainsi une augmentation dans la propagation des signaux aurait lieu (Okamoto et al., 1998 ; Smart et al., 1999 ; Ostrom et al., 2000).

2.3 La cavéoline et le cancer

L'association de la cavéoline avec plusieurs molécules de signalisation suggère qu'une absence ou une augmentation du niveau d'expression de la cavéoline pourrait dérégler le fonctionnement des cellules. Cependant, un lien direct entre le niveau d'expression de la cavéoline-1 et le développement des cellules tumorales est encore controversé.

Dans les cellules NIH-3T3 transformées avec l'oncogène H-ras ou v-abl, une diminution de la cavéoline et des cavéoles a été observée. De plus, ces

cellules présentent toutes les caractéristiques des cellules transformées (Koleske et al., 1995). Il a aussi été montré que les cellules NIH-3T3 qui sont transfectées avec un ARN anti-sense codant pour la cavéoline-1 induit la formation de tumeurs chez les souris immunodéficientes (Lee et al., 1998; Racine et al., 1999). L'utilisation d'un anti-sense pour réduire le niveau d'expression de la cavéoline-1 dans les cellules NIH-3T3 hyperactive la cascade des MAP kinases ras-p42/44 et induit la transformation des cellules (Galbiati et al., 1998). La réintroduction de la cavéoline recombinante dans les fibroblastes transformées ou les cellules cancéreuses du sein est suffisante pour inverser le phénotype transformé des cellules (Engelman et al., 1997; Lee et al., 1998). Plusieurs études ont constaté une diminution de la cavéoline-1 dans différentes lignées de cellules cancéreuses des poumons (Racine et al., 1999) et dans le cancer du colon (Bender et al., 2000). Par contre, d'autres études montrent qu'une augmentation du niveau d'expression de la cavéoline-1 est plutôt associée avec la progression du cancer de la prostate et du sein (Nasu et al., 1998; Yang et al., 1998; Yang et al., 1999; Li et al., 2001; Tahir et al., 2001).

Dans le cancer de la prostate, la forte expression de la cavéoline aurait pour effet de supprimer la capacité de c-myc d'induire l'apoptose (Timme et al., 2000). Il y a des évidences qui indiquent que la cavéoline et les cavéoles sont impliquées dans la régulation des signaux qui contrôlent l'apoptose. La cavéoline-1 interagit et inactive certaines molécules impliquées dans la survie et la prolifération cellulaire, tels que le récepteur PDGF et la phosphatidylinositol-3-

kinase (Liu et al., 1996; Yamamoto et al., 1999; Zundel et al., 2000). Les cavéolines 1 et 2 humaines sont localisées au niveau du chromosome 7 (q31.1) alors que la cavéoline-3 est située sur le chromosome 3 (p25). Les gènes codant pour les cavéolines 1 et 2 sont situés dans la région des génomes qui sont fréquemment perdus dans les tumeurs malignes. Cette région du chromosome 7 contiendrait un gène suppresseur de tumeur (Engelman et al., 1998a; Engelman et al., 1998b; Engelman et al., 1999; Razani et al., 2001b). Toutefois le rôle de la cavéoline-1 comme un agent suppresseur de tumeur demeure controversé.

Malgré une diminution du niveau d'expression de la cavéoline-1 dans plusieurs tumeurs, les souris nulles de la cavéoline-1 ne montrent pas une plus forte incidence pour le développement des carcinomes (Razani et al., 2001a). C'est seulement récemment qu'il a été montré que les souris nulles de la cavéoline-1 sont plus susceptibles de développer des tumeurs lorsqu'elles sont en contact avec des agents cancérigènes (Capozza et al., 2003) ou lorsque ces souris sont croisées avec des souris transgéniques qui ont tendance à développer des tumeurs (tumor-prone transgenic mice) (Williams et al., 2003).

2.4 Le phénotype des souris knockout de la cavéoline

Les cavéoles et les cavéolines sont impliquées dans de nombreuses activités cellulaires et pour étudier le rôle des cavéoles in vivo, des souris

knockout des différentes cavéolines ont été générées. Curieusement, les souris nulles des cavéolines-1, 2 et 3, ou des cavéolines-1 et 3 ensemble sont toujours viables et fertiles (Drab et al., 2001; Galbiati et al., 2001; Razani et al., 2001a; Park et al., 2002; Razani et al., 2002a). Une perte de la cavéoline-1 chez les souris nulles pour la cavéoline-1 montrent une absence des cavéoles dans les cellules endothéliales et adipeuses. De plus, en absence des cavéoles dans les cellules endothéliales, la transcytose de l'albumine est inhibée (Razani et al., 2001a; Schubert et al., 2001). Toutefois, un autre groupe a montré que la concentration de l'albumine dans le fluide cérébro-spinal est normal (Drab et al., 2001). Il est possible qu'en absence des cavéoles, une autre voie d'endocytose puisse être utilisée pour compenser l'absence de ces protéines.

Les souris knockout de la cavéoline-1 présentent de sévères anomalies pulmonaires, une vasodilatation excessive des vaisseaux sanguines due à l'hyperactivité de eNOS (Drab et al., 2001; Razani et al., 2001a; Zhao et al., 2002) et un dérèglement de l'homéostase des lipides (Razani et al., 2002a). Alors que les souris knockout de la cavéoline-2 présentent surtout des anomalies pulmonaires (Razani et al., 2002a). La malformation des tubules-T a été observé chez les souris knockout de la cavéoline-3 (Galbiati et al., 2001). Une cardiomyopathie sévère a été observée chez les souris qui ont subi une double délétion des cavéolines-1 et 3 (Park et al., 2002). La cavéoline est donc impliquée dans plusieurs fonctions cellulaires, mais elle n'est pas essentielle pour la survie des souris.

3. LE FACTEUR AUTOCRINE DE MOTILITÉ (AMF) ET SON RÉCEPTEUR (AMF-R)

3.1 Le facteur autocrine de motilité

L'AMF est une cytokine de 55 kDa en condition réduite et de 64 kDa en condition non-réduite. L'AMF a été isolé à partir des cellules de mélanomes humaines A2058 et il est connu pour son rôle dans la régulation de la motilité des cellules sécrétrices (Liotta et al., 1986). Comme la neuroleukine, le facteur de maturation, l'AMF est équivalente et à la phosphoglucose isomérase (PGI), une enzyme glycolytique (Chaput et al., 1988; Faik et al., 1988; Watanabe et al., 1996; Xu et al., 1996; Sun et al., 1999).

La PGI fait partie des protéines appelées "moonlighting proteins", des protéines qui peuvent performer plus d'une fonction tout dépendamment de leur localisation cellulaire (Jeffery, 1999). La PGI fonctionne comme une enzyme glycolytique qui convertit le glucose-6-phosphate en fructose-6-phosphate lorsqu'elle est localisée dans le cytoplasme et elle agit comme une cytokine lorsqu'elle est sécrétée (Chaput et al., 1988; Faik et al., 1988; Jeffery et al., 2000). La voie de secrétion de l'AMF est inconnue et une absence de peptide signal pour la sécrétion dans la séquence de l'AMF indique que l'AMF est possiblement secrété par une voie non-classique (Kuchler, 2000).

3.1.1 Les multiples fonctions de l'AMF/PGI

Comme cytokine et sous le nom de l'AMF, elle induit la migration cellulaire *in vitro* et la métastase *in vivo* (Liotta et al., 1986; Nabi et al., 1990; Silletti et al., 1991; Watanabe et al., 1991b; Watanabe et al., 1996). La cytokine neuroleukine induit la croissance et la survie des neurones sensoriels et spinales ainsi que des lymphocytes B (Gurney et al., 1986a; Gurney et al., 1986b). Elle agit également comme un facteur de maturation qui médie la différentiation des cellules précurseurs myéloïdes en monocytes matures (Xu et al., 1996).

Une déficience de la PGI chez l'humain cause une anémie hémolytique, une maladie qui est causée par un désordre génétique autosomal récessif (Kugler et al., 1998). Chez 64% des personnes atteintes de la maladie de l'arthrite rhumatoïde, des anticorps contre la PGI sont présents en concentration élevée dans le sérum et le fluide synovial. De plus, par immunohistochimie, une forte concentration de la PGI a été détectée à la surface des cellules endothéliales des artérioles indiquant que la liaison de la PGI avec son anticorps soit responsable de la maladie de l'arthrite rhumatoïde (Matsumoto et al., 1999; Schaller et al., 2001; Matsumoto et al., 2002).

Il a aussi été rapporté que la neuroleukine pourrait avoir un rôle dans la différenciation et la minéralisation des ostéoblastes (Zhi et al., 2001). Il existe

aussi des évidences qui montrent un rôle pour la PGI dans l'agglutination des spermatozoïdes (Yakirevich and Naot, 2000). Il a également été montré qu'une augmentation de l'expression de la neuroleukine et de l'AMF-R dans l'hippocampe des rats est associée avec l'apprentissage et la mémorisation. Une réduction de l'expression de la neuroleukine et de l'AMF-R est observé chez les rats plus âgés ainsi que ceux qui ont des difficultés d'apprentissage (Luo et al., 2002). De plus, une déficience de la PGI chez l'humain cause une anémie hémolytique qui pourrait être associé à un désordre neurologique (Kugler et al., 1998). Ces études montrent que l'AMF et son récepteur sont impliqués dans divers processus cellulaires et, par conséquent un contrôle du niveau d'expression de l'AMF et de son récepteur est très important pour le bon fonctionnement des cellules.

3.1.2 L'activité enzymatique et cytokine de l'AMF/PGI

Les structures cristallographiques de PGI de lapin et de Bacillus stearothermophilus montrent que le site actif de l'enzyme est conservé à travers l'évolution (Sun et al., 1999; Chou et al., 2000; Jeffery et al., 2000; Jeffery et al., 2001; Lee et al., 2001). Il a été rapporté que la 5-phospho-D-arabinonate (5PA) et la N-bromoacetylethanolamine phosphate (BAP), des inhibiteurs de l'activité enzymatique de la PGI, sont aussi capable d'inhiber la motilité des cellules murines tumorales du colon qui a été induite par l'activité cytokine de l'AMF.

Cette étude indique que le site de liaison de l'AMF avec son récepteur est à proximité du site enzymatique de la PGI (Sun et al., 1999; Chou et al., 2000).

Contrairement à la PGI de mammifère (lapin), la PGI bactérienne n'est pas endocytée dans les cellules NIH-3T3. De plus, la PGI bactérienne ou de la levure ne compétitionne pas avec la PGI de mammifère pour la liaison avec l'AMF-R et pour l'endocytose. Il a été rapporté que la PGI bactérienne et de la levure ne stimule pas la motilité des cellules NIH-3T3 (Amraei and Nabi, 2002). Cette étude montre que l'activité cytokine de la PGI est spécifique à la PGI des mammifères. Toutefois, une autre étude a montré que la PGI bactérienne stimule la motilité des cellules CT-26, et cette différence pourrait être expliquée par l'utilisation de différentes lignées cellulaires (Sun et al., 1999; Chou et al., 2000). La disparition des acides aminés 331 et 332 causée par une mutation de la PGI induit une perte de son activité enzymatique, mais son activité cytokine et sa capacité de se lier avec son récepteur AMF-R sont conservées (Tsutsumi et al., 2003a). Ces résultats indiquent que même si le site actif de l'enzyme est conservé durant l'évolution, il n'est pas suffisant pour son activité cytokine et pour sa liaison avec son récepteur.

3.2 Le récepteur du facteur autocrine de motilité

L'AMF-R ou gp78 est une glycoprotéine avec un poids moléculaire de 78 kDa qui a été identifié par l'augmentation de sa glycosylation lors du transfert

des cellules B16-F1 cultivées en monocouche comparée à une culture en suspension (Nabi and Raz, 1987). L'anticorps dirigé contre l'AMF-R est capable de stimuler à la fois la colonisation des poumons *in vivo* et la motilité cellulaire *in vitro* et ceci a permis l'identification de l'AMF-R comme étant le récepteur de l'AMF (Nabi et al., 1990; Silletti et al., 1991; Watanabe et al., 1991a; Watanabe et al., 1991b).

L'AMF-R est exprimé à la surface et à l'intérieur des cellules au niveau d'un compartiment tubulaire (Nabi et al., 1992). Par microscopie à fluorescence, le marquage de l'AMF-R se distingue des endosomes, des lysosomes, de l'appareil de Golgi, du réticulum de transition ainsi que du RE rugueux (RER) qui ont été respectivement marqués avec les anticorps contre le récepteur de transferrine, lamp-2, β-COP, ERGIC-53 et calnexine (Benlimame et al., 1995; Wang et al., 1997). Des études en microscopie électronique du marquage de l'AMF-R en post-enrobage dans les cellules MDCK, NIH-3T3 et Hela ont montrées que l'AMF-R est majoritairement localisé au niveau d'un organite membranaire lisse qui peut être sous une forme vésiculaire ou tubulaire (Benlimame et al., 1995). Une faible portion de l'AMF-R est aussi localisée à la membrane plasmique (Benlimame et al., 1998).

Les cellules MDCK traitées avec l'ilimaquinone, un métabolite des éponges de mer qui induit la fragmentation de l'appareil de Golgi (Takizawa et al., 1993), induit également une redistribution des tubules AMF-R en réseaux

fenestrés de tubules interconnectés qui sont morphologiquement équivalents au RE lisse (REL) (Wang et al., 1997). Il a également été montré que l'organisation des tubules AMF-R est dépendante des microtubules (Benlimame et al., 1995; Nabi et al., 1997). Par microscopie confocale, un double marquage de l'AMF-R avec la calnexine et la calréticuline, des marqueurs du RE, montrent que la majorité du marquage de l'AMF-R ne colocalise pas avec ces marqueurs. Toutefois, le marquage de l'AMF-R se retrouve souvent à proximité du marquage de la calnexine ou de la calréticuline (Wang et al., 2000).

Le REL est utilisé comme un lieu d'entreposage du calcium intracellulaire qui peut être libéré rapidement dans le cytoplasme suite à une stimulation de la cellule (Pozzan et al., 1994; Golovina and Blaustein, 1997). Concordant avec son rôle dans la régulation du calcium intracellulaire, dans les cellules MDCK, les tubules AMF-R sont étroitement associés avec les mitochondries et cette association est régulée par le calcium (Wang et al., 2000). Les tubules AMF-R sont donc des compartiments spécialisés du REL et l'AMF-R est un marqueur spécifique pour le REL.

3.2.1 L'AMF-R est une ubiquitine ligase E3

La dégradation des protéines aberrantes par le RE est médiée par la voie de l'ubiquitine et du protéasome. Le complexe de l'ubiquitine est formé par: 1) l'enzyme d'activation de l'ubiquitine (E1); 2) l'enzyme de conjugaison à

l'ubiquitine (E2); 3) une ligase qui lie l'ubiquitine à la protéine (E3). Ces enzymes vont ajouter des ubiquitines sur les protéines qui sont destinées à la dégradation par le protéosome. Il a également été montré qu'un domaine CUE (factor for coupling of ubiquitin conjugation to ER degradation) à la surface du RE est important pour le recrutement des enzymes E1 et E2 à la membrane du RE (Biederer et al., 1997).

L'AMF-R est un récepteur à sept domaines transmembranaire, auquel les domaines CUE et "ring-finger" ont été identifiés du côté C-terminal (Shimizu et al., 1999; Ponting, 2000). Il a récemment été montré que l'AMF-R est une ligase de l'ubiquitine E3 qui serait impliquée dans le processus de dégradation à partir du RE (Fang et al., 2001). La ligase E3 se lie directement à l'enzyme E2 et aux substrats CD3-δ. Cette ligase a pour fonction d'ubiquitiner les protéines nucléaires et cytoplasmiques qui sont destinées à la dégradation par le protéasome (Biederer et al., 1997). L'AMF-R est également impliqué dans la dégradation de l'apolipoprotéine B100 (Liang et al., 2003).

3.3 Le rôle de l'AMF et de l'AMF-R dans la motilité cellulaire et le cancer

La surexpression de l'AMF et de l'AMF-R est associée avec l'acquisition des capacités motiles et invasives des cellules tumorales (Silletti and Raz, 1996) et par conséquent, elle augmente le niveau d'agressivité des tumeurs et les métastases *in vivo* (Nakamori et al., 1994; Otto et al., 1994; Maruyama et al.,

1995; Hirono et al., 1996; Takanami et al., 1998 ; Taniguchi et al., 1998; Takanami et al., 2001; Niinaka et al., 2002; Takanami et al., 2002). Une forte concentration de l'AMF/PGI a été observée dans l'urine des personnes ayant des cancers de la vessie, gastro-intestinaux, colorectaux, des seins, des poumons et des reins. La présence de la PGI dans le sérum peut être utilisée comme marqueur pour la détection des personnes ayant des tumeurs malignes (Bodansky, 1954; Schwartz, 1973; Tor et al., 1981 ; Tor et al., 1982 ; Baumann et al., 1988; Guirguis et al., 1988 ; Guirguis et al., 1990 ; Filella et al., 1991; Patel et al., 1995; Baumgart et al., 1996).

L'AMF stimule la motilité des cellules sécrétrices de façon aléatoire ou directionnelle (Liotta et al., 1986). L'AMF induit la motilité cellulaire suite à sa liaison avec son récepteur qui est exprimé à la surface des cellules (Nabi et al., 1990; Silletti et al., 1991; Watanabe et al., 1991a). Cette motilité ce traduit par une activation de la protéine G sensible à la toxine de pertussis, la production d'inositol triphosphate, la phosphorylation du récepteur par des tyrosines kinases, l'activation de la protéine kinase C et la production du métabolite de la lipoxygénase 12-HETE (Stracke et al., 1987; Kohn et al., 1990; Watanabe et al., 1991a; Silletti et al., 1994). Il a été rapporté que l'AMF est un activateur de Rho et de Rac1 qui induit un réarrangement du cytosquelette et ce phénomène est associé avec une induction de la motilité cellulaire (Tsutsumi et al., 2002). La surexpression de l'AMF dans les cellules NIH-3T3 induit la secrétion de l'AMF et une diminution de l'expression de la cavéoline-1. De plus, cette surexpression

rend les cellules NIH-3T3 plus motiles, augmente la prolifération cellulaire et induit la transformation des cellules. Contrairement aux cellules NIH-3T3 de type sauvage, les cellules NIH-3T3 qui surexpriment l'AMF sont capables de former des tumeurs dans les souris nudes et une augmentation de microvaisseaux a été observée aux niveaux de ces tumeurs (Tsutsumi et al., 2003b).

L'AMF et l'AMF-R stimule la motilité cellulaire durant l'invasion et les métastases des cellules tumorales par l'activation des intégrines-β1 (Liotta et al., 1986). L'AMF augmente l'invasion des cellules de l'hépatome de façon autocrine en stimulant l'adhésion, la motilité et la sécrétion des métalloprotéases de la matrice 2 (MMP2) (Torimura et al., 2001). L'AMF est connu pour sa capacité de réguler la motilité et d'induire des métastases des cellules tumorales via son interaction avec son récepteur. Cette interaction peut également stimuler la motilité des cellules endothéliales et l'angiogénèse par une augmentation de l'expression de VEGF et de son récepteur (Funasaka et al., 2001; Funasaka et al., 2002).

4. LA TOXINE DE CHOLERA

CTX est une entérotoxine qui est libérée par la *cholerae Vibrio*. CTX est formée par les sous unité A et B. La sous-unité A est composée de deux peptides, A1 et A2, qui sont liés par un pont disulfure et le peptide A2 est lié à cinq sous-unités B (Middlebrook and Dorland, 1984; Spangler, 1992). Les sous-

unités B de CTX se lient de façon spécifique aux gangliosides de type GM1 (Critchley et al., 1982; Fishman, 1982) et CTX est endocytée vers l'appareil de Golgi puis, par un transport rétrograde vers le RE. Dans le RE, le peptide A1 est ensuite transloqué dans le cytoplasme pour activer l'adénylate cyclase et induire la production de l'AMP cyclique (Spangler, 1992; Lencer et al., 1993; Nambiar et al., 1993; Orlandi et al., 1993; Lencer et al., 1995; Majoul et al., 1996; Orlandi, 1997).

Des études en microscopie électronique ont localisé CTX au niveau des cavéoles dans plusieurs types cellulaires et suggèrent que les cavéoles soient impliquées dans l'endocytose de CTX (Montesano et al., 1982; Tran et al., 1987; Parton, 1994). La surexpression de la dynamine K44A, l'ajout du GTP-γ-S ou l'injection d'un anticorps contre la dynamine, ont tous pour effet de bloquer le détachement des cavéoles de la membrane plasmique et d'empêcher l'endocytose de CTX dans les cellules endothéliales ou dans les hépatocytes en culture (Schnitzer et al., 1996; Henley et al., 1998; Oh et al., 1998).

De plus, un traitement des cellules avec des agents pharmacologiques qui défont les cavéoles tels que la filipine, la nystatine et la mβCD, bloque l'endocytose de CTX (Orlandi and Fishman, 1998; Puri et al., 2001; Wolf et al., 2002). Un traitement avec la chlorpromazine, une déplétion du potassium ou l'expression d'un mutant de l'Eps15, afin de bloquer l'endocytose dépendante des vésicules de clathrine, ont peu d'effet sur le transport de CTX vers l'appareil

de Golgi et l'activation de CTX (Orlandi and Fishman, 1998; Nichols et al., 2001; Puri et al., 2001; Wolf et al., 2002). Une inhibition du transport intracellulaire avec l'expression d'un mutant de rab5 n'a pas bloqué l'internalisation de CTX vers l'appareil de Golgi (Nichols et al., 2001). Ces résultats indiquent que les cavéoles médient l'endocytose de CTX vers l'appareil de Golgi.

Toutefois, d'autres études ont montré que dans les cellules neuronales de l'hippocampe, l'internalisation de CTX est dépendante des vésicules de clathrine puisque son endocytose vers l'appareil de Golgi est inhibée par la chlorpromazine, alors que la filipine ou la mβCD n'a aucun effet sur l'internalisation de CTX (Sofer and Futerman, 1995; Shogomori and Futerman, 2001a). La mβCD aurait cependant pour effet de bloquer le transport de CTX des endosomes vers l'appareil de Golgi (Shogomori and Futerman, 2001b). Il a aussi été suggéré que dans les cellules CaCo2, CTX puisse être internalisé via une troisième voie qui serait médiée par les vésicules non-enrobées de clathrine dû à son indépendance à la dynamine et son insensibilité au traitement avec la filipine ou lors de la surexpression de la cavéoline-1 (Torgersen et al., 2001).

ARTICLE 1

Localization of autocrine motility factor receptor to caveolae and clathrin-independent internalization of its ligand to smooth endoplasmic reticulum

Benlimame, N., P.U. Le, and I.R. Nabi. (1998).

Mol. Biol. Cell. 9:1773-1786.

CONTRIBUTION DES AUTEURS

J'ai effectué les études de l'internalisation de l'AMF biotinylé et de la transferrine en microscopie à fluorescence (Figures 4 et 5). J'ai complété les études sur l'internalisation de l'AMF biotinylé en microscopie électronique (une partie de la figure 6). J'ai également participé aux discussions reliant les résultats, la rédaction et la révision de l'article.

Naciba Benlimame a effectué la majorité des expériences de microscopie électronique. Elle a localisé l'AMF-R dans les tubules AMF-R et dans les cavéoles par microscopie électronique et à fluorescence. Elle a également fait la conjugaison de l'AMF avec la biotine et elle a déterminé par immunobuvardage que l'AMF était bien biotinylé. De plus, par microscopie à fluorescence, elle a déterminé que l'AMF biotinylé soit bien reconnu par son récepteur. Elle a aussi commencé les études de l'internalisation de l'AMF biotinylé en microscopie électronique.

Ivan R. Nabi a principalement contribué à la rédaction de l'article.

LOCALIZATION OF AUTOCRINE MOTILITY FACTOR RECEPTOR TO CAVEOLAE AND CLATHRIN-INDEPENDENT INTERNALIZATION OF ITS LIGAND TO SMOOTH ENDOPLASMIC RETICULUM

Naciba Benlimame, Phuong U. Le and Ivan R. Nabi

Département de pathologie et biologie cellulaire, Université de Montréal, Montréal, Québec, Canada H3C 3J7

Running title: AMF-R endocytosis

Key words: endocytosis, AMF-R, cell motility, NIH-3T3 fibroblasts, HeLa

Corresponding author:

Dr. Ivan R. Nabi

Département de pathologie et biologie cellulaire

Université de Montréal

[redacted]

C. P. 6128, succursale A

Montréal, Québec

Canada H3C 3J7

Tel: (514) 343-6291

Fax: (514) 343-2459

E-mail:

Courier address:

Dr. Ivan R. Nabi

Département de pathologie et biologie cellulaire

Université de Montréal, Pavillion principale N-812

2900 Blvd. Edouard Montpetit

Montréal, Québec

Canada, H3T 1J4

ABSTRACT

Autocrine motility factor receptor (AMF-R) is a cell surface receptor which is also localized to a smooth subdomain of the endoplasmic reticulum (ER), the AMF-R tubule. By post-embedding immunoelectron microscopy, AMF-R concentrates within smooth plasmalemmal vesicles or caveolae in both NIH-3T3 fibroblasts and HeLa cells. By confocal microscopy, cell surface AMF-R labeled by the addition of anti-AMF-R antibody to viable cells at 4°C exhibits partial colocalization with caveolin confirming the localization of cell surface AMF-R to caveolae. Labeling of cell surface AMF-R by either anti-AMF-R antibody or biotinylated AMF (bAMF) exhibits extensive colocalization and after a pulse of 1-2 hours at 37°C, bAMF accumulates in densely labeled perinuclear structures as well as fainter tubular structures which colocalize with AMF-R tubules. After a subsequent 2-4 hour chase bAMF is localized predominantly to AMF-R tubules. Cytoplasmic acidification, blocking clathrin-mediated endocytosis, results in the essentially exclusive distribution of internalized bAMF to AMF-R tubules. By confocal microscopy, the tubular structures labeled by internalized bAMF show complete colocalization with AMF-R tubules. bAMF internalized in the presence of a 10-fold excess of unlabeled AMF labels perinuclear punctate structures, which are therefore the product of fluid phase endocytosis, but not AMF-R tubules demonstrating that bAMF targeting to AMF-R tubules occurs via a receptor-mediated pathway. By electron microscopy, bAMF internalized for 10 minutes is located to cell surface caveolae and after 30 minutes is present within smooth and rough ER tubules. AMF-R is therefore internalized via a receptor-mediated clathrin-independent pathway to smooth endoplasmic reticulum. The steady state localization of AMF-R to caveolae implicates these cell surface invaginations in AMF-R endocytosis.

INTRODUCTION

Expression of autocrine motility factor receptor (AMF-R) is associated with the acquisition of motile and metastatic properties by tumor cells (for review see (Silletti and Raz, 1996)). AMF-R expression correlates with the malignancy of human bladder, colon and gastric tumors (Nakamori *et al.*, 1994; Otto *et al.*, 1994; Hirono *et al.*, 1996). In cultured cells, AMF-R expression is decreased in contact inhibited A31-3T3 fibroblasts (Silletti and Raz, 1993) and increased following viral transformation of MDCK epithelial cells (Simard and Nabi, 1996). AMF-R is a cell surface receptor which mediates motility stimulation by its 55 kD polypeptide ligand, AMF, recently shown to be homologous to phosphohexose isomerase (Liotta *et al.*, 1986; Watanabe *et al.*, 1996). AMF is selectively secreted following transformation of NIH-3T3 cells and the presence of AMF activity in the urine of cancer patients correlates with tumor malignancy (Liotta *et al.*, 1986; Guirguis *et al.*, 1990).

Transduction of the AMF motility signal occurs via receptor phosphorylation, a pertussis-toxin sensitive G-protein, inositol phosphate production, tyrosine kinase and protein kinase C activation and production of the lipoxygenase metabolite 12-HETE (Silletti and Raz, 1996). AMF-R is localized not only to the plasma membrane but also to an intracellular tubular organelle, the AMF-R tubule (Nabi *et al.*, 1992; Benlimame *et al.*, 1995). AMF-R tubules are distinct from endosomes and lysosomes; by post-embedding immunoelectron microscopy AMF-R is present primarily in smooth tubules which extend from ribosome-studded cisternae however AMF-R tubules do not colocalize with ERGIC-53, a marker for the ER-Golgi intermediate compartment (Benlimame *et al.*, 1995; Wang *et al.*, 1997). Following treatment with ilimaquinone, which induces vesiculation of the Golgi apparatus (Takizawa *et al.*, 1993), AMF-R tubules acquire a fenestrated morphology typical of smooth

ER suggesting that the AMF-R tubule is a distinct smooth subdomain of the endoplasmic reticulum (Wang *et al.*, 1997). The intracellular distribution of this cell surface receptor to smooth ER implicates AMF-R recycling in its function in cell motility and tumor cell metastasis.

Actin cytoskeleton reorganization at the leading edge and de novo establishment of cell-substrate contacts results in lamellipodial extension and the consequent polarization of the motile cell (Rinnerthaler *et al.*, 1988; Condeelis, 1993; Stossel, 1993). The role of intracellular vesicular traffic in the establishment and maintenance of epithelial and neuronal polarity is well-established (Rodriguez-Boulan and Powell, 1992; Drubin and Nelson, 1996; Keller and Simons, 1997) and strongly supports a role for intracellular membrane traffic in the polarization of the motile cell and formation of a distinct plasma membrane domain, the leading lamella (Singer and Kupfer, 1986; Nabi *et al.*, 1992; Bretscher, 1996). Influenza HA and VSV G protein, polarized apically and basolaterally in epithelial cells and axonally and dendritically in neurons (Rodriguez-Boulan and Sabatini, 1978; Dotti and Simons, 1990), also follow distinct pathways to the cell surface in "unpolarized" fibroblasts (Yoshimoro *et al.*, 1996). Newly synthesized VSV G protein is targeted to the leading edge and cellular protrusions of motile fibroblasts and the directionality of its delivery was shown to be microtubule-dependent (Bergmann *et al.*, 1983; Rogalski *et al.*, 1984; Peränen *et al.*, 1996). Membrane recycling via coated pits has long been suggested to be implicated in cell spreading and cell motility (Bretscher, 1984). We show here that AMF-R is concentrated at the cell surface within smooth plasmalemmal vesicles or caveolae and that AMF is internalized via a non-clathrin pathway to intracellular smooth ER tubules. Our results implicate caveolae in transduction of the AMF motility signal as well as in the internalization of its receptor.

MATERIALS AND METHODS

Cells and Cell Culture

NIH-3T3 fibroblasts obtained from the ATCC were cloned and a highly spread clone was used for these studies. HeLa and NIH-3T3 cells were grown in an air-5% CO_2 incubator at constant humidity in Dulbecco's minimum essential medium (DMEM) containing non-essential amino acids, vitamins, glutamine and a penicillin-streptomycin antibiotic mixture (Gibco, Burlington, Ontario) supplemented with 5% fetal calf serum (Immunocorp, Montreal, Quebec) for HeLa or 10% calf serum (Gibco, Burlington, Ontario) for NIH-3T3 cells.

Antibodies and chemicals

Monoclonal antibody against AMF-R was used in the form of concentrated hybridoma supernatant (Nabi *et al.*, 1990). Rabbit anti-caveolin polyclonal antibody was purchased from Transduction Laboratories (Lexington, KY), rabbit anti-biotin antibody from Sigma (St. Louis, Missouri), and rat anti-LAMP-1 from the Developmental Studies Hybridoma Bank (University of Iowa, Iowa City). Secondary antibodies conjugated to either fluorescein, Texas Red or 12 nm gold particles and streptavidin conjugated to fluorescein or Texas Red were purchased from Jackson Immunoresearch Laboratories (West Grove, PA). Texas Red conjugated human diferric transferrin was kindly provided by Dr. Tim McGraw (Columbia University, New York, NY). Streptavidin conjugated to 10 nm gold particles was purchased from Sigma. The secondary antibodies were designed for use in multiple labeling studies and no interspecies cross-reactivity was detected. To detect antibodies to AMF-R, secondary antibodies specific for the μ chain of rat IgM were used.

Rabbit phosphohexose isomerase was purchased from Sigma and biotinylated with NHS-LC-biotin (Pierce, Rockford, Illinois) according to the manufacturer's instructions. To assess its purity, biotinylated phosphohexose

isomerase was separated by SDS-PAGE, transferred to nitrocellulose, probed with horseradish peroxidase conjugated streptavidin (Jackson Immunoresearch Laboratories) and revealed by chemiluminescence.

Immunofluorescence

Cells were plated on glass cover slips 2 days prior to each experiment at a concentration of 30,000 cells/35 mm dish. For AMF-R surface labeling, the cells were incubated in DMEM minus bicarbonate supplemented with 25 mM Hepes pH 7.2 and 2.5% serum for 15 min at 4°C prior to labeling with anti-AMF-R primary antibody or biotinylated AMF at 4°C for 30 min. The cells were washed at 4°C and then fixed with 3% paraformaldehyde in phosphate buffered saline (pH 7.4) supplemented with 0.1 mM Ca^{++} and 1 mM Mg^{++} (PBS/CM) for 15 min at room temperature. For caveolin labeling, after AMF-R surface labeling at 4°C and fixation as above, the cells were permeabilized with 0.2% Triton X-100 for 10 min, then extensively washed with PBS/CM containing 1% BSA. The cells were incubated with rabbit anti-caveolin polyclonal antibodies, washed, and then incubated with FITC goat anti-rat IgM to reveal anti-AMF-R and Texas Red donkey anti-rabbit IgG to reveal anti-caveolin. Cell surface labeling with biotinylated AMF was revealed with rabbit anti-biotin antibody and fluorescent anti-rabbit secondary antibody.

For the AMF internalization studies, NIH-3T3 cells were pulsed with biotinylated AMF (~250-500 µg/ml) and chased at 37°C for the indicated periods of time prior to fixation by the addition of precooled (-80°C) methanol/acetone directly to the cells. After fixation, internalized bAMF was revealed with Texas Red streptavidin and lysosomes and AMF-R tubules by anti-LAMP-1 and anti-AMF-R antibodies, respectively, followed by the corresponding FITC-conjugated secondary antibodies. Disruption of clathrin coated pits and vesicles by cytoplasmic acidification was performed essentially as previously described

(Heuser, 1989). NIH-3T3 cells were pretreated with acidification medium (DMEM containing 5% calf serum and 50mM MES pH 5.5) for 15 minutes at 37°C prior to addition of bAMF in acidification medium for one hour at 37°C. To ensure that cellular acidification blocked clathrin-mediated endocytosis, Texas Red transferrin (50 µg/ml) was added to cells in regular or acidification medium for 30 minutes at 37°C after which the cells were fixed with 3% paraformaldehyde.

After labeling the coverslips were mounted in Airvol (Air Products and Chemicals Inc., Allentown, PA) and viewed in a Zeiss Axioskop fluorescent microscope equipped with a 63X Plan Apochromat objective and selective filters. Confocal microscopy was performed with the 60X Nikon Plan Apochromat objective of a dual channel BioRad 600 laser scanning confocal microscope equipped with a krypton/argon laser and the corresponding dichroic reflectors to distinguish fluorescein and Texas Red labeling. Confocal images were printed using a Polaroid TX 1500 video printer.

Electron microscopy

Post-embedding immunolabeling for AMF-R was performed as previously described (Benlimame *et al.*, 1995). Cells grown on petri dishes were rinsed and incubated at 37°C in Ringer's solution for 15 minutes before fixing in Ringer's solution containing 2% paraformaldehyde and 0.2% glutaraldehyde for 30 minutes at 37°C. The fixed cells were rinsed in PBS/CM, scraped from the petri dish and collected by centrifugation. The cell pellet was post-fixed for 30 minutes with 1% osmium tetroxide in PBS/CM containing 1.5% potassium ferrocyanide (reduced osmium), dehydrated and embedded in LR-White resin. Ultra-thin sections (80 nm) were blocked with 2% BSA, 0.2 % gelatin in PBS/CM for 1 hour, and then incubated at room temperature with anti-AMF-R antibody for 1 hour followed by 12 nm gold conjugated goat anti-rat antibodies for 1 hour.

The sections were then stained with 5% uranyl acetate and examined in a Philips 300 electron microscope. The numerical density of gold particles associated with plasma membrane, caveolae, clathrin coated pits and vesicles, smooth tubules and vesicles, and rough ER was determined. The length of the limiting membrane of the indicated organelles was measured using a Sigma-Scan measurement system and the gold particles associated with these organelles counted. Rough ER was defined by the presence of a linear array of membrane-associated ribosomes. Smooth vesicles attached to the plasma membrane or within 100 nm of the plasma membrane were considered to be caveolae. Control labeling with non-immune rat IgM antibodies was analyzed similarly.

To follow the endocytic pathway of AMF by electron microscopy, biotinylated AMF was internalized as described for the fluorescence studies and detected by postembedding labeling with streptavidin conjugated to 10 nm gold as described above. No labeling was observed in the absence of biotinylated AMF.

RESULTS

Localization of AMF-R to cell surface caveolae

By post-embedding immunoelectron microscopy in NIH-3T3 and HeLa cells, AMF-R is primarily localized to smooth intracellular membranous tubules (Figure 1 A,D), similar in morphology to those previously described in MDCK cells (Benlimame *et al.*, 1995). At the cell surface, AMF-R label localizes to smooth invaginations of the plasma membrane morphologically equivalent to caveolae (Figure 1 B,C,E,F). Quantification of the labeling revealed that the predominant AMF-R label is localized to smooth tubules and vesicles, flat regions of the plasma membrane and caveolae (Table 1). While specific label was previously detected in the rough ER of MDCK cells (Benlimame *et al.*, 1995), the density of labeling of rough ER tubules in NIH-3T3 and HeLa cells is reduced and at control levels. The density of AMF-R labeling of caveolae is equal to that of intracellular smooth tubules and vesicles in NIH-3T3 cells and greater than that of intracellular smooth tubules and vesicles in HeLa cells and essentially no AMF-R label is found within clathrin coated pits and vesicles. The density of AMF-R labeling in caveolae is increased relative to flat regions of the plasma membrane. However, based on the total number of gold particles at the plasma membrane, only 13 % of cell surface AMF-R in NIH-3T3 and 26 % in HeLa cells is found within caveolae.

To assess whether cell surface AMF-R colocalizes with caveolin, viable NIH-3T3 cells were surface labeled for AMF-R by the addition of anti-AMF-R antibodies to viable cells at 4°C (Nabi *et al.*, 1992) and then double immunofluorescently labeled after fixation and permeabilization with antibodies to caveolin (Figure 2). While the punctate AMF-R surface label (Figure 2 A) did not completely colocalize with the finer caveolin labeling (Figure 2 B), confocal microscopy clearly revealed distinct points and patterns labeled for both cell

surface AMF-R and caveolin (Figure 2 C, yellow). Peripheral regions densely labeled for both AMF-R and caveolin were frequently observed. The partial colocalization of cell surface AMF-R with caveolin is consistent with the fact that, based on the EM data, only 13 % of cell surface AMF-R was localized within the caveolae of NIH-3T3 cells.

Internalization of AMF

The ligand for AMF-R, AMF, is homologous to phosphohexose isomerase (Watanabe *et al.*, 1996). Phosphohexose isomerase (referred to here as AMF) was biotinylated and after separation by SDS-PAGE revealed a single major band after revelation of the blots with streptavidin-HRP (Figure 3 A). Cell surface labeling of NIH-3T3 cells by the addition of both biotinylated AMF (bAMF) (Figure 3 B) and anti-AMF-R at 4°C (Figure 3 C) revealed a high degree of colocalization (Figure 3 D, yellow) demonstrating that AMF and antibodies to AMF-R recognize the same receptor. The presence of spots labeled exclusively with either bAMF or anti-AMF-R may be due to the fact that the two were added together and may compete for the same site. Indeed, the addition of excess cold AMF prior to cell surface labeling with anti-AMF-R significantly reduced binding of the antibody (our unpublished observations), as previously demonstrated by immunoblot (Nabi *et al.*, 1990).

Pulse labeling of NIH-3T3 cells with bAMF for one or two hours resulted in the ability to detect both punctate structures as well as fainter tubular structures which colocalized with AMF-R tubules (Figure 4 A,B). Under these conditions, the extent of punctate and tubular labeling varied between cells. Fibrillar labeling of bAMF was also observed and has been determined to be localized to the cell surface (our unpublished results). An extended chase of 2 or 4 hours after a two hour pulse resulted in decreased punctate labeling and the accumulation of bAMF labeling in tubular structures which colocalized with AMF-

R tubules (Figure 4 C,D). The vast majority of the cells exhibited predominantly intracellular tubular labeling as well as cell surface fibrillar labeling. Following treatment of cells with acidified medium (pH 5.5) and disruption of clathrin coated pits and vesicles (Heuser, 1989), bAMF internalized for one hour is localized to intracellular AMF-R tubules (Figure 4 E,F). In the acidified medium, internalized transferrin did not cluster in the perinuclear recycling compartment demonstrating that the acidification procedure did indeed disrupt clathrin-mediated endocytosis (Figure 4 G, H). bAMF is therefore internalized via a clathrin-independent endocytic pathway to the smooth ER.

The colocalization of bAMF labeled tubules with AMF-R tubules was confirmed by confocal microscopy (Figure 5). Following a 1 hour bAMF internalization, internalized bAMF is localized to tubular structures which colocalize with AMF-R tubules (Figure 5 A-C) as well as to punctate structures which exhibit partial colocalization with LAMP-1 positive lysosomes (Figure 5 D-F). As seen here, the intense punctate labeling can hide the fainter tubular labeling of bAMF in some cells (Figure 4 A, 5 D). In acidification medium, the vast majority of bAMF labeling, aside from cell surface fibrils, is localized to tubules which colocalize with AMF-R tubules (Figure 5 G-I). bAMF internalized for 1 hour in the presence of 10-fold excess unlabeled AMF is localized only to punctate structures and no labeling of AMF-R tubules can be detected (Figure 5 J-L). While the extent of tubular labeling of bAMF varies between cells under control conditions (Figure 5 A, D), in the presence of excess unlabeled AMF the localization of bAMF to AMF-R tubules is never observed (Figure 5 J-L). bAMF internalization to intracellular AMF-R tubules therefore occurs via a receptor-mediated process. The inability of excess AMF to block bAMF internalization to punctate perinuclear structures, which exhibit partial colocalization with LAMP-1 labeled lysosomes (our unpublished observations), demonstrates that this

labeling is not saturable and corresponds to non-specific fluid phase uptake. The disappearance of lysosomal labeling following extended chase times (Figure 4 C,D) is therefore most likely due to lysosomal degradation of fluid phase internalized bAMF.

The location of biotinylated AMF internalized at 37°C was determined by post-embedding electron microscopy with streptavidin-10 nm gold. Following a 10 minute pulse biotinylated AMF could be detected in caveolae (Figure 6 A,B). Following a 30 minute pulse, both caveolae and intracellular smooth and rough ER elements were labeled (Figure 6 C-G). Dense structures morphologically equivalent to lysosomes are also labeled and presumably correspond to the perinuclear structures densely labeled for internalized bAMF by immunofluorescence (Figure 6 H,I).

DISCUSSION

AMF-R localization to caveolae

Caveolae are smooth plasmalemmal vesicles whose cytoplasmic surface presents a spiral coat containing the 21 kD caveolae-specific phosphoprotein caveolin (Rothberg *et al.*, 1992). By post-embedding immunoelectron microscopy AMF-R is localized to morphologically identifiable caveolae as well as to smooth ER tubules (Figure 1; Table 1). In contrast to polarized epithelial MDCK cells (Benlimame *et al.*, 1995), labeling of rough ER tubules was not above background in either NIH-3T3 or HeLa cells indicating that AMF-R is a specific marker for smooth ER in these two cell types. The localization of AMF-R to caveolae was confirmed by the colocalization of cell surface AMF-R, labeled by the addition of anti-AMF-R to viable cells at 4°C, with caveolin by confocal fluorescence microscopy (Figure 2). By both postembedding immunoelectron microscopy and confocal double labeling with caveolin, only a minor portion of cell surface AMF-R actually distributes to caveolae identified either morphologically or by the presence of caveolin. Whether flat plasma membrane regions to which AMF-R is localized are equivalent to cholesterol-rich Triton X-100 insoluble glycolipid rafts (Brown and Rose, 1992; Sargiacomo *et al.*, 1993; Chang *et al.*, 1994) is difficult to assess due to the large quantity of AMF-R localized to intracellular tubules (61% in NIH-3T3 and 74% in HeLa as per Table 1). Based on the labeling of AMF-R by immunoelectron microscopy, only about 5% of total cellular AMF-R is actually localized to caveolae (Table 1).

Transduction of the AMF motility signal is mediated by a pertussis-toxin sensitive G protein, phosphorylation of AMF-R and both protein kinase C and tyrosine kinase activities (Stracke *et al.*, 1987; Nabi *et al.*, 1990; Watanabe *et al.*, 1991; Timar *et al.*, 1993; Kanbe *et al.*, 1994). Heterotrimeric G proteins, including the pertussis toxin sensitive $G_{\alpha i2}$ protein, have been shown to

associate with caveolar domains (Sargiacomo *et al.*, 1993; Chang *et al.*, 1994; Schnitzer *et al.*, 1995) although results from another study indicate that heterotrimeric G proteins do not associate with an immunoisolated caveolae fraction (Stan *et al.*, 1997). The AMF-R sequence codes for a putative Type 1 membrane protein and contains within its cytoplasmic domain a consensus sequence for a G-protein activator motif (Watanabe *et al.*, 1991; Okamoto and Nishimoto, 1992; Silletti *et al.*, 1996). The association of AMF-R with caveolae could serve to facilitate its interaction with heterotrimeric G-proteins following receptor activation. Caveolin was originally identified as a phosphorylated substrate of Rous sarcoma virus tyrosine kinase and tyrosine kinase activities have been localized to caveolae (Glenney, 1989; Sargiacomo *et al.*, 1993; Shenoy-Scaria *et al.*, 1994; Robbins *et al.*, 1995; Liu *et al.*, 1997). The flattening of caveolae at the plasma membrane is stimulated by the protein kinase C stimulator PMA and the okadaic acid mediated internalization of caveolae is blocked by the kinase inhibitor staurosporine suggesting that protein kinase C activity may be associated with caveolae (Smart *et al.*, 1993; Parton *et al.*, 1994). The involvement of heterotrimeric G proteins as well as tyrosine kinase and protein kinase C activities in transduction of the AMF motility signal is consistent with the localization of AMF-R to cell surface caveolae.

Clathrin-independent internalization of AMF-R to smooth ER tubules

The presence of AMF-R both at the cell surface and within an intracellular ER-associated organelle suggested that the receptor recycles between these two cellular sites (Nabi *et al.*, 1992; Benlimame *et al.*, 1995). Caveolae have been implicated in transcytosis in endothelial cells (Palade *et al.*, 1979; Ghitescu *et al.*, 1986; Schnitzer *et al.*, 1994) and interaction between caveolae and smooth ER has been proposed for the vascular endothelium (Bundgaard, 1991). The inositol triphosphate receptor-like protein has been

localized to caveolae and this protein is expressed not only at the plasma membrane but also within the ER (Fujimoto *et al.*, 1992; Sharp *et al.*, 1992). The fact that the density of AMF-R labeling within caveolae is equivalent to (NIH-3T3) or greater than (HeLa) that of smooth vesicles and tubules is consistent with the concentration of AMF-R within caveolae prior to vesicle budding and fusion with AMF-R tubules (Table 1).

AMF exhibits sequence identity to phosphohexose isomerase (Watanabe *et al.*, 1996). Biotinylated phosphohexose isomerase or bAMF colocalizes with cell surface AMF-R labeled with antibodies to AMF-R at 4°C and is endocytosed by cells at 37°C to tubules which colocalize with smooth ER AMF-R tubules by confocal microscopy and to morphologically identifiable ER tubules by electron microscopy. bAMF internalization to smooth ER tubules is a receptor-mediated process as it can be blocked by the presence of excess unlabeled AMF. Cellular acidification, which specifically blocks clathrin mediated endocytosis, disrupts the internalization of transferrin, but not that of bAMF to AMF-R tubules, demonstrating that AMF-R is internalized to AMF-R tubules via a non-clathrin endocytic pathway. Under the conditions used in these experiments, internalization of bAMF to lysosomal structures is also observed. This lysosomal labeling is observed even in the presence of excess unlabeled AMF indicating that it is not receptor-mediated and due to fluid phase uptake. We have therefore identified a clathrin-independent AMF-R-mediated endocytic pathway which targets bAMF to the endoplasmic reticulum. The localization of AMF-R and internalized bAMF to cell surface caveolae by electron microscopy implicates these smooth invaginations of the plasma membrane in the endocytosis of AMF-R to the smooth ER subdomain for which it is a marker (Benlimame *et al.*, 1995; Wang *et al.*, 1997).

Role of caveolae in AMF-R internalization

Whether caveolae are involved in endocytic processes in non-endothelial remains a controversial subject. The transient opening and shutting of caveolae at the cell surface, in the absence of dissociation from the plasma membrane, creates localized ligand concentrations which enter the cell via a process called potocytosis (Anderson *et al.*, 1992). Caveolin-positive intracellular endocytic structures have been identified in elicited macrophages (Kiss and Geuze, 1997). Clathrin-independent internalization of the cholecystokinin receptor, apparently via caveolae, targeted the receptor only to subplasmalemmal vesicles (Roettger *et al.*, 1995). Non-clathrin mediated endocytosis has been identified in non-endothelial cells for a number of ligands, among them cholera toxin, ricin and the ß-adrenergic receptor (Montesano *et al.*, 1982; Sandvig *et al.*, 1987; Raposo *et al.*, 1989). Cholera toxin and ricin are targeted to the same endosomes as transferrin receptor (Tran *et al.*, 1987; Hansen *et al.*, 1993) indicating that ligands internalized via clathrin and non-clathrin invaginations can follow the endosomal/lysosomal pathway. Disruption of clathrin polymerization does not influence the extent of bulk flow internalization (Cupers *et al.*, 1994) and whether these clathrin-independent internalization routes involve caveolae or clathrin-coated pits without the clathrin remains to be determined (van Deurs *et al.*, 1993).

The receptor-mediated endocytosis of bAMF not to endosomes and lysosomes but to the ER certainly suggests that bAMF endocytosis is not mediated by uncoated clathrin vesicles. Internalization of anti-AMF-R mAb to intracellular tubular structures has also been visualized in metastatic K1735 melanoma cell variants (Silletti *et al.*, 1994). Furthermore, alternate internalization routes involving caveolae have been described. Treatment of A431 cells with the phosphatase inhibitor okadaic acid enhances the ability of caveolae labeled with cholera toxin to dissociate from the plasma membrane;

internalized cholera toxin was targeted to intracellular tubular profiles and clusters of caveolae, some of which were not labeled by fluid phase uptake (Parton *et al.*, 1994). Cholesterol depletion results in the internalization of caveolin and its recycling via the ER, ERGIC, and the Golgi apparatus to the plasma membrane (Smart *et al.*, 1994; Conrad *et al.*, 1995). SV40 virus associates with caveolae and is internalized via smooth plasmalemmal vesicles to smooth tubules which are extensions of the ER (Kartenbeck *et al.*, 1989; Anderson *et al.*, 1996; Stang *et al.*, 1997). The internalization pathway of SV40 to the ER (Kartenbeck *et al.*, 1989) is therefore remarkably similar to that of AMF-R described here and the localization of both AMF-R and SV40 to cell surface caveolae certainly implicates caveolae in this ER directed endocytic pathway. It can be argued that the caveolae to which AMF-R is localized at steady state are not necessarily involved in its clathrin-independent endocytosis, however we would argue that the best interpretation of our data is that AMF-R internalization to the ER occurs via caveolae. Distinct receptor-mediated caveolae internalization pathways have been described in endothelial cells of the rete mirabile (Bendayan and Rasio, 1996), and it is quite conceivable that morphologically indistinguishable but functionally distinct subpopulations of caveolae exist. AMF activation of AMF-R may stimulate both transduction of the AMF motility stimulating signal and internalization of AMF-R, perhaps within the same cell surface caveolar domain.

The identification of caveolin as a phosphorylated substrate for the src tyrosine kinase is indicative of a role for caveolae in cellular transformation (Glenney, 1989). AMF-R localization to these cell surface structures further implicates them in tumor cell malignancy and metastasis. Oncogene transformation of NIH-3T3 results in the loss of caveolin expression and disappearance of caveolae (Koleske *et al.*, 1995) while treatment with the tumor

promotor PMA also results in the disappearance of caveolar invaginations (Smart *et al.*, 1993). In lymphocytes or Sf21 insect cells which lack caveolin and caveolae, the formation of cell surface caveolae as well as intracellular caveolar vesicles can be induced following transfection with caveolin suggesting that caveolin expression is necessary for caveolar invagination (Fra *et al.*, 1995; Li *et al.*, 1996). Alternatively, caveolin may serve to stabilize caveolar invaginations at the cell surface.

AMF recycling and cell motility

The established role of AMF-R in cell motility and metastasis implicates AMF-R internalization, and subsequent recycling to the cell surface, in the motile process (Nabi *et al.*, 1992; Silletti and Raz, 1996). The potential involvement of a caveolae-mediated recycling pathway via an ER-associated organelle in cell motility complements the previously described role for coated pit internalization and recycling (Bretscher, 1984; Altankov and Grinnell, 1993), the targeting of Golgi-derived membrane vesicles (Bergmann *et al.*, 1983; Rogalski *et al.*, 1984; Peränen *et al.*, 1996), and the recycling of early endosomal or lysosomal proteins (Bretscher, 1989; Garrigues *et al.*, 1994; Hopkins *et al.*, 1994) in cellular movement. The extension of lamellipodia by the motile cell requires the continual generation of de novo polarized membrane domains. The targeting of the bulk of intracellular membrane traffic towards the site of formation of new membrane domains, the leading edge, is therefore not surprising. Targeting of intracellular membrane traffic is in large part mediated by the microtubule cytoskeleton (Kelly, 1990; Cole and Lippincott-Schwartz, 1995) whose role in determining the directionality of membrane traffic to the leading edge as well as the directionality of cell movement has been demonstrated (Rogalski *et al.*, 1984; Tanaka *et al.*, 1995). AMF-R tubules associate with a pericentriolar microtubule subdomain enriched in stabilized microtubules in Moloney sarcoma

virus transformed epithelial MDCK cells supporting a role for microtubules in the intracellular targeting of AMF-R tubules (Nabi *et al.*, 1997). The role of microtubules in the directionality of lamellipodial extension and cell movement may be to target intracellular membrane traffic to the leading edge of the motile cell. It should however be noted that while cells in serum culture are continually motile, cell motility in situ occurs in response to specific motile stimuli (Stoker and Gherardi, 1991). A recycling pathway stimulated by a cytokine, such as AMF, may represent a motility specific membrane targeting pathway.

ACKNOWLEDGEMENTS

These studies were made possible by the prior work of Avraham Raz and Hideomi Watanabe. We thank Danièle Simard for her early contributions to this project and M. Bendayan, L. Ghitescu and M. Desjardins for helpful discussions throughout the work and for critical reading of the text. The technical assistance of Ginette Guay and the photographic work of Jean Leveillé are sincerely appreciated. This work was supported by grants from the Medical Research Council of Canada, the National Cancer Institute of Canada and by an establishment grant from FCAR.

REFERENCES

Altankov, G., and Grinnell, F. (1993). Depletion of intracellular potassium disrupts coated pits and reversibly inhibits cell polarization during fibroblast spreading. J. Cell Biol. *120*, 1449-1459.

Anderson, H.A., Chen, Y., and Norkin, L.C. (1996). Bound simian virus 40 translocates to caveolin-enriched membrane domains, and its entry is inhibited by drugs that selectively disrupt caveolae. Molec. Biol. Cell *7*, 1825-1834.

Anderson, R.G.W., Kamen, B.A., Rothberg, K.G., and Lacey, S.W. (1992). Potocytosis: Sequestration and transport of small molecules by caveolae. Science *253*, 410-411.

Bendayan, M., and Rasio, E.A. (1996). Transport of insulin and albumin by the microvascular endothelium of the rete mirabile. J. Cell Sci. *109*, 1857-1864.

Benlimame, N., Simard, D., and Nabi, I.R. (1995). Autocrine motility factor receptor is a marker for a distinct tubular membrane organelle. J. Cell Biol. *129*, 459-471.

Bergmann, J.E., Kupfer, A., and Singer, S.J. (1983). Membrane insertion at the leading edge of motile fibroblasts. Proc. Natl. Acad. Sci. U.S.A. *80*, 1367-1371.

Bretscher, M.S. (1984). Endocytosis: relation to capping and cell locomotion. Science *226*, 681-686.

Bretscher, M.S. (1989). Endocytosis and recycling of the fibronectin receptor in CHO cells. EMBO J. *8*, 1341-1348.

Bretscher, M.S. (1996). Getting membrane flow and the cytoskeleton to cooperate in moving cells. Cell *87*, 601-606.

Brown, D.A., and Rose, J.K. (1992). Sorting of GPI-anchored proteins in glycolipid enriched membrane subdomains during transport to the apical cell surface. Cell *68*, 533-544.

Bundgaard, M. (1991). The three-dimensional organization of smooth endoplasmic reticulum in capillary endothelia: Its possible role in regulation of free cytosolic calcium. J. Struct. Biol. *107*, 76-85.

Chang, W.-J., Ying, Y.-s., Rothberg, K.G., Hooper, N.M., Turner, A.J., Gambliel, H.A., De Gunzberg, J., Mumby, S.M., Gilman, A.G., and Anderson, R.G.W. (1994). Purification and characterization of smooth muscle caveolae. J. Cell Biol. *126*, 127-138.

Cole, N.B., and Lippincott-Schwartz, J. (1995). Organization of organelles and membrane traffic by microtubules. Curr. Opin. Cell Biol. *7*, 55-64.

Condeelis, J. (1993). Life at the leading edge: The formation of cell protrusions. Ann. Rev. Cell Biol. *9*, 411-444.

Conrad, P.A., Smart, E.J., Ying, Y.-S., Anderson, R.G.W., and Bloom, G.S. (1995). Caveolin cycles between plasma membrane caveolae and the Golgi

complex by microtubule-dependent and microtubule-independent steps. J. Cell Biol. *131*, 1421-1433.

Cupers, P., Veithen, A., Kiss, A., Baudhuin, P., and Courtoy, P.J. (1994). Clathrin polymerization is not required for bulk-phase endocytosis in rat fetal fibroblasts. J. Cell Biol. *127*, 725-735.

Dotti, C.G., and Simons, K. (1990). Polarized sorting of viral glycoproteins to the axons and dendrites of hippocampal neurons in culture. Cell *62*, 63-72.

Drubin, D.G., and Nelson, W.J. (1996). Origins of cell polarity. Cell *84*, 335-344.

Fra, A.M., Williamson, E., Simons, K., and Parton, R.G. (1995). *De novo* formation of caveolae in lymphocytes by expression of VIP21-caveolin. Proc. Natl. Acad. Sci. U.S.A. *92*, 8655-8659.

Fujimoto, T., Nakade, S., Miyawaki, A., Mikoshiba, K., and Ogawa, K. (1992). Localization of 1,4,5-triphosphate receptor-like protein in plasmalemmal caveolae. J. Cell Biol. *119*, 1507-1513.

Garrigues, J., Anderson, J., Hellström, K.E., and Hellström, I. (1994). Anti-tumor antibody BR96 blocks cell migration and binds to a lysosomal membrane glycoprotein on cell surface microspikes and ruffled membranes. J. Cell Biol. *125*, 129-142.

Ghitescu, L., Fixman, A., Simionescu, M., and Simionescu, N. (1986). Specific binding sites for albumin restricted to plasmalemmal vesicles of continuous

capillary endothelium: receptor-mediated transcytosis. J. Cell Biol. *102*, 1304-1311.

Glenney, J.R. (1989). Tyrosine phosphorylation of a 22-kDa protein is correlated with transformation by Rous Sarcoma virus. J. Biol. Chem. *264*, 20163-20166.

Guirguis, R., Javadpour, N., Sharareh, S., Biswas, C., el-Amin, W., Mansur, I., and Kim, J.S. (1990). A new method for evaluation of urinary autocrine motility factor and tumor cell collagenase stimulating factor as markers for urinary tract cancers. J. Occup. Med. *32*, 846-853.

Hansen, S.H., Sandvig, K., and van Deurs, B. (1993). Molecules internalized by clathrin-independent endocytosis are delivered to endosomes containing transferrin receptors. J. Cell Biol. *123*, 89-97.

Heuser, J. (1989). Effects of cytoplasmic acidification on clathrin lattice morphology. J. Cell Biol. *108*, 401-411.

Hirono, Y., Fushida, S., Yonemura, Y., Yamamoto, H., Watanabe, H., and Raz, A. (1996). Expression of autocrine motility factor receptor correlates with disease progression in human gastric cancer. Br. J. Cancer *74*, 2003-2007.

Hopkins, C.R., Gibson, A., Shipman, M., Strickland, D.K., and Trowbridge, I.S. (1994). In migrating fibroblasts, recycling receptors are concentrated in narrow tubules in the pericentriolar area, and then routed to the plasma membrane of the leading lamella. J. Cell Biol. *125*, 1265-1274.

Kanbe, K., Chigara, M., and Watanabe, H. (1994). Effects of protein kinase inhibitors on the cell motility stimulated by autocrine motility factor. Biochimia Biophysica Acta *1222*, 395-399.

Kartenbeck, J., Stukenbrok, H., and Helenius, A. (1989). Endocytosis of simian virus 40 into the endoplasmic reticulum. J. Cell Biol. *109*, 2721-2729.

Keller, P., and Simons, K. (1997). Post-Golgi biosynthetic trafficking. J. Cell Sci. *110*, 3001-3009.

Kelly, R.B. (1990). Microtubules, membrane traffic, and cell organization. Cell *61*, 5-7.

Kiss, A.L., and Geuze, H.J. (1997). Caveolae can be alternative endocytic structures in elicited macrophages. Eur. J. Cell Biol. *73*, 19-27.

Koleske, A.J., Baltimore, D., and Lisanti, M.P. (1995). Reduction of caveolin and caveolae in oncogenically transformed cells. Proc. Natl. Acad. Sci. U.S.A. *92*, 1381-1385.

Li, S., Song, K.S., Koh, S.S., Kikuchi, A., and Lisanti, M.P. (1996). Baculovirus-based expression of mammalian caveolin in Sf21 insect cells. J. Biol. Chem. *271*, 28647-28654.

Liotta, L.A., Mandler, R., Murano, G., Katz, D.A., Gordon, R.K., Chiang, P.K., and Schiffman, E. (1986). Tumor cell autocrine motility factor. Proc. Natl. Acad. Sci. U.S.A. *83*, 3302-3306.

Liu, P., Ying, Y.-s., and Anderson, R.G.W. (1997). Platelet-derived growth factor activated mitogen-activated protein kinase in isolated caveolae. Proc. Natl. Acad. Sci. U.S.A. *94*, 13666-13670.

Montesano, R., Roth, J., Robert, A., and Orci, L. (1982). Non-coated membrane invaginations are involved in binding and internalization of cholera and tetanus toxins. Nature (Lond.) *296*, 651-653.

Nabi, I.R., Guay, G., and Simard, D. (1997). AMF-R tubules concentrate in a pericentriolar microtubule domain following transformation of MDCK epithelial cells. J. Histochem. Cytochem. *45*, 1351-1363.

Nabi, I.R., Watanabe, H., and Raz, A. (1990). Identification of B16-F1 melanoma autocrine motility-like factor receptor. Cancer Res. *50*, 409-414.

Nabi, I.R., Watanabe, H., and Raz, A. (1992). Autocrine motility factor and its receptor: Role in cell locomotion and metastasis. Cancer Met. Rev. *11*, 5-20.

Nakamori, S., Watanabe, H., Kameyama, M., Imaoka, S., Furukawa, H., Ishikawa, O., Sasaki, Y., Kabuto, T., and Raz, A. (1994). Expression of autocrine motility factor receptor in colorectal cancer as a predictor for disease recurrence. Cancer *74*, 1855-1862.

Okamoto, T., and Nishimoto, I. (1992). Detection of G protein-activator regions in M_4 subtype muscarinic, cholinergic, and α_2-adrenergic receptors based upon characteristics in primary structure. J. Biol. Chem. *267*, 8342-8346.

Otto, T., Birchmeier, W., Schmidt, U., Hinke, A., Schipper, J., Rübben, H., and Raz, A. (1994). Inverse relation of E-cadherin and autocrine motility factor receptor expression as a prognostic factor in patients with bladder carcinomas. Cancer Res. *54*, 3120-3123.

Palade, G., Simionescu, M., and Simionescu, N. (1979). Structural aspects of the permeability of the microvascular endothelium. Acta Physiol. Scand. *463*, 11-32.

Parton, R.G., Joggerst, B., and Simons, K. (1994). Regulated internalization of caveolae. J. Cell Biol. *127*, 1199-1215.

Peränen, J., Auvinen, P., Virta, H., Wepf, R., and Simons, K. (1996). Rab8 promotes polarized membrane transport through reorganization of actin and microtubules in fibroblasts. J. Cell Biol. *135*, 153-167.

Raposo, G., Dunia, I., Delavier-Klutchko, C., Kaveri, S., Strosberg, A.D., and Benedetti, E.L. (1989). Internalization of beta-adrenergic receptor in A431 cells involves non-coated vesicles. Eur. J. Cell Biol. *50*, 340-352.

Rinnerthaler, G., Geiger, B., and Small, J.V. (1988). Contact formation during fibroblast locomotion: involvement of membrane ruffles and microtubules. J. Cell Biol. *106*, 747-760.

Robbins, S.M., Quintrell, N.A., and Bishop, J.M. (1995). Myristolation and differential palmitoylation of the HCK protein-tyrosine kinases govern their

attachment to membranes and association with caveolae. Mol. Cell Biol. *15*, 3507-3515.

Rodriguez-Boulan, E., and Powell, S.K. (1992). Polarity of epithelial and neuronal cells. Ann. Rev. Cell Biol. *8*, 395-427.

Rodriguez-Boulan, E., and Sabatini, D. (1978). Asymmetric budding of viruses in epithelial monolayers: a model system for the study of epithelial polarity. Proc. Natl. Acad. Sci. U.S.A. *75*, 5071-5075.

Roettger, B.F., Rentsch, R.U., Pinon, D., Holicky, E., Hadac, E., Larkin, J.M., and Miller, L.J. (1995). Dual pathways of internalization of the cholecystokinin receptor. J. Cell Biol. *128*, 1029-1041.

Rogalski, A.A., Bergmann, J.E., and Singer, S.J. (1984). Effect of microtubule assembly status on the intracellular processing and surface expression of an integral protein of the plasma membrane. J. Cell Biol. *99*, 1101-1109.

Rothberg, K.G., Heuser, J.E., Donzell, W.C., Ying, Y.-S., Glenney, J.R., and Anderson, R.G.W. (1992). Caveolin, a protein component of caveolae membrane coats. Cell *68*, 673-682.

Sandvig, K., Olsnes, S., Petersen, O.W., and van Deurs, B. (1987). Acidification of the cytosol inhibits endocytosis from coated pits. J. Cell Biol. *105*, 679-689.

Sargiacomo, M., Sudol, M., Tang, Z., and Lisanti, M.P. (1993). Signal transducing molecules and GPI-linked proteins form a caveolin-rich insoluble complex in MDCK cells. J. Cell Biol. *122*, 789-808.

Schnitzer, J.E., Liu, J., and Oh, P. (1995). Endothelial caveolae have the molecular transport machinery for vesicle budding, docking, and fusion including the VAMP, NSF, SNAP, annexins, and GTPases. J. Biol. Chem. *270*, 14399-14404.

Schnitzer, J.E., Oh, P., Pinney, E., and Allard, J. (1994). Filipin-sensitive caveolae-mediated transport in endothelium: Reduced transcytosis, scavenger endocytosis, and capillary permeability of select macromolecules. Proc. Natl. Acad. Sci. U.S.A. *127*, 1217-1232.

Sharp, A.H., Snyder, S.H., and Nigam, S.K. (1992). Inositol 1,4,5-triphosphate receptors. Localization in epithelial tissue. J. Biol. Chem. *267*, 7444-7449.

Shenoy-Scaria, A.M., Dietzen, D.J., Kwong, J., Link, D.C., and Lublin, D.M. (1994). Cysteine^3 of Src family protein tyrosine kinases determines palmitoylation and localization in caveolae. J. Cell Biol. *126*, 353-363.

Silletti, S., Paku, S., and Raz, A. (1996). Tumor autocrine motility factor responses are mediated through cell contact and focal adhesion rearrangement in the absence of new tyrosine phosphorylation in metastatic cells. Am. J. Pathol. *148*, 1649-1660.

Silletti, S., and Raz, A. (1993). Autocrine motility factor is a growth factor. Biochem. Biophys. Res. Comm. *194*, 446-457.

Silletti, S., and Raz, A. (1996). Regulation of autocrine motility factor receptor expression in tumor cell locomotion and metastasis. Curr. Topics Microbiol. Immunol. *213/II*, 137-169.

Silletti, S., Timar, J., Honn, K.V., and Raz, A. (1994). Autocrine motility factor induces differential 12-lipoxygenase expression and activity in high and low-metastatic K1735 melanoma cell variants. Cancer Res. *54*, 5752-5756.

Simard, D., and Nabi, I.R. (1996). Inverse relation of autocrine motility factor receptor and E-cadherin expression following transformation of epithelial MDCK cells. Biochem. Biophys. Res. Comm. *219*, 122-127.

Singer, S.J., and Kupfer, A. (1986). The directed migration of eukaryotic cells. Ann. Rev. Cell Biol. 2, 337-365.

Smart, E.J., Foster, D., Ying, Y.-S., Kamen, B.A., and Anderson, R.G.W. (1993). Protein kinase C activators inhibit receptor-mediated potocytosis by preventing internalization of caveolae. J. Cell Biol. *124*, 307-313.

Smart, E.J., Ying, Y.-S., Conrad, P.A., and Anderson, R.G.W. (1994). Caveolin moves from caveolae to the Golgi apparatus in response to cholesterol oxidation. J. Cell Biol. *127*, 1185-1197.

Stan, R.-V., Roberts, W.G., Predescu, D., Ihida, K., Saucan, L., Ghitescu, L., and Palade, G.E. (1997). Immunoisolation and partial characterization of endothelial plasmalemmal vesicles (caveolae). Molec. Biol. Cell *8*, 595-605.

Stang, E., Kartenbeck, J., and Parton, R.G. (1997). Major histocompatibility complex class I molecules mediate association of SV40 with caveolae. Molec. Biol. Cell *8*, 47-57.

Stoker, M., and Gherardi, E. (1991). Regulation of cell movement: the motogenic cytokines. Biochimia Biophysica Acta *1072*, 81-102.

Stossel, T.P. (1993). On the crawling of animal cells. Science *260*, 1086-1094.

Stracke, M.L., Guirguis, R., Liotta, L.A., and Schiffman, E. (1987). Pertussis toxin inhibits stimulated motility independently of the adenylate cyclase pathway in human melanoma cells. Biochem. Biophys. Res. Comm. *146*, 339-345.

Takizawa, P.A., Yucei, J.K., Veit, B., Faulkner, D.J., Deerinck, T., Soto, G., Ellisman, M., and Malhotra, V. (1993). Complete vesiculation of Golgi membranes and inhibition of protein transport by a novel sea sponge metabolite, ilimaquinone. Cell *73*, 1079-1090.

Tanaka, E., Ho, T., and Kirschner, M.W. (1995). The role of microtubule dynamics in growth cone motility and axonal growth. J. Cell Biol. *128*, 139-155.

Timar, J., Silletti, S., Bazaz, R., Raz, A., and Honn, K.V. (1993). Regulation of melanoma cell motility by the lipoxygenase metabolite 12(S)-HETE[1]. Int. J. Cancer *55*, 1003-1010.

Tran, D., Carpentier, J.L., Sawano, F., Gorden, P., and Orci, L. (1987). Ligands internalized through coated or noncoated invaginations follow a common intracellular pathway. Proc. Natl. Acad. Sci. U.S.A. *84*, 7957-7961.

van Deurs, B., Kaae Holm, P., Sandvig, K., and Hansen, S.H. (1993). Are caveolae involved in clathrin-independent endocytosis? Trends Cell Biol. *3*, 249-251.

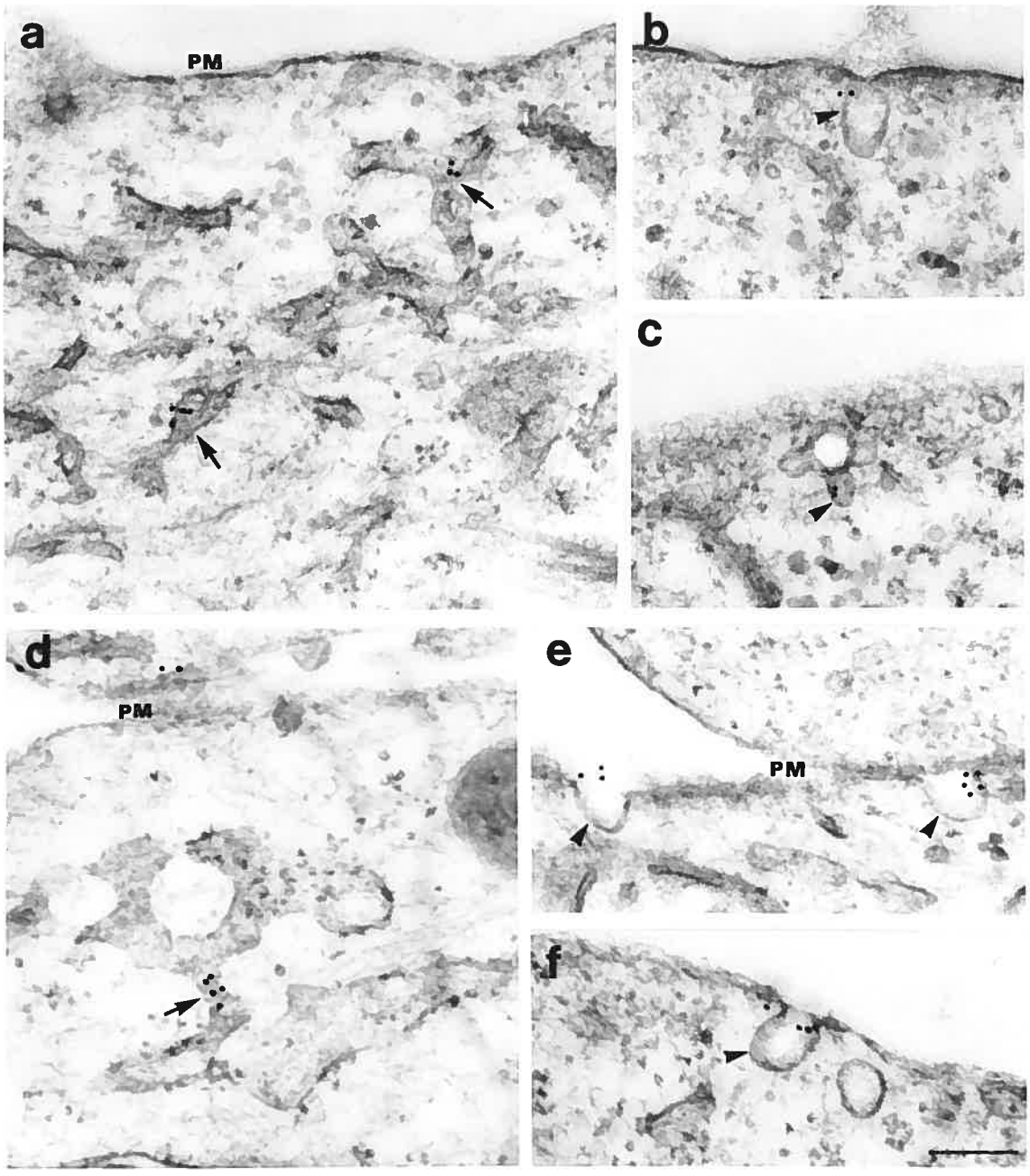
Wang, H.-J., Benlimame, N., and Nabi, I.R. (1997). The AMF-R tubule is a smooth ilimaquinone-sensitive subdomain of the endoplasmic reticulum. J. Cell Sci. *110*, 3043-3053.

Watanabe, H., Carmi, P., Hogan, V., Raz, T., Silletti, S., Nabi, I.R., and Raz, A. (1991). Purification of human tumor cell autocrine motility factor and molecular cloning of its receptor. J. Biol. Chem. *266*, 13442-13448.

Watanabe, H., Takehana, K., Date, M., Shinozaki, T., and Raz, A. (1996). Tumor cell autocrine motility factor is the neuroleukin/phosphohexose isomerase polypeptide. Cancer Res. *56*, 2960-2963.

Yoshimoro, T., Keller, P., Roth, M.G., and Simons, K. (1996). Different biosynthetic transport routes to the plasma membrane in BHK and CHO cells. J. Cell Biol. *133*, 247-256.

Figure 1: Electron microscopic localization of AMF-R in NIH-3T3 fibroblasts and HeLa cells. HeLa (A,B,C) and NIH-3T3 (D,E,F) cells were post-embedding immunolabeled with anti-AMF-R and 12-nm gold-conjugated anti-rat IgM secondary antibodies. Typical AMF-R labeling of smooth tubules (A,D, arrows) and cell surface caveolae (B,C,E,F, arrowheads) is shown. PM: plasma membrane. Bar = 0.2 μm.


a
PM
b
c
d
PM
e
PM
f

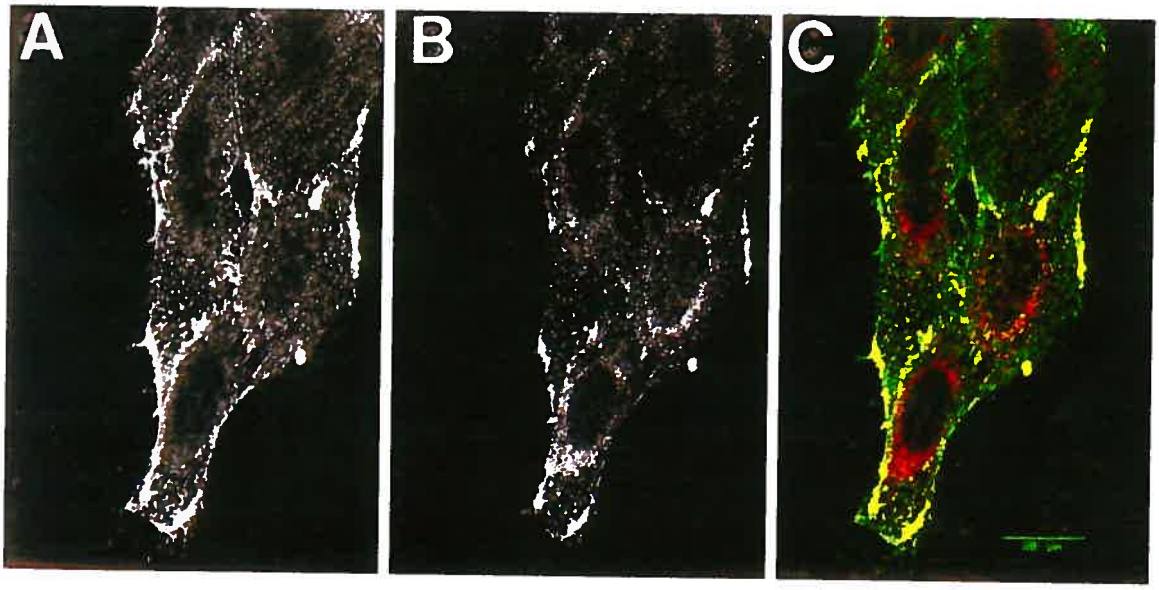


Figure 2. Colocalization of AMF-R and caveolin by confocal microscopy. Viable NIH-3T3 cells were labeled for cell surface AMF-R at 4°C (A) and for caveolin after fixation and permeabilization (B). To demonstrate the colocalization of AMF-R and caveolin, confocal images from both fluorescent channels were superimposed (panel C; AMF-R in green and caveolin in red) and colocalization appears in yellow. Bar, 20 μm.

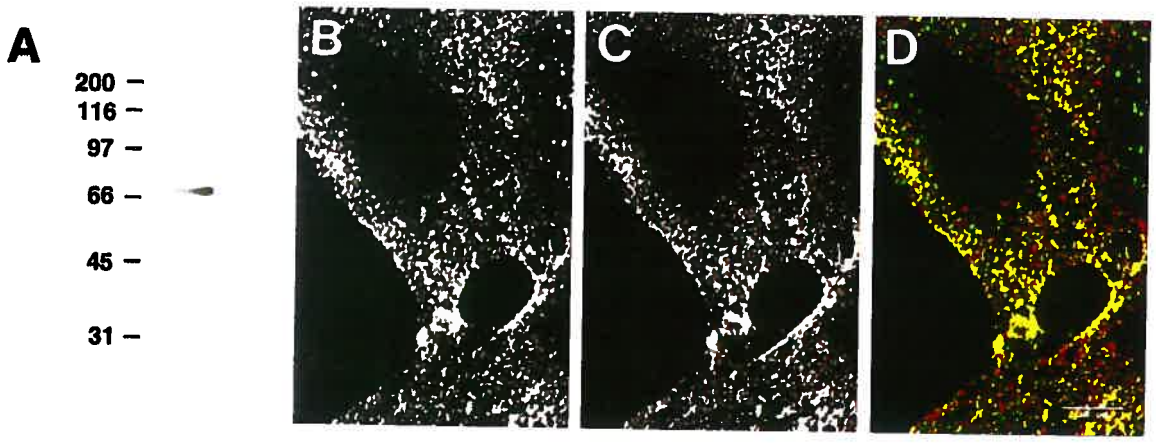
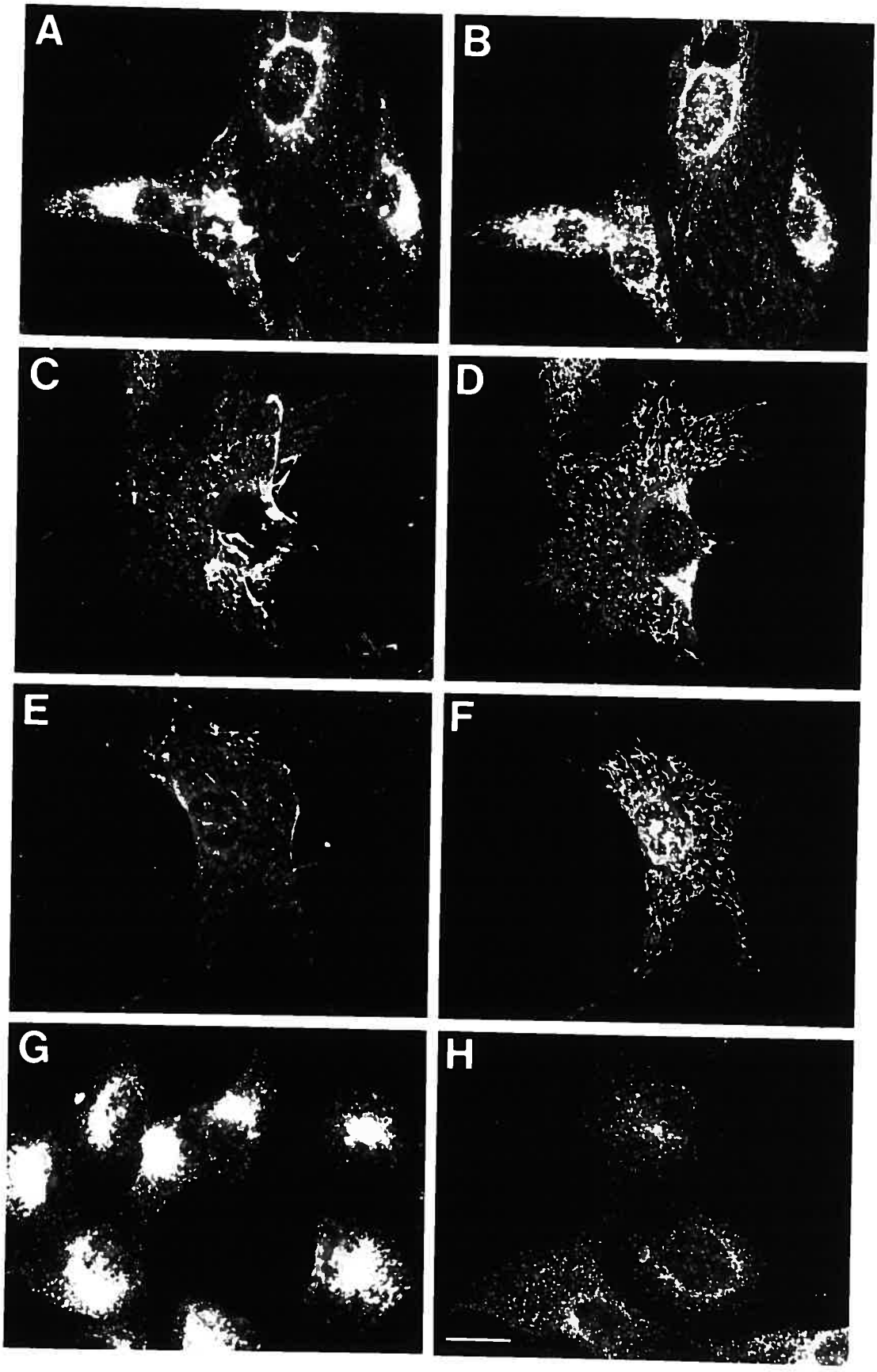


Figure 3. bAMF and anti-AMF-R mAb colocalize on the cell surface. bAMF migrated as a single band in protein blots revealed with HRP-streptavidin (A). Confocal imaging of cell surface labeling of viable NIH-3T3 cells at 4°C with bAMF (B) or anti-AMF-R antibody (C). Confocal images from both fluorescent channels were superimposed (panel D; bAMF in green and AMF-R in red) and revealed a significant degree of colocalization in yellow. Bar, 20 μm.

Figure 4: Internalization of bAMF to AMF-R tubules. NIH-3T3 cells were pulse labeled with bAMF at 37°C for one hour (A, B), for two hours and chased for 4 hours (C, D) or for one hour in medium acidified to pH 5.5 to disrupt clathrin-mediated endocytosis (E, F). After fixation with methanol/acetone, cells were double labeled with Texas Red-streptavidin to reveal bAMF (A, C, E) and anti-AMF-R mAb and FITC-conjugated anti-rat secondary antibody to reveal AMF-R labeling (B, D, F). To ensure that cellular acidification disrupted clathrin-mediated endocytosis of transferrin receptor, NIH-3T3 cells were incubated at 37°C with Texas Red transferrin for 30 minutes in regular medium (G) or in medium acidified to pH 5.5 (H). Bar = 20 μm.


A
B
C
D
E
F
G
H

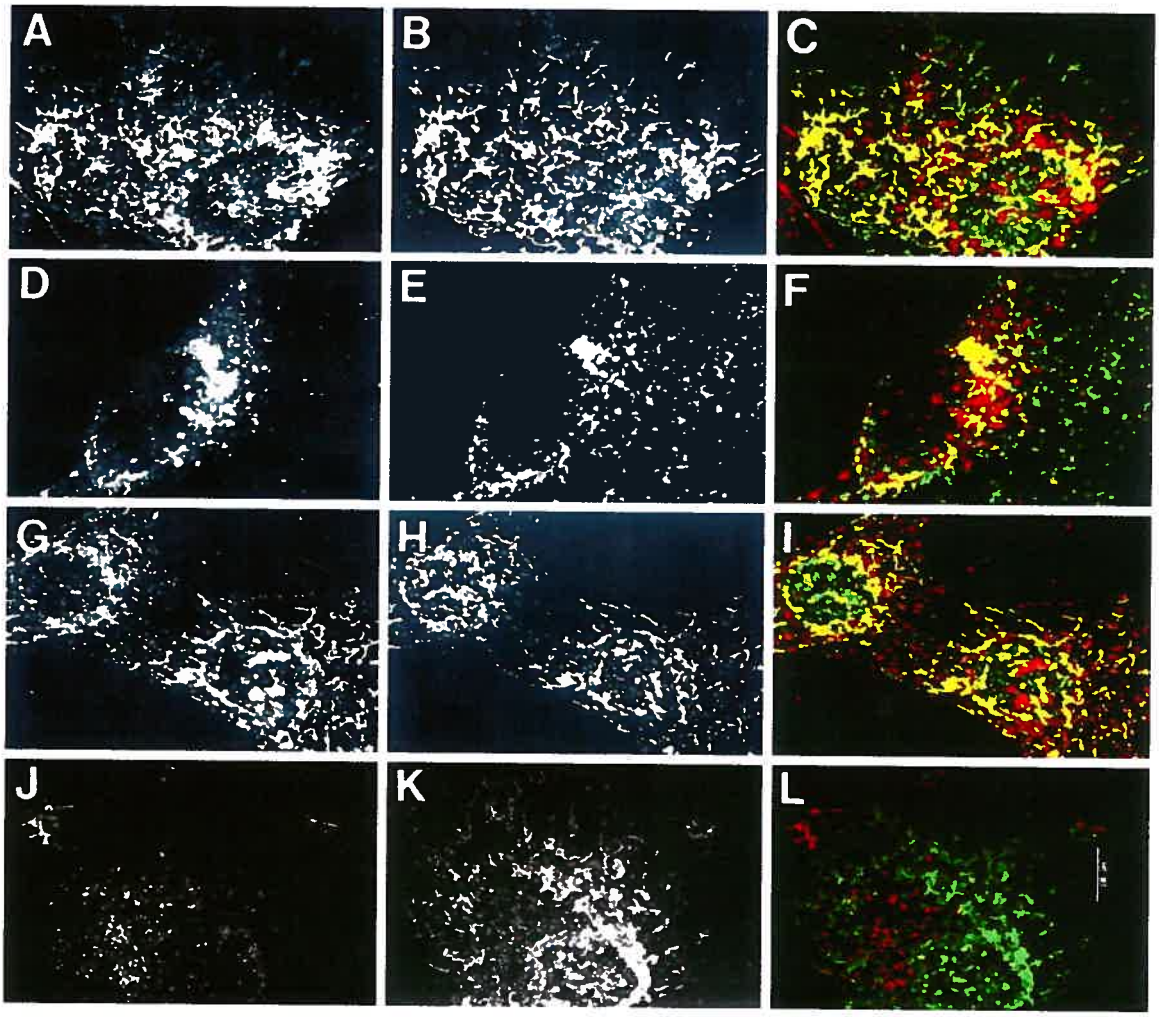


Figure 5. Localization of internalized bAMF to AMF-R tubules by confocal microscopy. NIH-3T3 cells were pulse labeled with bAMF at 37°C for 1 h in regular medium (A-F) for 1 h in medium acidified to pH 5.5 to disrupt clathrin-mediated endocytosis (G-I), or in regular medium in the presence of 10-fold excess unlabeled AMF (J-L) before fixation with methanol/acetone. bAMF was revealed with Texas Red-streptavidin (A, D, G, and J) and AMF-R (B, H, and K) or LAMP-1 (E) labeled with the appropriate primary antibodies and FITC-conjugated secondary antibodies. Confocal images from both fluorescent channels were superimposed (panels C, I, and L, bAMF in red and AMF-R in green; panel F, bAMF in red and LAMP-1 in green) and colocalization appears in yellow. Bar, 10 μm.

Figure 6: Electron microscopy of the internalization pathway of bAMF. NIH-3T3 cells were pulsed with bAMF at 37°C for 10 (A,B,H) or 30 minutes (C,D,E,F,G,I). The localization of bAMF was revealed by postembedding labeling with 10 nm gold-conjugated streptavidin. After 10 minutes, bAMF is localized to cell surface caveolae (A,B). After a 30 minute pulse, bAMF is localized to caveolae and smooth vesicles (C,D) and also appears in intracellular membranous tubules (E,F,G) including distinctive smooth (E) and rough (F) ER elements. bAMF labeling of dense lysosomal structures is also detected (H,I). Bar = 0.1 μm.

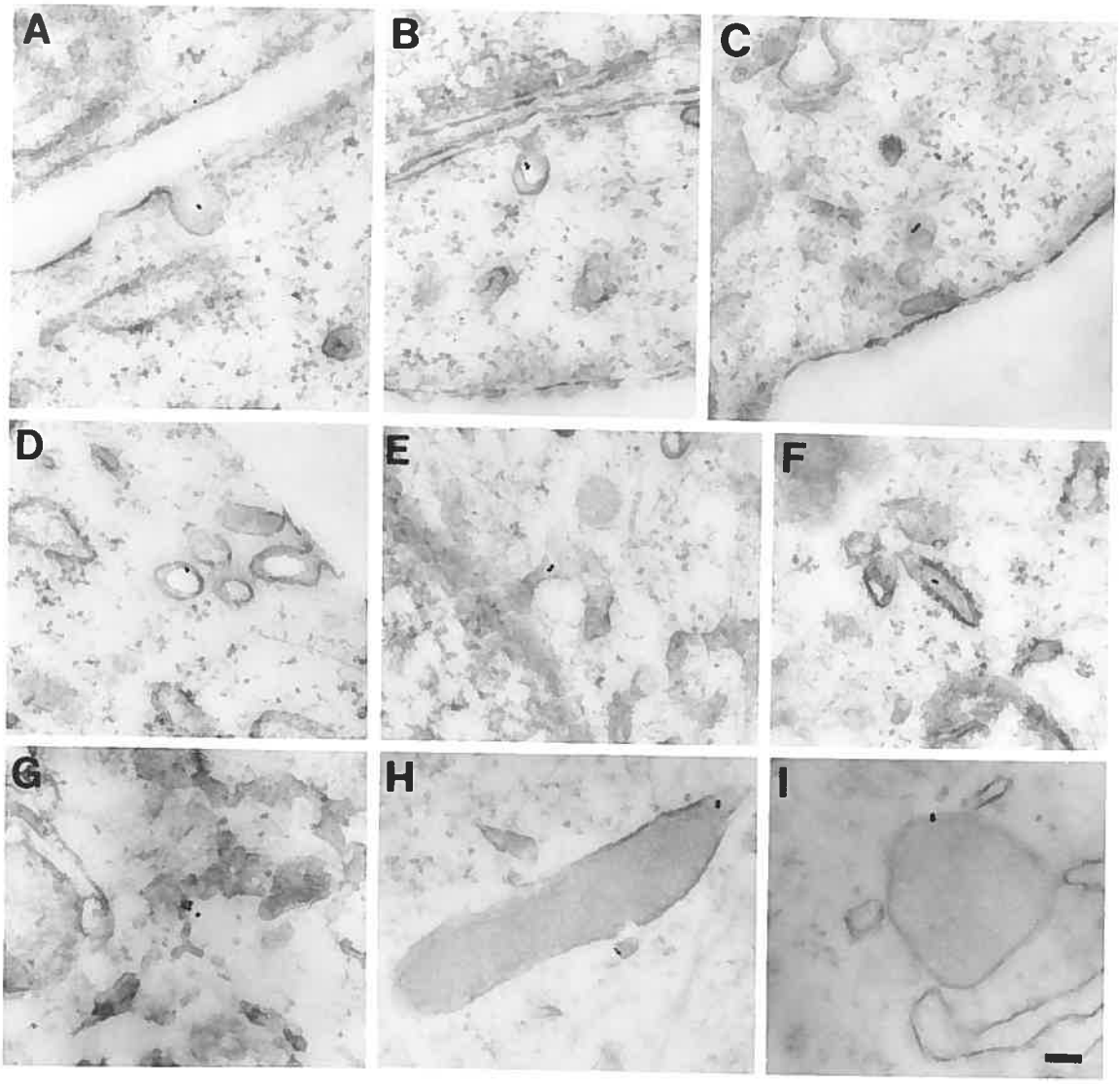

A
B
C
D
E
F
G
H
I

Table 1: Localization of AMF-R in HeLa and NIH-3T3 cells by immunoelectron microscopy

	Smooth tubules and vesicles	Rough endoplasmic reticulum	Flat plasma membrane	Caveolae	Clathrin-coated pits and vesicles
HeLa					
AMF-R					
# gold particles	660	34	147	52	3
µm membrane	328.5	187.5	245.6	14.6	19.3
gold particles/µm	**2.01± 0.15**	**0.18 ± 0.04**	**0.60 ± 0.08**	**3.56 ± 0.53**	**0.16± 0.13**
Control	83	6	25	3	1
# gold particles	307.5	95.2	211.0	16.1	5.0
µm membrane	**0.27 ± 0.08**	**0.06 ± 0.03**	**0.12 ± 0.05**	**0.19 ± 0.10**	**0.20 ± 0.21**
gold particles/µm					
NIH-3T3					
AMF-R					
# gold particles	640	74	296	44	2
µm membrane	432.6	308.9	308.8	28.8	34.7
gold particles/µm	**1.48 ± 0.10**	**0.24 ± 0.06**	**0.96 ± 0.10**	**1.53 ± 0.30**	**0.06 ± 0.06**
Control	33	7	10	4	2
# gold particles	303.1	109.8	138.1	31.7	6.2
µm membrane	**0.11 ± 0.02**	**0.06 ± 0.03**	**0.07 ± 0.03**	**0.13 ± 0.06**	**0.32 ± 0.18**
gold particles/µm					

Gold particles associated with the indicated membrane organelles were counted and the density per µm membrane length determined. Control labeling was determined using a nonimmune rat IgM antibody (Benlimame *et al.*, 1995).

ARTICLE 2

Clathrin-mediated endocytosis and recycling of autocrine motility factor receptor to fibronectin fibrils is a limiting factor for NIH-3T3 cell motility.

Le, P.U., N. Benlimame, A. Lagana, A. Raz, and I.R. Nabi., (2000).

J. Cell Sci. 113:3227-3240.

CONTRIBUTION DES AUTEURS

J'ai effectué toutes les expériences (à part la Figure 4) qui ont conduit à la publication de cet article . J'ai également participé à la rédaction de ce papier.

Naciba Benlimame a effectué la cointernalisation de l'AMF avec le FITC-dextran (Figure4).

Annaïck Lagana a aidé à la mise au point des conditions avec lesquelles nous pouvions "quencher" l'AMF en surface pour montrer que l'AMF recyclé provient de l'intérieur de la cellule.

Avraham Raz nous a fournis l'anticorps anti-AMF et il a participé à la rédaction et aux discussions reliées à la publication de l'article.

Ivan R. Nabi a principalement participé à la rédaction de l'article.

CLATHRIN-MEDIATED ENDOCYTOSIS AND RECYCLING OF AUTOCRINE MOTILITY FACTOR RECEPTOR TO FIBRONECTIN FIBRILS IS A LIMITING FACTOR FOR NIH-3T3 CELL MOTILITY

Phuong U. Le, Naciba Benlimame, Annick Lagana, Avraham Raz* and Ivan R. Nabi

Département de pathologie et biologie cellulaire, Université de Montréal, Montréal, Québec, Canada H3C 3J7; *Karmanos Cancer Institute, Detroit, Michigan 48201, U.S.A.

Key words: endocytosis, motility, caveolae, fibronectin, AMF-R

Short title: Endocytosis and recycling of AMF-R

Corresponding author:

Dr. Ivan R. Nabi

Département de pathologie et biologie cellulaire
Université de Montréal
C. P. 6128, succursale A
Montréal, Québec
Canada, H3C 3J7

Tel.: (514) 343-6291
Fax: (514) 343-5755
[redacted]

Courier address:

Département de pathologie et biologie cellulaire
Université de Montréal, Pavillion principal N-812
2900 Blvd. Edouard Montpetit
Montréal, Québec
Canada, H3T 1J4

SUMMARY

Autocrine motility factor receptor (AMF-R) is internalized via a clathrin-independent pathway to smooth endoplasmic reticulum tubules (Benlimame, N., P. U. Le, and I. R. Nabi. 1998. Molec. Biol. Cell 9:1773-1786). This endocytic pathway is shown here to be inhibited by methyl-ß-cyclodextrin (mßCD) implicating caveolae or caveolae-like structures in AMF internalization to smooth ER. AMF-R is also internalized via a clathrin-dependent pathway to a transferrin receptor-negative, LAMP-1/lgpA-negative endocytic compartment identified by electron microscopy as a multivesicular body (MVB). Endocytosed AMF recycles to cell surface fibrillar structures which colocalize with fibronectin; AMF-R recycling is inhibited at 20°C, which blocks endocytosis past the early endosome, but not by mßCD demonstrating that AMF-R recycling to fibronectin fibrils is mediated by clathrin-dependent endocytosis to MVBs. Microtubule disruption with nocodazole did not affect delivery of bAMF to cell surface fibrils indicating that recycling bAMF traverses the MVB but not a later endocytic compartment. Plating NIH-3T3 cells on an AMF coated substrate did not specifically affect cell adhesion but prevented bAMF delivery to cell surface fibronectin fibrils and reduced cell motility. AMF-R internalization and recycling via the clathrin-mediated pathway are therefore rate-limiting for cell motility. This recycling pathway to the site of deposition of fibronectin may be implicated in the de novo formation of cellular attachments or the remodeling of the extracellular matrix during cell movement.

INTRODUCTION

The repeated extension of pseudopodia of the motile cell requires the continual supply to the leading edge of the molecular components necessary for renewal and stabilization of the pseudopodial domain. Such a need should necessarily implicate protein recycling in the motile process (Bretscher, 1996; Nabi, 1999). In particular, the formation of adhesion contacts at the leading edge must require the directed delivery of adhesion molecules, including integrins, in order to stabilize de novo pseudopodial protrusions. Exocytosis of VAMP-3 containing vesicles has recently been reported at the site of phagosome formation indicating that localized exocytosis from an endosomal compartment is associated with polarized pseudopodial protrusion (Bajno et al., 2000). In neutrophils, calcineurin-dependent integrin recycling has been shown to be necessary for pseudopodial protrusion and cell movement (Hendey et al., 1992; Lawson and Maxfield, 1995). Expression of PKCα is associated with both increased endocytosis and recycling of ß1 integrin and with increased migration of MCF-7 breast carcinoma cells (Ng et al., 1999). Disruption of clathrin-mediated endocytosis by potassium depletion results in the loss of fibroblast polarization and motility (Altankov and Grinnell, 1993). Similarly, clathrin-minus *Dictyostelium* mutants exhibit loss of cell polarity, reduced cell motility and, curiously, increased pseudopodial protrusion from the posterior half of the cell implicating clathrin and, presumably, clathrin-dependent endocytosis in the polarization of pseudopodial protrusion as well as in uropod stabilization (Wessels et al., 2000). These studies support a role for endocytosis and recycling in cell motility however the precise nature of any such motility related pathways remains to be further clarified.

Receptor-mediated endocytosis via clathrin-coated vesicles from the cell surface to endosomal compartments and subsequently to degradative

lysosomes is well-characterized. The early endosome or sorting endosome segregates recycling receptors from the lysosomal directed pathway and delivers them back to the cell surface via the recycling compartment. The multivesicular body (MVB) is an intermediate between early endosomal compartments and late endosomes (Gruenberg and Maxfield, 1995; Mellman, 1996). The MVB presents a limiting membrane and multiple internal vesicles and recycling from the MVB to the cell surface has been described (Felder et al., 1990). In addition, non-clathrin endocytosis has been described to deliver cargo to early endosomes (Sandvig and van Deurs, 1991; Lamaze and Schmid, 1995) and appears to be distinct from caveolae as this pathway is not inhibited by methyl-ß-cyclodextrin (mßCD), a cholesterol extracting agent which disrupts caveolae (Rodal et al., 1999). A clathrin-independent pathway which delivers endocytic cargo to smooth ER has also been described and has been proposed to be mediated by caveolae based on morphological criteria (Kartenbeck et al., 1989; Benlimame et al., 1998). In particular, autocrine motility factor receptor (AMF-R), a membrane receptor localized to smooth ER tubules and caveolae, internalizes its ligand (AMF) via a clathrin-independent pathway to the smooth ER in NIH-3T3 fibroblasts (Benlimame et al., 1995; Wang et al., 1997; Benlimame et al., 1998).

Autocrine motility factor (AMF), a member of the ectoenzyme/exoenzyme family and also known as neuroleukin, maturation factor or phosphohexose isomerase (Watanabe et al., 1996; Sun et al., 1999), and its receptor, AMF-R, have well-established roles in transduction of a motility signal stimulating the motile and metastatic abilities of normal and tumor cells (Silletti and Raz, 1996). The stable expression of AMF-R within intracellular smooth ER tubules and its ability to internalize its ligand suggested that endocytosis of this receptor is associated with its function in cell motility (Benlimame et al., 1998; Nabi, 1999).

We show here that the non-clathrin internalization of AMF is inhibited by mßCD demonstrating that this ER endocytic pathway is mediated by caveolae or caveolae-like structures. AMF-R is also endocytosed via a clathrin-dependent pathway which delivers the AMF/AMF-R complex to MVBs from which it recycles to cell surface fibronectin fibrils. Plating of cells on an AMF coated substrate prevents AMF/AMF-R complex recycling and reduces the motility of NIH-3T3 cells demonstrating that AMF-R internalization and recycling are rate-limiting for cell motility. This recycling pathway to cell surface fibronectin fibrils could be involved in the de novo formation of cellular attachments or in the remodeling of the extracellular matrix (ECM) during cell movement.

MATERIALS AND METHODS

Antibodies and reagents

Monoclonal antibody against AMF-R was used in the form of concentrated hybridoma supernatant (Nabi et al., 1990). Monoclonal mouse anti-human TfR antibodies were purchased from Zymed Laboratories, Inc. (San Francisco, CA), rat anti-LAMP-1 from the Developmental Studies Hybridoma Bank (University of Iowa, Iowa City, IA), mouse anti-fibronectin from Transduction Laboratories (Mississauga, ON) and mouse anti-tubulin from ICN (Costa Mesa, CA). Anti-AMF antibodies were as previously described (Niinaka et al., 1998). Secondary antibodies and streptavidin conjugated to fluorescein or Texas Red were purchased from Jackson Immunoresearch Laboratories (West Grove, PA). Nanogold-streptavidin and the HQ silver™ Enhancement Kit was purchased from Nanoprobes, Inc. (Stony Brook, NY). Lysine fixable FITC dextran (10,000 MW) was purchased from Molecular Probes Inc. (Eugene, OR). Streptavidin, d-biotin, bovine plasma fibronectin and poly-D-lysine were purchased from Sigma (Oakville, ON). Rabbit phosphohexose isomerase (referred to as AMF) was purchased from Sigma and biotinylated with NHS-LC-biotin (Pierce, Rockford, IL) according to the manufacturer's instructions. mßCD and nocodazole were purchased from Sigma. Bovine serum albumin (BSA) solution (7.5%) was purchased from Canadian Life Technologis (Burlington, ON).

Cell culture

NIH-3T3 fibroblasts obtained from the American Type Culture Collection (1658-CRL) were cloned and a highly spread clone was used for these studies (Benlimame et al., 1998). NIH-3T3 cells were grown in complete medium consisting of DME supplemented with 10% calf serum, non-essential amino

acids, vitamins, glutamine, and a penicillin-streptomycin antibiotic mixture (Canadian Life Technologies).

Immunofluorescence labeling

30,000 NIH-3T3 cells were plated on glass cover slips for 2 days before each experiment unless otherwise indicated. For the AMF internalization studies, NIH-3T3 cells were pulsed with 25 µg/ml of biotinylated AMF (bAMF) in complete medium at 37°C, washed and then chased in complete medium. Disruption of clathrin-coated pits and vesicles by cytoplasmic acidification (Heuser, 1989) was performed as described previously (Benlimame et al., 1998). mßCD treatment of NIH-3T3 cells was performed essentially as previously described (Baorto et al., 1997). NIH-3T3 cells were pretreated with 0.5 mM mßCD in serum-free medium supplemented with 1% BSA for 30 minutes at 37°C and bAMF internalization in the presence of 0.5 mM mßCD was performed in the same medium; the cells were subsequently chased in complete medium. Colocalization of internalized bAMF with anti-AMF-R or anti-LAMP-1 antibodies was performed following fixation with precooled (-80°C) methanol/acetone prior to labeling with Texas Red-streptavidin and the appropriate FITC-conjugated secondary antibodies (Benlimame et al., 1998). Colocalization of internalized bAMF with anti-TfR was performed following fixation with 3% paraformaldehyde and permeabilization with 0.2% Triton X-100 prior to labeling with Texas Red-streptavidin and the FITC-conjugated anti-mouse secondary antibodies. For the bAMF cointernalization with FITC-dextran, NIH-3T3 cells were incubated with bAMF (250 µg/ml) and FITC-dextran (5 mg/ml) for 15 min at 37°C and after the indicated times of chase, the cells were washed three times with PBS supplemented with 0.1 mM Ca^{++} and 1 mM Mg^{++}

(PBS/CM), fixed with 3% paraformaldehyde, and permeabilized with 0.2% Triton X-100 prior to labeling with Texas Red-streptavidin to reveal bAMF.

In order to label only cell surface exposed bAMF, AMF-R or fibronectin, viable cells were labeled in medium which did not contain bicarbonate supplemented with 25 mM Hepes and 5% calf serum (bicarbonate-free medium) at 4°C with Texas Red-streptavidin and the appropriate primary antibodies, fixed with 3% paraformaldehyde and then labeled with FITC-conjugated secondary antibodies (Benlimame et al., 1998). To quench cell surface bAMF, NIH-3T3 cells were pulse labeled with bAMF for the indicated time and then placed at 4°C and incubated with 50 µg/ml of unconjugated streptavidin diluted in bicarbonate-free medium for 10 minutes. The cells were then washed 3 times with complete medium supplemented with 5 µg/ml of d-biotin in order to block free sites of bound unconjugated streptavidin, washed 3 more times with complete medium to remove the free biotin, and then chased for 30 minutes at 37°C prior to cell surface labeling as described above. For the 20°C block, cells were pulsed with bAMF (25 µg/ml) for 30 minutes or 3 hours at 20°C in bicarbonate-free medium. To disrupt microtubules, NIH-3T3 cells were pretreated for 30 minutes with 10 µM nocodazole and then pulsed with bAMF (25 µg/ml) for 30 minutes at 37°C in the presence of nocodazole.

After labeling, the coverslips were mounted in Airvol (Air Products and Chemicals, Allentown, PA) and viewed in a Zeiss Axioskop fluorescent microscope equipped with a 63X Plan Apochromat objective and selective filters. Confocal microscopy was performed with the 60X Nikon Plan Apochromat objective of a dual channel Bio-Rad MRC-600 laser scanning confocal microscope equipped with a krypton/argon laser and the corresponding dichroic reflectors to distinguish fluorescein and Texas Red labeling. To quantify cellular expression of cell surface fibrils labeled for bAMF or AMF-R, 50 cells

were selected at random from at least three experiments and the number of cells presenting fibrillar labeling counted.

Electron microscopy

NIH-3T3 fibroblasts grown on tissue culture dishes were pulsed and chased with bAMF as described for the fluorescence studies and then processed for electron microscopy as previously described (Benlimame et al., 1998). Ultrathin sections (80 nm) were incubated in saturated sodium periodate solution for 20 min at 22°C. The sections were blocked with 1% BSA in PBS and then incubated with nanogold-streptavidin for 30 minutes. The sections were washed three times with PBS, three times with deionized water and then the nanogold labeling was amplified by silver enhancement for 7 min at 22°C. The sections were contrasted with uranyl acetate and lead citrate and examined in a Zeiss CEM902 electron microscope. In the absence of nanogold-streptavidin labeling, silver particles due to non-specific silver enhancement were not observed. In the absence of bAMF internalization, specific labeling of MVBs was not observed.

Substrate coating

AMF, BSA, fibronectin, and polylysine were immobilized on the surface of Nunc Lab-Tek permanox chamber slides (Canadian Life Technologies) or plastic 12- and 96-well tissue culture plates (Falcon) by incubation with the respective solutions at the indicated concentrations for 1 hour at room temperature. AMF, BSA, or fibronectin solutions in PBS were added to the various plastic supports at the same concentration. To correct for the variable surface areas, the volume added was proportional to the surface area of the well and substrate coating concentrations are indicated as $\mu g/cm^2$. Poly-D-lysine coating (10 $\mu g/cm^2$) was performed overnight at 4°C. Where indicated, dishes were incubated in complete medium overnight at 37°C prior to AMF coating.

After incubation with the substrate, the dishes were washed rapidly 3 times, incubated at room temperature for 30 minutes and then washed 3 more times with complete medium in order to remove free protein and block non-specific sites.

Cell adhesion and motility assays

Cell adhesion was measured by adding 20,000 cells to coated or uncoated wells of 96-well plates for 30 minutes at 37°C after which the medium was removed and the wells rinsed once gently with fresh medium. Substrate attached cells were fixed by the addition of 50 µl 50%TCA to 200 µl medium in each well. After incubation at 4°C for at least 60 minutes, the plates were rinsed with water and dried; cells were colored with 0.2% sulforhodamine, rinsed with 1% acetic acid, dried and the cell-associated sulforhodamine solubilized with 100 mM Tris and read in a microplate reader (MR600; Dynatech Instruments Inc., California) at 490 nM, essentially as previously described (Skehan et al., 1990). For each condition, OD values from 8 wells were averaged and the average of 8 identical wells to which cells were not added was substracted.

To measure cell motility, 20,000 NIH-3T3 fibroblasts were plated on plastic tissue culture wells, uncoated or coated with AMF, BSA, polylysine or fibronectin, for one hour in complete medium in a CO_2 incubator before the medium was replaced with bicarbonate-free DMEM containing 25 mM Hepes pH 7.3 supplemented with 10% calf serum. A layer of mineral oil was placed over the medium and the cells placed on a 37°C stage. Time lapse images were collected every 60 minutes for 15 hours with the 10X objective of an inverted Zeiss Axiovert microsope equipped with a Princeton MicroView CCD camera and Northern Eclipse image analysis software. Cell movement was tracked by plotting nuclear location every hour and the distance travelled from 2-15 hours after plating was measured. Data for a total of 30 cells was accumulated from at

least three experiments for each condition. To measure the extent of cell spreading, the contour of cells in the time lapse images collected 60 minutes after cell plating was traced and cell area determined using Northern Eclipse image analysis software. Data of 18 cells was accumulated from three experiments for each condition.

RESULTS

Dual pathways of AMF-R internalization

Receptor-mediated internalization of biotinylated AMF (bAMF) via a clathrin-independent pathway to the smooth ER has been previously demonstrated in NIH-3T3 fibroblasts (Benlimame et al., 1998). Following a 60 minute bAMF pulse, internalized bAMF is localized to both AMF-R labeled smooth ER tubules and punctate structures (Figure 1 A-C) as previously described (Benlimame et al., 1998). bAMF internalization in the presence of mßCD, a cholesterol extracting agent which disrupts caveolae expression (Ohtani et al., 1989; Kilsdonk et al., 1995; Klein et al., 1995; Hailstones et al., 1998; Orlandi and Fishman, 1998; Rodal et al., 1999), prevents bAMF internalization to smooth ER tubules but not to the punctate structures which are distinct from smooth ER AMF-R tubules (Figure 1 D-F). The ability of mßCD to inhibit clathrin-independent internalization to smooth ER tubules indicates that caveolae or caveolae-like structures are involved in this endocytic route.

bAMF internalization in medium acidified to pH 5.5, which blocks clathrin-mediated endocytosis (Heuser, 1989), does not affect bAMF internalization to smooth ER AMF-R tubules but prevents its internalization to the perinuclear punctate structures (Figure 1 G-I). The punctate labeling of internalized bAMF was previously interpreted as fluid phase due to our inability to completely prevent this endocytic route with an excess of unlabeled AMF (Benlimame et al., 1998). However, an excessively high concentration of bAMF (250-500 µg/ml) was used for those studies. We have repeated the bAMF internalization with only 25 µg/ml bAMF and under these conditions a ten-fold excess of unlabeled AMF completely blocks bAMF internalization (Figure 1 J-L) demonstrating that both the clathrin-dependent and -independent pathways are receptor-mediated.

Clathrin-mediated bAMF internalization to multivesicular bodies

In order to identify the specific organelle to which bAMF is internalized via the clathrin-dependent pathway, we performed bAMF internalization in the presence of mßCD to block the caveolae-mediated pathway to smooth ER tubules. mßCD has been described to reduce the clathrin-mediated endocytosis of transferrin and EGF, however at the mßCD concentration used in this study (0.5 mM) the effect was minimal (Rodal et al., 1999; Subtil et al., 1999) and we observed no qualitative effects on the clathrin-mediated endocytosis of bAMF. After a 15 minute bAMF pulse and 30 minute chase, bAMF labeled cells were colabeled with markers for early endosomes (TfR) or for late endosomes and lysosomes (LAMP-1). The punctate structures to which bAMF is localized can be distinguished from early endosomes labeled for the TfR (Figure 2 A-C) and from late endosomes and lysosomes labeled for LAMP-1 (Figure 2 D-F). Previously, internalized bAMF was described to partially colocalize with LAMP-1 (Benlimame et al., 1998) however the high bAMF concentration (250-500 µg/ml) used may have resulted in some late endosomal or lysosomal delivery of bAMF via fluid phase endocytosis. The results presented here suggest that the punctate structures to which bAMF is internalized are neither early endosomes, late endosomes nor lysosomes.

Using the same conditions of bAMF internalization (15 minute pulse in the presence of mßCD and 30 minute chase) for EM and postembedding labeling with streptavidin nanogold, the majority of bAMF labeled structures are MVBs which contain few internal vesicles (Figure 3). MVBs have been previously identified as an intermediate compartment between early and late endosomes (Hopkins and Trowbridge, 1983; Dunn et al., 1986; Gruenberg et al., 1989), which is consistent with the ability to distinguish bAMF labeling from that of both the TfR and LAMP-1.

bAMF associates with fibronectin cell surface sites

To distinguish bAMF internalization from endocytosis to lysosomes, we cointernalized bAMF and FITC-dextran, a fluid phase marker. Complete colocalization of cointernalized bAMF and FITC-dextran was observed following a 15 minute pulse labeling (Figure 4 A-C). The short nature of the pulse restricted our ability to visualize bAMF internalization to smooth ER tubules, first visualized after a 30 minute bAMF pulse and best seen after a 60 minute bAMF pulse. Following a 15 minute chase, internalized bAMF and FITC-dextran remain colocalized (Figure 4 D-F) however after a 30 minute chase the bAMF labeling dissociates from that of FITC-dextran (Figure 4 G-I). After a 60 minute chase, bAMF no longer associates with FITC-dextran positive endocytic organelles and exhibits a highly fibrillar localization (Figure 4 J-L). After 2 hours of chase, bAMF is completely associated with fibrils (Figure 4 M-O). The dissociation of bAMF from the fluid phase endocytic pathway of FITC-dextran confirms that bAMF internalization to MVBs is receptor-mediated.

The fibrillar labeling of bAMF internalized and chased for 2 hours can be labeled with Texas Red-streptavidin added to viable cells at 4°C showing that this fibrillar labeling is localized at the cell surface of NIH-3T3 fibroblasts (Figure 5 A). This cell surface labeling was further shown to exhibit complete colocalization with fibronectin (Figure 5 A, B), a secreted ECM component (Yamada and Olden, 1978; Mosher, 1984). To ensure that Texas Red-streptavidin labeling of fibronectin fibrils is truly due to the distribution of bAMF to these sites, cells which had internalized and recycled bAMF were double labeled with anti-AMF antibodies (Niinaka et al., 1998). Anti-AMF antibody labeled fibronectin fibrils demonstrating that AMF is indeed localized to these cell surface fibrils (Figure 5 C,D). To show that bAMF internalizes and recycles to cell surface fibronectin fibrils together with its receptor, cells were surface labeled with Texas-Red streptavidin to label cell surface bAMF and with anti-

AMF-R antibodies to label cell surface AMF-R. AMF-R exhibits a punctate distribution as previously reported (Benlimame et al., 1998) but can also be localized to fibrillar sites to which bAMF is localized (Figure 5 E,F). Endogenous AMF is expressed in the BALB/c 3T3-A31 cell line (Niinaka et al., 1998) and cell surface labeled at 4°C with antibodies to AMF (Figure 5 G) and AMF-R (Figure 5 H) show that AMF and AMF-R associate with cell surface fibrils in the absence of a bAMF pulse at 37°C. While the extent of fibrillar labeling varied between cells, the majority of cells could be observed to express AMF and AMF-R labeled cell surface fibrils.

AMF recycles to fibronectin fibrils via clathrin-mediated endocytosis to MVBs

To demonstrate that bAMF fibrillar labeling is indeed the result of bAMF recycling from intracellular sites and not a consequence of the redistribution of cell surface AMF-R, residual cell surface bAMF was quenched by the addition of 50 µg/ml unconjugated streptavidin at 4°C for 10 minutes after the bAMF pulse label and before the chase at 37°C. To ensure that the unconjugated streptavidin eliminated the cell surface labeling, NIH-3T3 fibroblasts were pulse labeled with bAMF for 30 minutes at 37°C, conditions which result in extensive cell surface bAMF fibrils (Figure 6 A, B). As seen in Figure 6 C, incubation with streptavidin at 4°C was able to completely eliminate bAMF cell surface labeling. Subsequently, NIH-3T3 cells were pulsed with bAMF for 15 minutes, after which no fibrillar cell surface bAMF is detected (Figure 4), and then incubated with unconjugated streptavidin at 4°C before a 30 minute chase at 37°C. Rinsing the cells with biotin prior to the chase was necessary to visualize bAMF recycling, evidently in order to block free binding sites of the unconjugated streptavidin, and extending the 4°C incubation also reduced the extent of recycling. Nevertheless, distinct fibronectin fibrils labeled for bAMF were observed on

49±3 % of cells after the streptavidin quench (compared to 82±3 % of cells that were not quenched) clearly indicating that the association of bAMF with cell surface fibrils is a consequence of bAMF recycling following endocytosis (Figure 6 E, F).

To determine which endocytic pathway mediates recycling of bAMF to cell surface fibronectin fibrils, we endocytosed bAMF at 20°C, a temperature block that prevents endocytic traffic between the early and late endosome (Dunn et al., 1980; Hopkins and Trowbridge, 1983; Griffiths et al., 1988). In contrast to the cell surface fibrillar bAMF labeling of cells pulse labeled for 30 minutes at 37°C (Figure 7 A), cells pulse labeled for either 30 minutes or 3 hours at 20°C exhibit no surface fibril-associated bAMF (Figure 7 C,E). However, bAMF internalization to smooth ER AMF-R tubules is still visualized in permeabilized cells after a 3 hour bAMF pulse at 20°C (not shown). Furthermore, the presence of mßCD does not affect bAMF recycling to fibronectin fibrils (Figure 7 G, H). bAMF recycling to cell surface fibronectin fibrils is therefore due to clathrin-dependent endocytosis of bAMF.

The MVB is equivalent to the endosomal carrier vesicle (ECV) which mediates early to late endosomal traffic (Gruenberg and Maxfield, 1995). Depolymerization of microtubules blocks endocytic traffic from early to late endosomes at the level of the MVB/ECV (De Brabander et al., 1988; Gruenberg et al., 1989; Aniento et al., 1993). To determine that bAMF recycling to the cell surface is mediated by MVBs and not a later endocytic compartment, NIH-3T3 cells were treated with 10 µM nocodazole to depolymerize microtubules and then pulse labeled with bAMF for 30 minutes at 37°C. In the presence of nocodazole (Figure 8), bAMF is internalized to smooth ER AMF-R tubules and MVBs (Figure 8 C, D) in an equivalent fashion to cells exhibiting intact microtubules (Figure 8 A, B). Recycling of bAMF to fibronectin fibrils is not

disrupted by microtubule depolymerization indicating that it is not mediated by a late endosomal compartment (Figure 8 E, F).

AMF recycling to fibronectin fibrils regulates cell motility

In contrast to the ability of bAMF to recycle to cell surface fibronectin fibrils on uncoated Lab-Tek wells (Figure 9 A, B), bAMF does not recycle to cell surface fibrils on an AMF coated substrate (Figure 9 C, D). Plating of the cells on a polylysine coated substrate, effectively immobilizing the cells (Figure 10), did not prevent bAMF recycling to fibronectin fibrils (Figure 9 E, F). The AMF substrate also inhibited the association of AMF-R with fibronectin fibrils to a similar extent (Figure 9 G, H). Labeling of permeabilized cells plated on an AMF coated substrate showed that bAMF was internalized to smooth ER AMF-R tubules but not to punctate MVBs (not shown) indicating that the presence of substrate attached AMF selectively inhibits ligand stimulated receptor internalization and recycling via the clathrin-dependent pathway.

The number of cells exhibiting bAMF or AMF-R labeled fibrils on a polylysine coated substrate (bAMF = 78±3%; AMF-R = 68±8%) was essentially equivalent to that on an uncoated substrate (bAMF = 78±4%; AMF-R = 60±9%). However, decreased expression of bAMF and AMF-R labeled fibrils correlated with increased AMF substrate concentrations. No cells plated on an AMF substrate at a concentration of 260 $\mu g/cm^2$ exhibited bAMF or AMF-R labeled fibrils. On 26 $\mu g/cm^2$ AMF coated substrate, only 14±3 and 8±4 % of cells presented bAMF or AMF-R labeled fibrils, respectively, while on 2.6 $\mu g/cm^2$ AMF coated substrate 40±7% and 31±3% of cells presented bAMF or AMF-R labeled fibrils, respectively. The ability to specifically inhibit bAMF recycling on AMF coated substrates allowed us to determine the role of the bAMF recycling pathway in cell motility. The motility of NIH-3T3 cells was determined by time lapse videomicroscopy over 13 hours. On dishes coated with AMF solutions

varying from 2.6 to 560 μg/cm^2, the motility of NIH-3T3 cells is reduced by more than 50%; increasing AMF concentrations resulted in greater inhibition of motility however the effect is saturating as cell motility on 560 μg/cm^2 AMF was equivalent to that on 260 μg/cm^2 AMF (Figure 10 A). Inhibition of cell motility is therefore directly correlated with inhibition of bAMF and AMF-R recycling to fibronectin fibrils by the AMF coated substrate.

On polylysine coated plates, effectively immobilizing the cells, the basal motility detected due to nuclear movement was significantly lower ($p < 10^{-5}$) than that of cells plated on 260 or 560 μg/cm^2 AMF indicating that the AMF substrate does not completely immobilize the cells. To control for the possibility that the AMF coating prevented the attachment of soluble serum factors thereby inhibiting cell motility, plates were coated with 260 μg/cm^2 BSA or incubated with serum containing medium overnight before applying AMF; the BSA coating did not influence cell motility and precoating the plates with serum did not affect the ability of a subsequent AMF coating to reduce the motility of the cells. An immobilized AMF substrate therefore specifically inhibits both clathrin-dependent AMF recycling and cell motility.

Substrate adhesivity is a critical determinant of cell motility and increasing substrate concentrations of fibronectin is associated with inhibition of cell motility (Palacek et al., 1997). The reduced motility of cells on AMF substrate could therefore be due to cell immobilization due to increased adhesivity. To determine if AMF acts as an adhesion molecule, we measured cell motility on fibronectin coated substrates of different concentrations and compared the adhesivity of NIH-3T3 cells to AMF and fibronectin substrates which induced a similar degree of motility inhibition. Substrate coating with 5 μg/cm^2 fibronectin stimulated cell motility however 50 μg/cm^2 fibronectin inhibited cell motility to a similar extent as substrate coating with 260 μg/cm^2

AMF and 100 $\mu g/cm^2$ fibronectin immobilized the cells as observed for the polylysine coated substrate (Figure 10 A). Using a microtitre plate based adhesion assay, we measure cell adhesivity to AMF, fibronectin, BSA and polylysine coated substrates. As shown in Figure 10 B, 260 $\mu g/cm^2$ of AMF substrate coating did not increase cell adhesivity greater than an equivalent concentration of BSA. Furthermore, for all concentrations of AMF used, coating the substrate with an equivalent concentration of BSA resulted in the same or greater cell adhesion (not shown). The presence of AMF does not therefore specifically enhance cell adhesion.

Cell adhesion to all concentrations of fibronectin, including 5 $\mu g/cm^2$ fibronectin which is associated with increased cell motility, was greater than to AMF coated substrates (Figure 10 B). In particular, at concentrations where equivalent motility inhibition on AMF or fibronectin substrates was observed (2.6 $\mu g/cm^2$ AMF vs. 20 $\mu g/cm^2$ fibronectin; 260 $\mu g/cm^2$ AMF vs 50 $\mu g/cm^2$ fibronectin), cell adhesion to the fibronectin coated substrate was significantly greater than to the AMF coated substrate ($p < 10^{-5}$). Furthermore, spreading of newly plated cells on AMF coated substrates was equivalent to a BSA coated substrate and not much increased relative to cells plated on uncoated plastic although spreading on fibronectin coated substrates was significantly greater (Figure 10 C).

DISCUSSION

Dual pathways of AMF-R internalization

AMF-R is a cell surface receptor which has also been localized to a smooth subdomain of the endoplasmic reticulum (Benlimame et al., 1995; Wang et al., 1997). At the cell surface AMF-R was localized to caveolae and we previously demonstrated that bAMF is internalized via a clathrin-independent pathway to smooth ER tubules (Benlimame et al., 1998). The absence of bAMF internalization to smooth ER in the presence of mßCD (Figure 1) confirms that the internalization of the AMF/AMF-R complex to smooth ER AMF-R tubules is mediated by caveolae or caveolae-like structures. mßCD is a cholesterol extracting reagent which disrupts the expression of caveolae and glycolipid rafts (Ohtani et al., 1989; Kilsdonk et al., 1995; Klein et al., 1995; Hailstones et al., 1998; Rodal et al., 1999). mßCD and filipin disrupt cholera toxin internalization and mßCD blocks the internalization of E. coli by macrophages demonstrating that cholestrol extracting agents are able to disrupt caveolae mediated internalization (Baorto et al., 1997; Orlandi and Fishman, 1998). mßCD exerts minimal influence on ricin endocytosis which is internalized via a non-clathrin pathway (Rodal et al., 1999). The ability of mßCD to inhibit AMF internalization to smooth ER demonstrates that this internalization pathway, similar to that described for SV40 virus (Kartenbeck et al., 1989), is not equivalent to the clathrin-independent ricin pathway. These data support the existence of two distinct clathrin-independent endocytic pathways: a non-clathrin pathway to endosomes and a caveolae-mediated pathway to smooth ER.

AMF is also internalized via an acid-inhibited clathrin-mediated pathway to a perinuclear endosomal compartment (Benlimame et al., 1998). While the specificity of this pathway was not previously established (Benlimame et al., 1998), we show here that an excess of unlabeled AMF can inhibit both the

caveolae and clathrin-mediated internalization of bAMF demonstrating that both pathways are receptor-mediated (Figure 11). The cholecystokinin receptor is also internalized via both clathrin and caveolae pathways; the caveolae internalization pathway delivers the receptor to submembrane vesicles which do not internalize further into the cell and which recycle the receptor to the cell surface (Roettger et al., 1995). The AMF-R sequence is highly conserved and codes for a novel seven-transmembrane domain receptor (Shimizu et al., 1999) whose activity is pertussis-toxin sensitive (Nabi et al., 1990). Dual pathways of ligand-receptor internalization may be related to differential receptor sensitivity to motogenic or mitogenic stimuli, as observed for AMF (Silletti and Raz, 1993).

Recycling of bAMF to fibronectin fibrils is mediated by the MVB

The ability of the 20°C block but not mßCD to prevent bAMF recycling demonstrates that clathrin-mediated endocytosis of bAMF to the MVB and not caveolae-mediated endocytosis to SER is responsible for bAMF recycling to the cell surface (Figure 11). Whether the bAMF/AMF-R complex can recycle from the smooth ER to the cell surface, perhaps in response to a localized signal, remains to be determined (Nabi, 1999). The identification of the clathrin-dependent bAMF endocytic compartment by electron microscopy as spherical vesicles containing internal membranes equivalent to multivesicular bodies (MVBs) (Figure 3) is consistent with the ability to distinguish bAMF positive endocytic structures from TfR labeled early endosomes (Gruenberg and Maxfield, 1995) and LAMP-1 labeled late endosomes (Geuze et al., 1988; Griffiths et al., 1988). The MVB is equivalent to the ECV (Gruenberg and Maxfield, 1995) and the inability of microtubule depolymerization to disrupt bAMF recycling indicates that the recycling pathway does not require ECV/MVB to late endosome targeting (Gruenberg et al., 1989; Bomsel et al., 1990; Aniento et al., 1993). MVBs have previously been shown to be the site of

sorting of cell surface recycling of EGF receptor (EGF-R); kinase active EGF-R is localized to internal membrane vesicles of the MVB while kinase-dead EGF-R remains on the limiting membrane of the MVB and recycles to the cell surface (Felder et al., 1990). The MVBs labeled for bAMF could be equivalent to a sorting endosome at an early stage of maturation which retains recycling ability but also presents few internal vesicles (Trowbridge et al., 1993).

Recycling of bAMF via MVB to the cell surface delivers the receptor-ligand complex to extracellular fibronectin fibrils and is, to our knowledge, the first demonstrated recycling pathway of a motility factor receptor to sites of ECM deposition. In our experiments, NIH-3T3 cells were plated in the absence of exogenous fibronectin such that the fibronectin observed in these cultures was necessarily derived from the cells. It is not however clear whether fibronectin and recycling bAMF are cosecreted. The removal of fibronectin from areas of cell-substrate contact and its internalization and recycling have been proposed to be involved in the reorganization of the ECM and of cell-substrate attachments (Avnur and Geiger, 1981; Grinnell, 1986). A portion of the fibronectin receptor is internalized via clathrin-dependent endocytosis to an endosomal compartment from which its recycles back to the cell surface (Raub and Kuentzel, 1989). Recycling of ß1 integrin via MVBs and the recycling compartment has been recently described and proposed to contribute to cell migration (Ng et al., 1999).

Fibrillar ECM contact sites have been described to form in fibroblasts (Chen and Singer, 1982) and to be associated with the fibronectin receptor or α5ß1 integrin (Chen et al., 1985; Singer et al., 1988). These fibrillar ECM contact or fibrillar adhesions are enriched in α5ß1 integrin and tensin and are distinct from focal contacts which are labeled for vinculin, paxillin and phosphotyrosine (Zamir et al., 1999). It has been recently reported that

fibronectin fibrils are formed by the translocation of α5ß1 integrin from focal contacts to the fibrillar ECM contact sites along actin filaments via its association with tensin, a principal component of these ECM fibrils (Katz et al., 2000; Pankov et al., 2000; Zamir et al., 2000).

The nature of the association of the AMF/AMF-R complex with fibronectin is not yet clear, however free bAMF was not observed to associate with fibronectin (not shown). The activated AMF/AMF-R complex may associate directly with fibronectin or serve as a chaperone to other proteins, such as the fibronectin receptor, that regulate cell adhesion to the ECM. The AMF-R recycling pathway could function to deliver integrins or fibronectin to extracellular cell adhesion sites. AMF activation of its receptor has been shown to increase adhesion and spreading of metastatic murine melanoma (B16a) cells on fibronectin due to the up-regulation of surface αIIbß1 and α5ß1 integrins (Timar et al., 1996). Furthermore, AMF differentially influences the adhesion, spreading and migration of low and high metastatic murine K1735 melanoma cells on the ECM components fibronectin, laminin and collagen type IV via alterations of focal contact architecture (Silletti et al., 1998a). In low metastatic cells, ECM reorganization induced by AMF stimulation has been suggested to modulate cell motility (Silletti et al., 1998b). AMF activation of the AMF-R recycling pathway could be actively involved in the remodeling of the fibronectin ECM of motile cells by regulating fibril formation or turnover.

Recycling of AMF/AMF-R and cell motility

The rapid movement of cells over a substrate requires that an essential aspect of cell motility must be the directed delivery of the necessary molecular machinery to the site of pseudopodial extension, a process that must necessarily implicate the same vesicular trafficking mechanisms implicated in the polarization of other cell types (Nabi, 1999). On an AMF substrate, we

observe the reduction of NIH-3T3 fibroblast motility that is associated with the inhibition of AMF/AMF-R recycling to fibronectin fibrils but not with increased cell adhesion and spreading. In addition, the motility of cells plated on saturating AMF substrate concentrations remains significantly higher than that of cells immobilized on polylysine or on 100 $\mu g/cm^2$ fibronectin indicating that the AMF substrate is not immobilizing the cell but rather slowing down cell movement. The AMF coated substrate is therefore not reducing cell motility by enhancing substrate adhesivity but rather due to specific interaction of the immobilized ligand with its receptor selectively inhibiting the clathrin-dependent recycling pathway of the AMF/AMF-R complex to cell surface fibronectin fibrils. A direct relationship between reduction of cell motility and of AMF/AMF-R recycling was observed. Inhibition of this recycling pathway is not a consequence of reduced cell motility as cells immobilized on polylysine still exhibit efficient recycling of AMF to cell surface fibrils. The clathrin-dependent AMF/AMF-R recycling pathway is therefore not a determining factor but rather a limiting factor for cellular displacement. AMF-R mediated recycling to sites of ECM deposition may be a necessary element of the establishment and stabilization of de novo cell-substrate adhesive contacts during cell movement.

AMF-R function in cell motility therefore involves signal transduction of a motility signal as well as internalization and recycling of its ligand-receptor complex. Whether AMF/AMF-R recycling to fibronectin fibrils is involved in fibril formation and/or turnover or rather acts to regulate de novo cell adhesion to sites of ECM deposition remains to be determined.

ACKNOWLEDGEMENTS

We thank Gaetan Mayer for his assistance with the nanogold labeling, Ginette Guay for technical assistance and Jean Leveillé and Gaston Lambert for the preparation of the figures. This study was supported by grants from the Medical Research Council of Canada (IRN) and NIH CA-51714 (AR).

Altankov, G. and Grinnell, F. (1993). Depletion of intracellular potassium disrupts coated pits and reversibly inhibits cell polarization during fibroblast spreading. *J. Cell Biol.* **120**, 1449-1459.

Aniento, F., Emans, N., Griffiths, G. and Gruenberg, J. (1993). Cytoplasmic dynein-dependent vesicular transport from early to late endosomes. *J. Cell Biol.* **123**, 1373-1387.

Avnur, Z. and Geiger, B. (1981). The removal of extracellular fibronectin from areas of cell-substrate contact. *Cell* **25**, 121-32.

Bajno, L., Peng, X. R., Schreiber, A. D., Moore, H. P., Trimble, W. S. and Grinstein, S. (2000). Focal Exocytosis of VAMP3-containing Vesicles at Sites of Phagosome Formation. *J Cell Biol* **149**, 697-706.

Baorto, D. M., Gao, Z., Malviya, R., Dustin, M. L., van der Merwe, A., Lublin, D. M. and Abraham, S. N. (1997). Survival of enterobacteria in macrophages relies on glycolipid traffic. *Nature (Lond.)* **389**, 636-639.

Benlimame, N., Le, P. U. and Nabi, I. R. (1998). Localization of autocrine motility factor receptor to caveolae and clathrin-independent internalization of its ligand to smooth endoplasmic reticulum. *Molec. Biol. Cell* **9**, 1773-1786.

Benlimame, N., Simard, D. and Nabi, I. R. (1995). Autocrine motility factor receptor is a marker for a distinct tubular membrane organelle. *J. Cell Biol.* **129**, 459-471.

Bomsel, M., Parton, R., Kuznetsov, S. A., Schroer, T. A. and Gruenberg, J. (1990). Microtubule- and motor-dependent fusion in vitro between apical and basolateral endocytic vesicles from MDCK cells. *Cell* **62**, 719-731.

Bretscher, M. S. (1996). Getting membrane flow and the cytoskeleton to cooperate in moving cells. *Cell* **87**, 601-606.

Chen, W. T., Hasegawa, E., Hasegawa, T., Weinstock, C. and Yamada, K. M. (1985). Development of cell surface linkage complexes in cultured fibroblasts. *J Cell Biol* **100**, 1103-14.

Chen, W. T. and Singer, S. J. (1982). Immunoelectron microscopic studies of the sites of cell-substratum and cell-cell contacts in cultured fibroblasts. *J Cell Biol* **95**, 205-22.

De Brabander, M., Nuydens, R., Geerts, H. and Hopkins, C. R. (1988). Dynamic behavior of the transferrin receptor followed in living epidermoid carcinoma (A431) cells with Nanovid microscopy. *Cell Motil. Cytoskel.* **9**, 30-47.

Dunn, W. A., Connolly, T. P. and Hubbard, A. L. (1986). Receptor-mediated endocytosis of epidermal growth factor by rat hepatocytes: Receptor pathway. *J. Cell Biol.* **102**, 24-36.

Dunn, W. A., Hubbard, A. L. and Aronson, N. N. J. (1980). Low temperature selectively inhibits fusion between pinocytic vesicles and lysosomes during heterophagy of ^{125}I-asialofetuin by the perfused rat liver. *J. Biol. Chem.* **255**, 5971-5978.

Felder, S., Miller, K., Moehren, G., Ullrich, A., Schlessinger, J. and Hopkins, C. R. (1990). Kinase activity controls the sorting of the epidermal growth factor receptor within the multivesicular body. *Cell* **61**, 623-634.

Geuze, H. J., Stoorvogel, W., Strous, G. J., Slot, J. W., Bleekemolen, J. E. and Mellman, I. (1988). Sorting of mannose-6-phosphate receptors and lysosomal membrane proteins in endocytic vesicles. *J. Cell Biol.* **107**, 2491-2501.

Griffiths, G., Hoflack, B., Simons, K., Mellman, I. and Kornfeld, S. (1988). The mannose-6-phosphate receptor and the biogenesis of lysosomes. *Cell* **52**, 329-341.

Grinnell, F. (1986). Focal adhesion sites and the removal of substratum-bound fibronectin. *J. Cell Biol.* **103**, 2697-706.

Gruenberg, J., Griffiths, G. and Howell, K. E. (1989). Characterization of the early endosome and putative endocytic carrier vesicles in vivo and with an assay of vesicle fusion in vitro. *J. Cell Biol.* **108**, 1301-1316.

Gruenberg, J. and Maxfield, F. R. (1995). Membrane transport in the endocytic pathway. *Curr. Opin. Cell Biol.* **7**, 552-563.

Hailstones, D., Sleer, L. S., Parton, R. G. and Stanley, K. K. (1998). Regulation of caveolin and caveolae by cholesterol in MDCK cells. *J. Lipid Res.* **39**, 369-379.

Hendey, B., Klee, C. B. and Maxfield, F. R. (1992). Inhibition of neutrophil chemokinesis on vitronectin by inhibitors of calcineurin. *Science* **258**, 296-299.

Heuser, J. (1989). Effects of cytoplasmic acidification on clathrin lattice morphology. *J. Cell Biol.* **108**, 401-411.

Hopkins, C. R. and Trowbridge, I. S. (1983). Internalization and processing of transferrin and the transferrin receptor in human carcinoma A431 cells. *J. Cell Biol.* **97**, 508-521.

Kartenbeck, J., Stukenbrok, H. and Helenius, A. (1989). Endocytosis of simian virus 40 into the endoplasmic reticulum. *J. Cell Biol.* **109**, 2721-2729.

Katz, B.-Z., Zamir, E., Bershadsky, A., Kam, Z., Yamada, K. M. and Geiger, B. (2000). Physical state of the extracellular matrix regulates the structure and molecular composition of cell-matrix adhesions. *Molec. Biol. Cell* **11**, 1047-1060.

Kilsdonk, E. P. C., Yancey, P. G., Stoudt, G. W., Bangerter, F. W., Johnson, W. J., Phillips, M. C. and Rothblat, G. H. (1995). Cellular cholesterol efflux mediated by cyclodextrins. *J. Biol. Chem.* **270**, 17250-17256.

Klein, U., Gimpl, G. and Fahrenholz, F. (1995). Alteration of the myometrial plasma membrane cholesterol content with ß-cyclodextrin modulates the binding affinity of the oxytocin receptor. *Biochem.* **34**, 13784-13793.

Lamaze, C. and Schmid, S. L. (1995). The emergence of clathrin-independent pinocytic pathways. *Curr. Opin. Cell Biol.* **7**, 573-80.

Lawson, M. A. and Maxfield, F. R. (1995). Ca^{2+}- and calcineurin-dependent recycling of an integrin to the front of migrating neutrophils. *Nature (Lond.)* **377**, 75-79.

Mellman, I. (1996). Endocytosis and molecular sorting. *Annu. Rev. Cell Dev. Biol.* **12**, 575-626.

Mosher, D. F. (1984). Physiology of fibronectin. *Ann. Rev. Med.* **35**, 561-75.

Nabi, I. R. (1999). The polarization of the motile cell. *J. Cell Sci.* **112**, 1803-1811.

Nabi, I. R., Watanabe, H. and Raz, A. (1990). Identification of B16-F1 melanoma autocrine motility-like factor receptor. *Cancer Res.* **50**, 409-414.

Ng, T., Shima, D., Squire, A., Bastiaens, P. I., Gschmeissner, S., Humphries, M. J. and Parker, P. J. (1999). PKCa regulates ß1 integrin-dependent cell motility through association and control of integrin traffic. *The EMBO Journal* **18**, 3909-23.

Niinaka, Y., Paku, S., Haga, A., Watanabe, H. and Raz, A. (1998). Expression and secretion of neuroleukin/phosphohexose isomerase/maturation factor as autocrine motility factor by tumor cells. *Cancer Res.* **58**, 2667-2674.

Ohtani, Y., Irie, T., Uekama, K., Fukunaga, K. and Pitha, J. (1989). Differential effects of a-, ß-, and g-cyclodextrins on human erythrocytes. *Eur. J. Biochem.* **186**, 17-22.

Orlandi, P. A. and Fishman, P. H. (1998). Filipin-dependent inhibition of cholera toxin: evidence for toxin internalization and activation through caveolae-like domains. *J. Cell Biol.* **141**, 905-15.

Palacek, S. P., Loftus, J. C., Ginsberg, M. H., Lauffenberger, D. A. and Horwitz, A. F. (1997). Integrin-ligand binding properties govern cell migration speed through cell-substratum adhesiveness. *Nature (Lond.)* **385**, 537-540.

Pankov, R., Cukierman, E., Katz, B. Z., Matsumoto, K., Lin, D. C., Lin, S., Hahn, C. and Yamada, K. M. (2000). Integrin dynamics and matrix assembly: tensin-dependent translocation of alpha(5)beta(1) integrins promotes early fibronectin fibrillogenesis. *J Cell Biol* **148**, 1075-90.

Raub, T. J. and Kuentzel, S. L. (1989). Kinetic and morphological evidence for endocytosis of mammalian cell integrin receptors by using an anti-fibronectin receptor ß subunit monoclonal antibody. *Exp. Cell Res.* **184**, 407-426.

Rodal, S. K., Skretting, G., Garred, Ø., Vilhardt, F., van Deurs, B. and Sandvig, K. (1999). Extraction of cholesterol with methyl-ß-cyclodextrin perturbs formation of clathrin-coated endocytic vesicles. *Molec. Biol. Cell* **10**, 961-974.

Roettger, B. F., Rentsch, R. U., Pinon, D., Holicky, E., Hadac, E., Larkin, J. M. and Miller, L. J. (1995). Dual pathways of internalization of the cholecystokinin receptor. *J. Cell Biol.* **128**, 1029-1041.

Sandvig, K. and van Deurs, B. (1991). Endocytosis without clathrin. *Cell Biol. Int. Rep.* **15**, 3-8.

Shimizu, K., Tani, M., Watanabe, H., Nagamachi, Y., Niinaka, Y., Shiroishi, T., Ohwada, S., Raz, A. and Yokota, J. (1999). The autocrine motility factor receptor gene encodes a novel type of seven transmembrane protein. *FEBS Lett.* **456**, 295-300.

Silletti, S., Paku, S. and Raz, A. (1998a). Autocrine motility factor and the extracellular matrix. I. Coordinate regulation of melanoma cell adhesion, spreading and migration involves focal contact reorganization. *Int. J. Cancer* **76**, 120-128.

Silletti, S., Paku, S. and Raz, A. (1998b). Autocrine motility factor andthe extracellular matrix. II. Degradation or remodeling of substratum components directs the motile response of tumor cells. *Int. J. Cancer* **76**, 129-135.

Silletti, S. and Raz, A. (1993). Autocrine motility factor is a growth factor. *Biochem. Biophys. Res. Comm.* **194**, 446-457.

Silletti, S. and Raz, A. (1996). Regulation of autocrine motility factor receptor expression in tumor cell locomotion and metastasis. *Curr. Topics Microbiol. Immunol.* **213/II**, 137-169.

Singer, II, Scott, S., Kawka, D. W., Kazazis, D. M., Gailit, J. and Ruoslahti, E. (1988). Cell surface distribution of fibronectin and vitronectin receptors depends on substrate composition and extracellular matrix accumulation. *J. Cell Biol.* **106**, 2171-82.

Skehan, P., Storeng, R., Scudiero, D., Monks, A., McMahon, J., Vistica, D., Warren, J. T., Bokesch, H., Kenney, S. and Boyd, M. R. (1990). New colorimetric cytotoxicity assay for anticancer-drug screening. *J. Natl. Cancer Inst.* **82**, 1107-12.

Subtil, A., Gaidarov, I., Kobylarz, K., Lampson, M. A., Keen, J. H. and McGraw, T. E. (1999). Acute cholesterol depletion inhibits clathrin-coated pit budding. *Proc. Natl. Acad. Sci. U.S.A.* **96**, 6775-80.

Sun, Y. J., Chou, C. C., Chen, W. S., Wu, R. T., Meng, M. and Hsiao, C. D. (1999). The crystal structure of a multifunctional protein: phosphoglucose isomerase/autocrine motility factor/neuroleukin. *Proc. Natl. Acad. Sci. U.S.A.* **96**, 5412-5417.

Timar, J., Trikha, M., Szekeres, K., Baza, R., Tovari, J., Silletti, S., Raz, A. and Honn, K. V. (1996). Autocrine motility factor signals integrin-mediated metastatic melanoma cell adhesion and invasion. *Cancer Res.* **56**, 1902-1908.

Trowbridge, I. S., Collawn, J. F. and Hopkins, C. R. (1993). Signal-dependent membrane protein trafficking in the endocytic pathway. *Ann. Rev. Cell Biol.* **9**, 129-161.

Wang, H.-J., Benlimame, N. and Nabi, I. R. (1997). The AMF-R tubule is a smooth ilimaquinone-sensitive subdomain of the endoplasmic reticulum. *J. Cell Sci.* **110**, 3043-3053.

Watanabe, H., Takehana, K., Date, M., Shinozaki, T. and Raz, A. (1996). Tumor cell autocrine motility factor is the neuroleukin/phosphohexose isomerase polypeptide. *Cancer Res.* **56**, 2960-2963.

Wessels, D., Reynolds, J., Johnson, O., Voss, E., Burns, R., Daniels, K., Garrard, E., O'Halloran, T. J. and Soll, D. R. (2000). Clathrin plays a novel role in the regulation of cell polarity, pseudopod formation, uropod stability and motility in Dictyostelium. *J. Cell Sci.* **113**, 21-36.

Yamada, K. M. and Olden, K. (1978). Fibronectins - adhesive glycoproteins of cell surface and blood. *Nature (Lond.)* **275**, 179-184.

Zamir, E., Katz, B. Z., Aota, S., Yamada, K. M., Geiger, B. and Kam, Z. (1999). Molecular diversity of cell-matrix adhesions. *J Cell Sci* **112**, 1655-69.

Zamir, E., Katz, M., Posen, Y., Erez, N., Yamada, K. M., Katz, B. Z., Lin, S., Lin, D. C., Bershadsky, A., Kam, Z. and Geiger, B. (2000). Dynamics and segregation of cell-matrix adhesions in cultured fibroblasts. *Nat Cell Biol* **2**, 191-196.

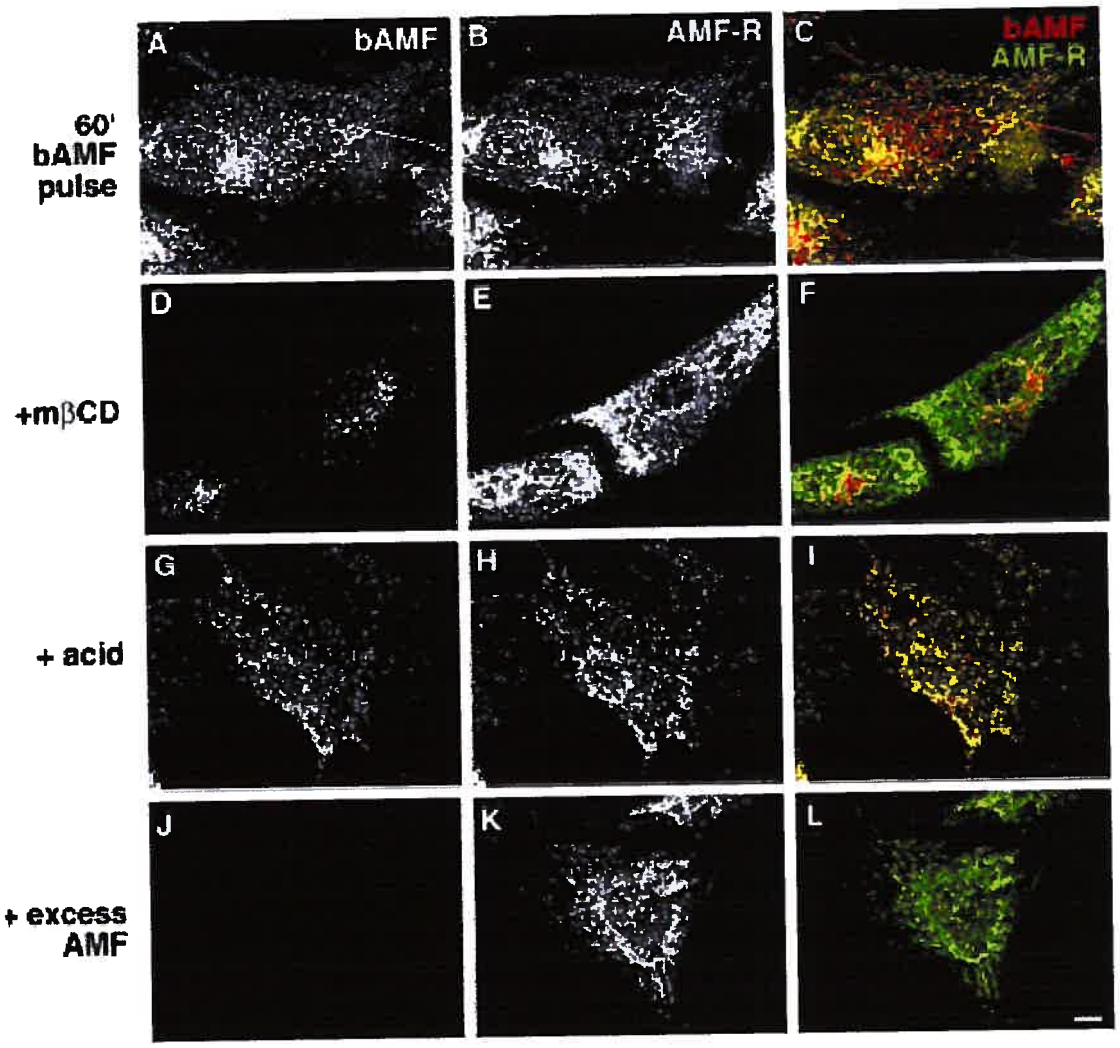


Figure 1: Dual internalization pathways of AMF-R. NIH-3T3 cells were pulse labeled with 25 μg/ml bAMF for 60 minutes at 37°C in complete medium (A-C), in serum-free medium supplemented with 1% BSA and 0.5 mM mßCD (D-F), in complete medium acidified to pH 5.5 (G-I), or in complete medium supplemented with a 10-fold excess of unlabeled AMF (J-L). After fixation with methanol/acetone. bAMF was revealed with Texas Red-streptavidin (A, D, G, J) and AMF-R tubules labeled with anti-AMF-R mAb and FITC-conjugated secondary antibodies (B, E, H, K). FITC and Texas Red confocal images were merged and colocalization appears in yellow (C, F, I, L). Bar = 10 μm.

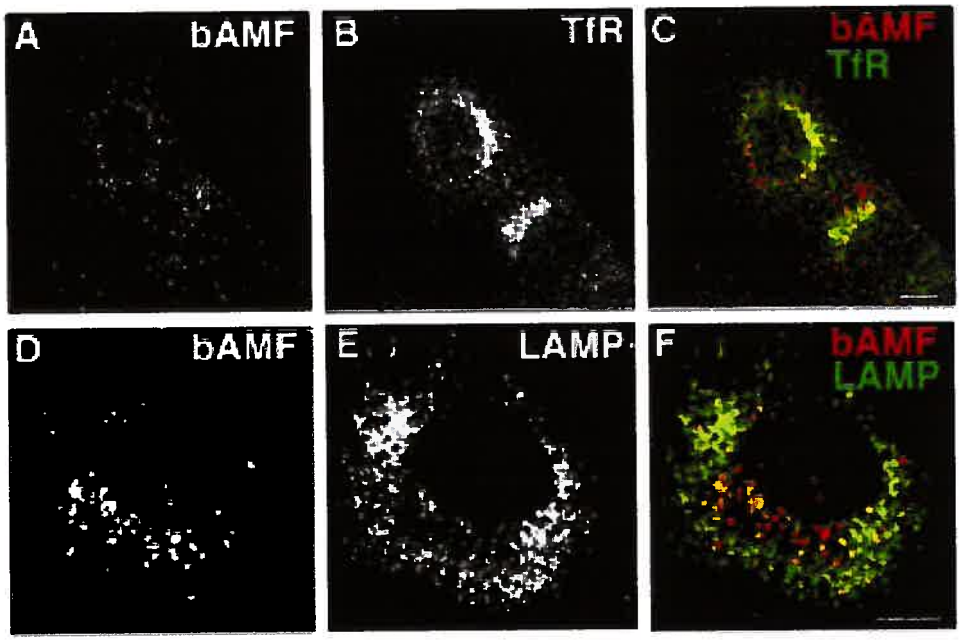
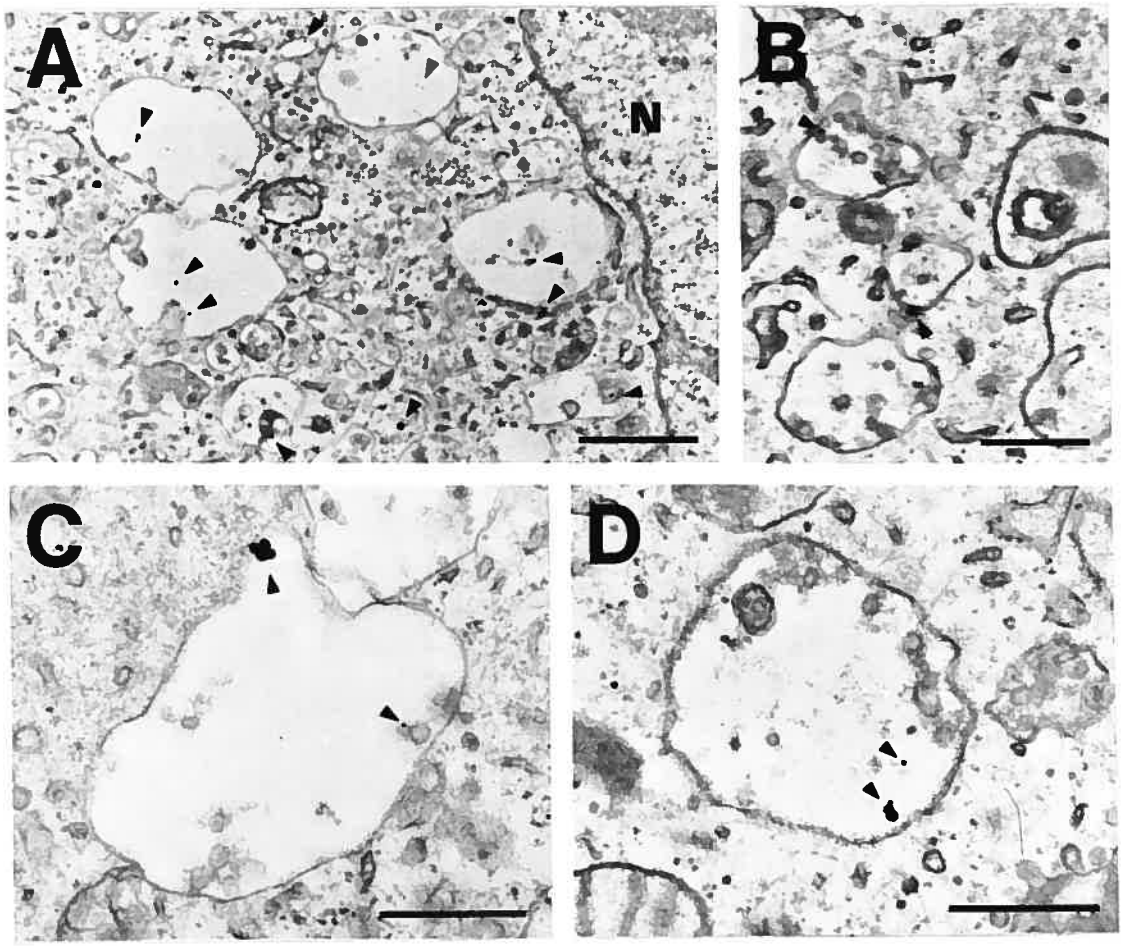


Figure 2: Clathrin-mediated bAMF endocytosis delivers bAMF to a TfR and LAMP-1 negative compartment. NIH-3T3 cells were pulse labeled with 25 μg/ml bAMF for 15 minutes at 37°C in the presence of 0.5 mM of mßCD and chased for 30 minutes in complete medium. The cells were either fixed with 3% paraformaldehyde and permeabilized with 0.2% TX-100 (A-C) and then double labeled with Texas-red streptavidin to reveal bAMF (A) and anti-TfR and FITC-conjugated secondary antibodies (B) or fixed with methanol/acetone (D-F) and double labeled with Texas Red-streptavidin to reveal bAMF (D) and anti-LAMP-1 and FITC-conjugated secondary antibodies (E). Confocal images from both fluorescent channels were merged (C, F) and colocalization appears in yellow. Bar = 10 μm.

Figure 3: Electron microscopy of bAMF internalization to the MVBs. NIH-3T3 fibroblasts were pulsed with 25 µg/ml bAMF for 15 minutes at 37°C in the presence of 0.5 mM of mßCD and chased for 30 minutes in complete medium (A-D). The localization of bAMF was revealed by postembedding labeling with streptavidin-nanogold following by silver enhancement. Arrowheads point to silver particles. Internalized bAMF is predominantly localized to MVBs. N: nucleus. Bar = 0.5 µm.


A
N
B
C
D

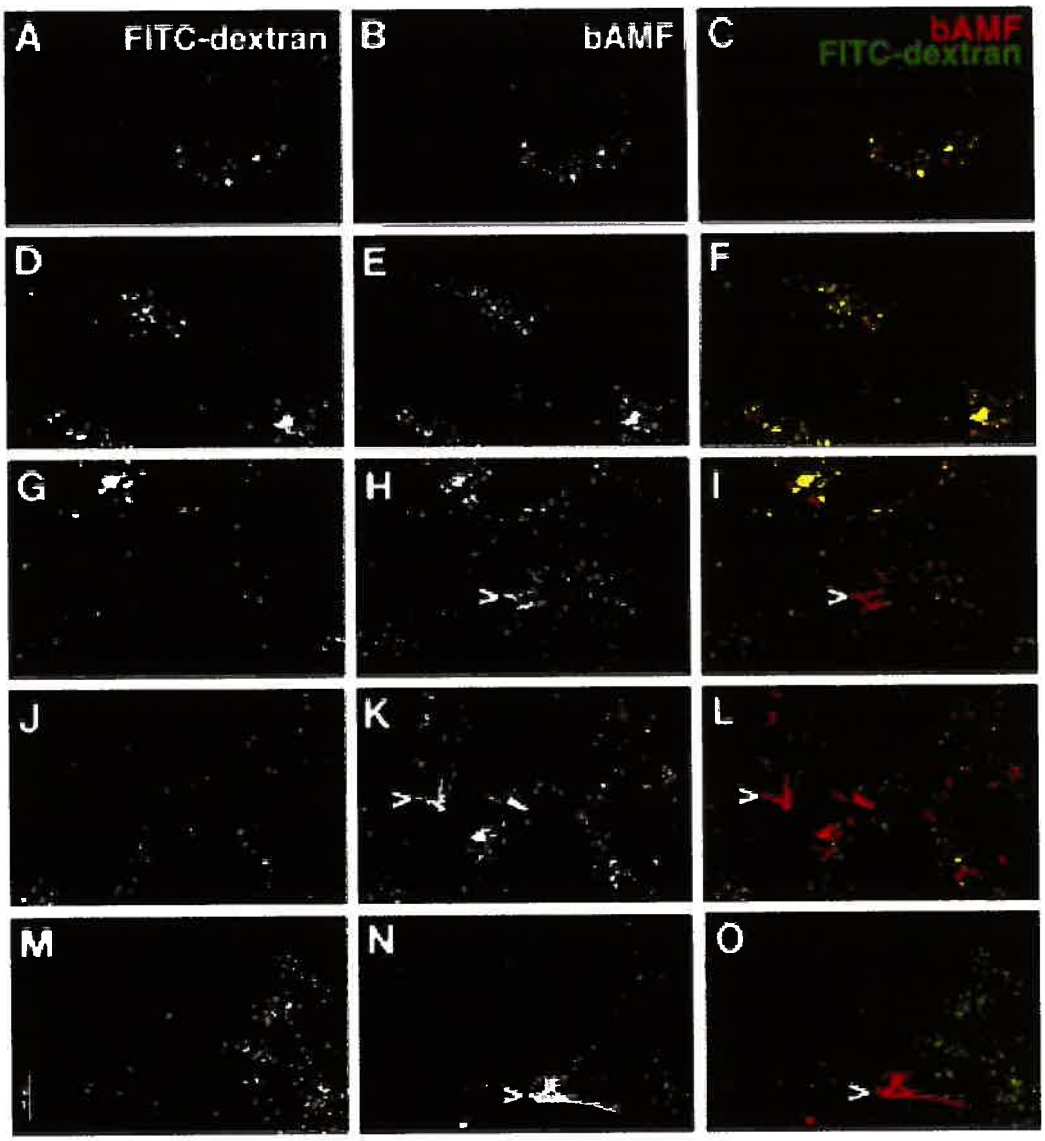


Figure 4: Cointernalization of bAMF with the fluid phase marker FITC-dextran. NIH-3T3 cells were pulse labeled with 250 μg/ml bAMF and 5 mg/ml FITC-dextran for 15 minutes at 37°C and either not chased (A-C) or chased for 15 (D-F), 30 (G-I), 60 (J-L) or 120 minutes (M-O). In these representative confocal images, FITC-dextran labeling (A, D, G, J, M) appears in green in the merged images (C, F, I, L, O) and AMF labeling (B, E, H, K, N) appears in red in the merged images (C, F, I, L, O). The arrows point to fibrillar labeling of bAMF. Bar (M) = 10 μm.

Figure 5: Colocalization of recycling bAMF with fibronectin. NIH-3T3 cells were pulse labeled with 25 µg/ml bAMF for 30 minutes and chased for 2 hours in complete medium (A-F) or not pulsed with bAMF (G-H). Cell surface expression was selectively labeled by incubating viable cells at 4°C with Texas-Red streptavidin to detect cell surface bAMF (A, C, E) and with antibodies to fibronectin (B), AMF (D, G), or AMF-R (F, H) and either FITC (B,D,F,H) or Texas Red (G) conjugated secondary antibodies. Arrows indicate cell surface fibrils. Bar = 20 µm.

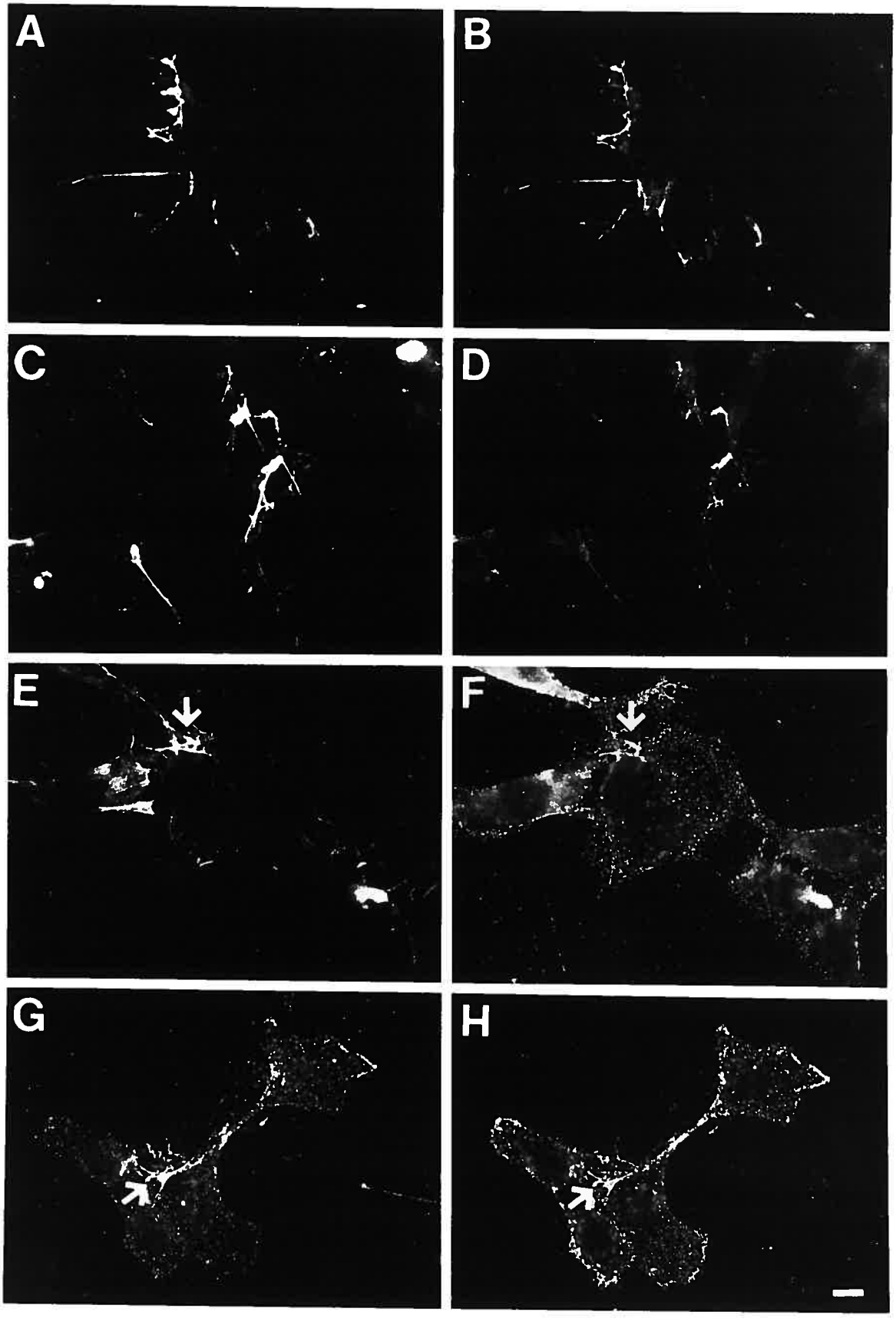

A
B
C
D
E
F
G
H

Figure 6: Endocytosed bAMF is delivered to cell surface fibrils. NIH-3T3 fibroblasts were pulse labeled with 50 µg/ml bAMF for 30 minutes at 37°C and were incubated at 4°C for 10 minutes in bicarbonate-free medium without (A, B) or with 50 µg/ml unconjugated streptavidin (C, D) effectively quenching the expression of cell surface fibrils. Subsequently, cells were pulse labeled with 50 µg/ml bAMF for only 15 minutes at 37°C, quenched with 50 µg/ml of streptavidin at 4°C for 10 minutes and then chased for 30 minutes at 37°C (E, F). Viable cells were surface labeled at 4°C for bAMF using Texas Red-streptavidin (A, C, E) and for fibronectin using anti-fibronectin and FITC conjugated secondary antibodies (B, D, F). Bar = 20 µm.

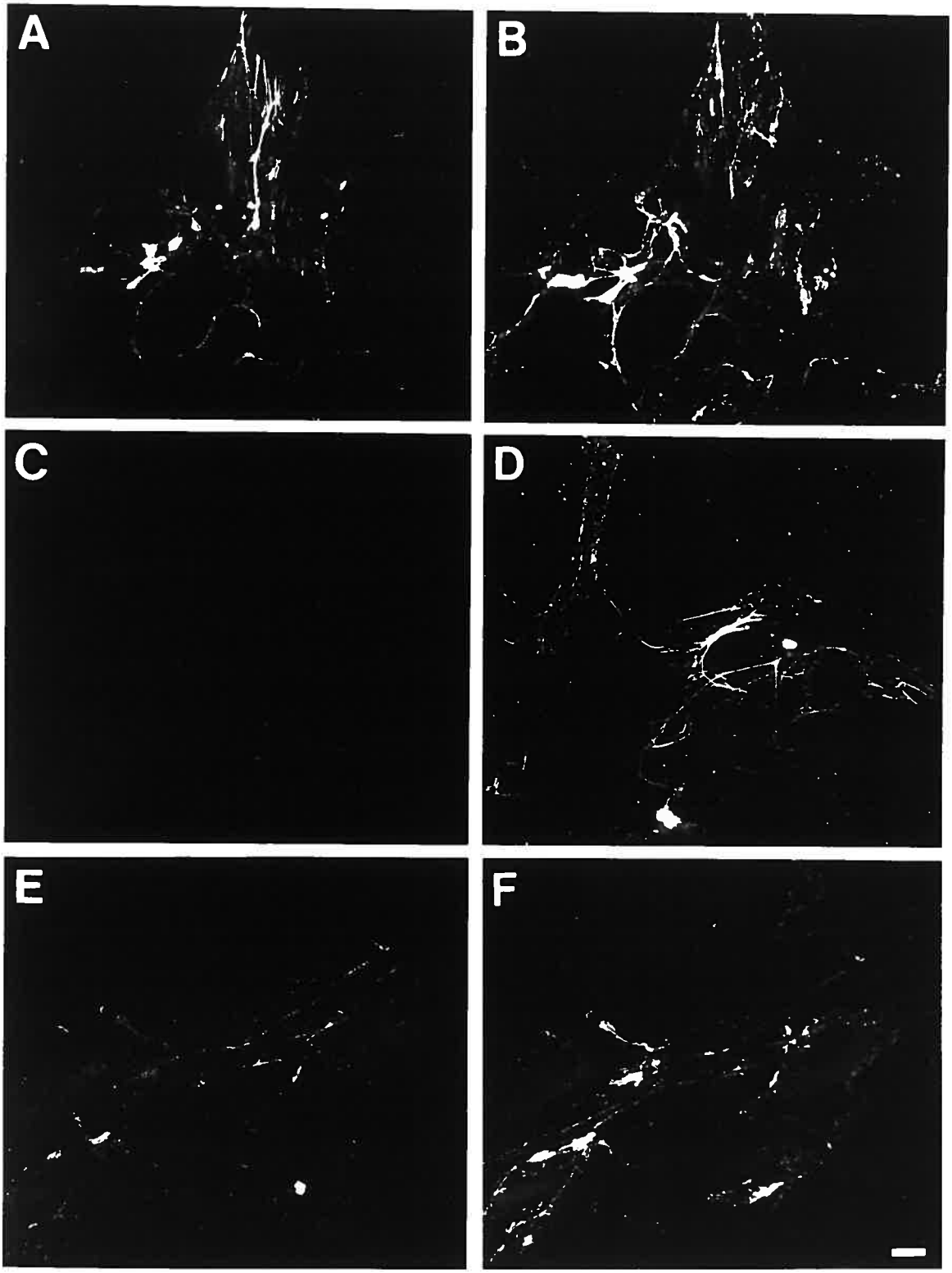

A
B
C
D
E
F

Figure 7: bAMF recycling to cell surface fibrils is blocked at 20°C but not by mßCD. NIH-3T3 cells were pulse labeled with 25 µg/ml bAMF for 30 minutes at 37°C (A, B), for 30 minutes at 20°C (C, D), for 3 hours at 20°C (E, F), or for 30 minutes in the presence of mßCD (G, H). Viable cells were surface labeled at 4°C for bAMF using Texas Red-streptavidin (A, C, E, and G) and for fibronectin using anti-fibronectin and FITC-conjugated secondary antibodies (B, D, F, and H). Bar = 20 µm.

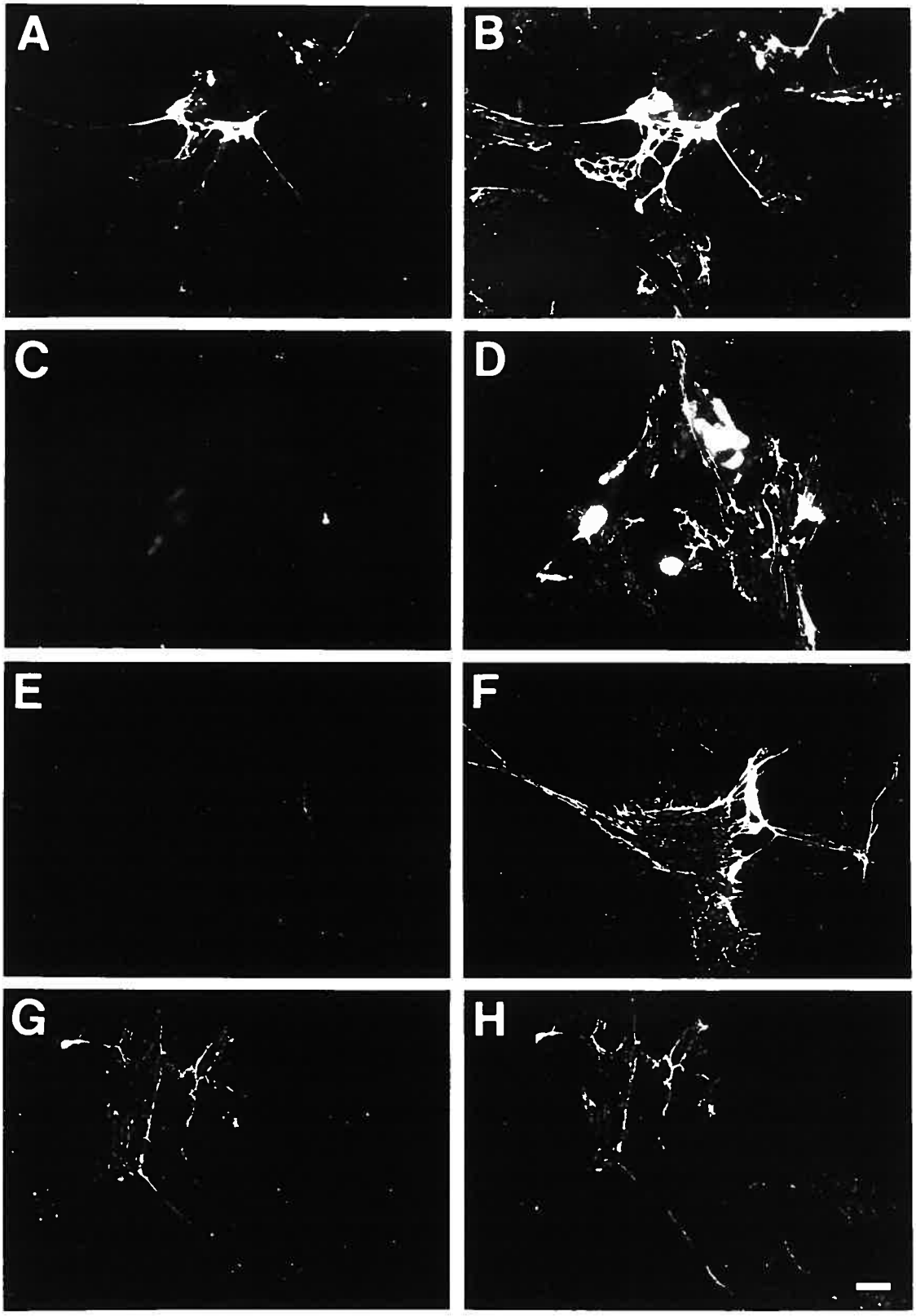

A
B
C
D
E
F
G
H

Figure 8: Microtubule depolymerization does not prevent bAMF recycling. NIH-3T3 fibroblasts were pulse labeled with 25 µg/ml bAMF for 30 minutes in complete medium (A, B) or in complete medium supplemented with 10 µM nocodazole (C-F). After fixation with methanol/acetone, bAMF was revealed with Texas Red-streptavidin (A, C) and tubulin with anti-tubulin antibody and FITC-conjugated secondary antibodies (B, D). Alternatively, cell surface bAMF (E) and cell surface fibronectin (F) were labeled by the addition of Texas Red-streptavidin and anti-fibronectin antibodies, respectively, to viable cells at 4°C and anti-fibronectin antibodies revealed by FITC-conjugated secondary antibodies (F). Bar = 20 µm.

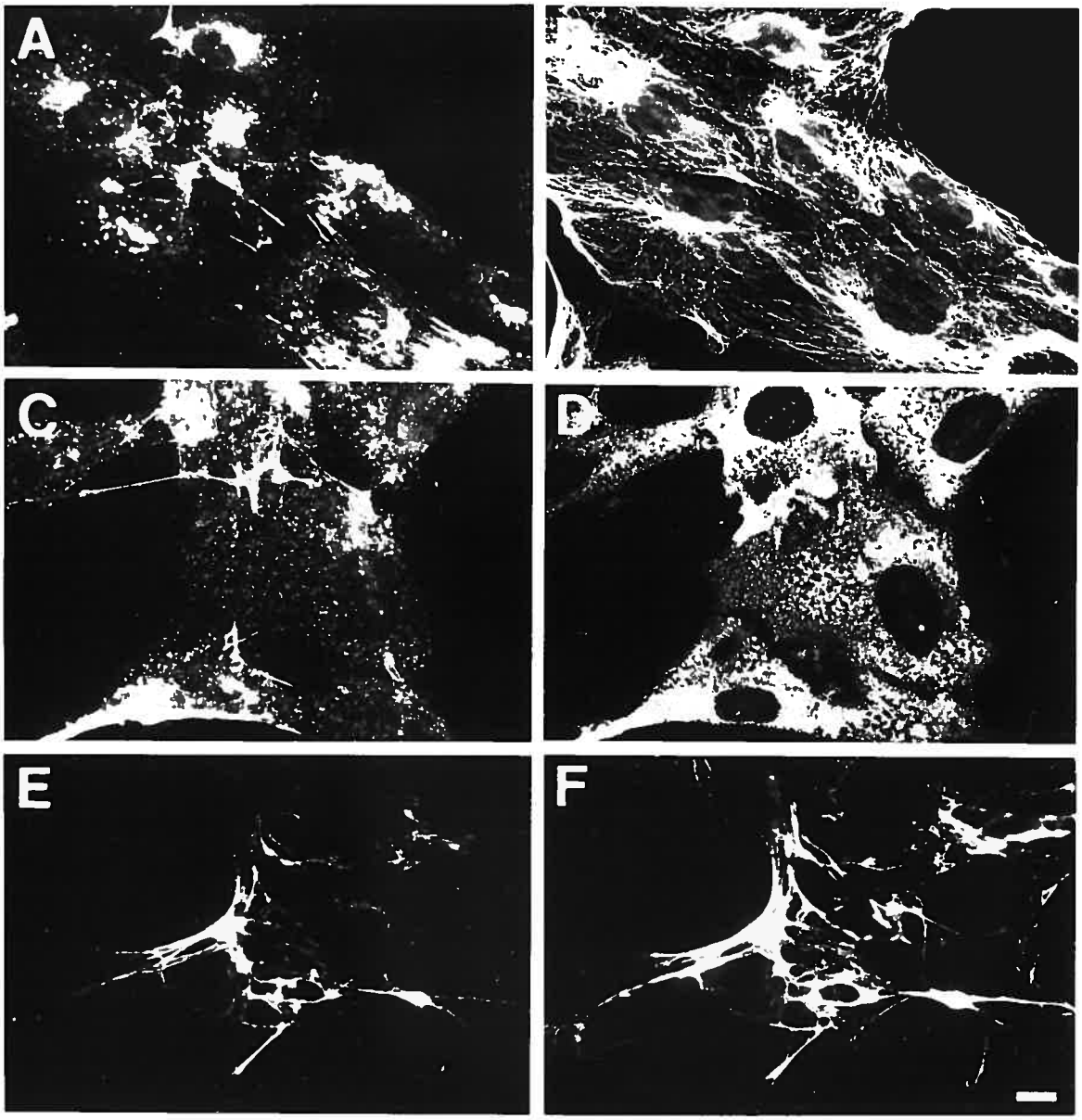
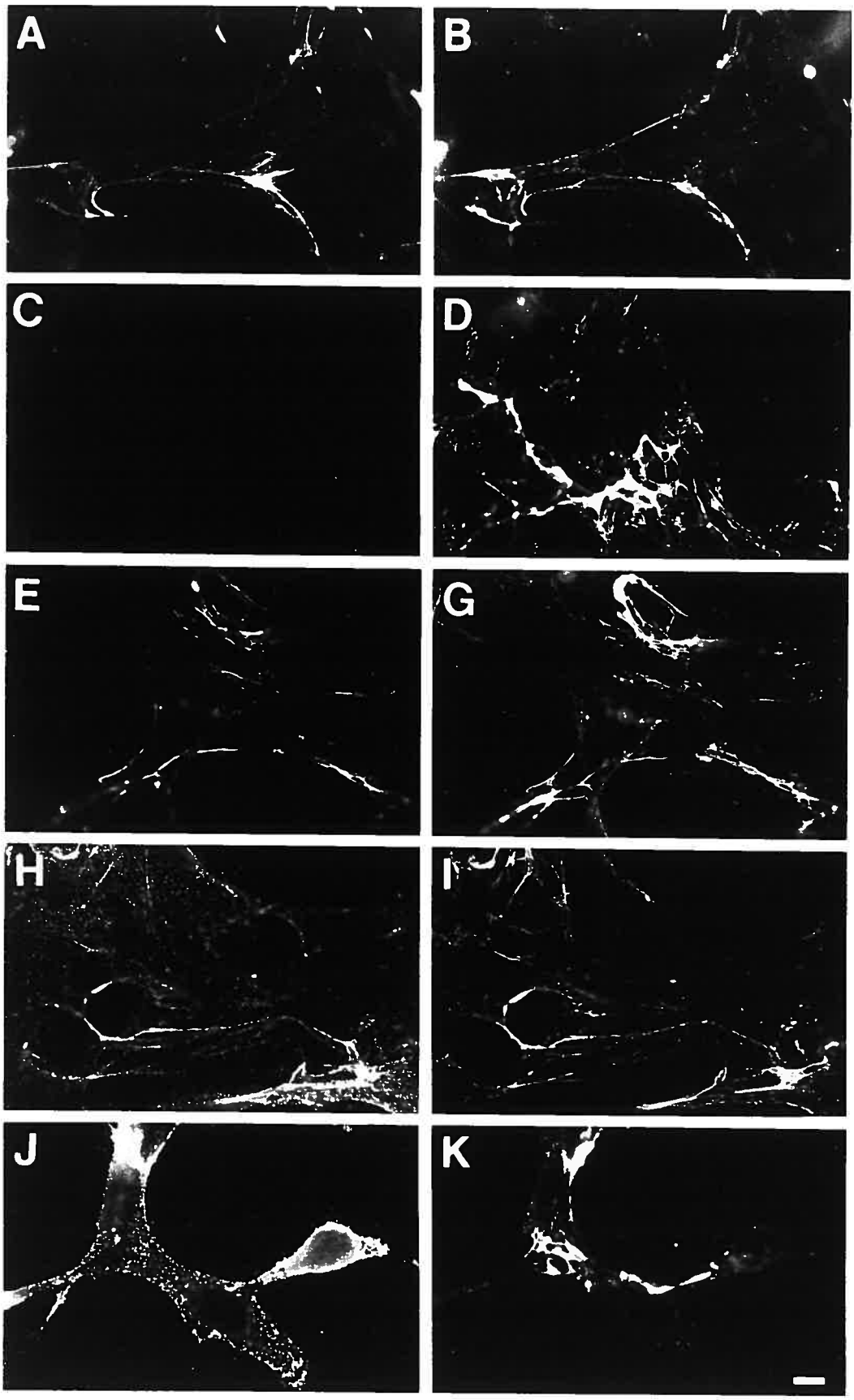

A
C
D
E
F

Figure 9: Substrate attached AMF inhibits AMF-R recycling to cell surface fibronectin fibrils. NIH-3T3 cells were plated on Lab-Tek wells that were either left uncoated (A, B, G, H), coated with 260 μg/cm^2 AMF (C, D, I, J) or coated with 10 μg/cm^2 of polylysine (E, F) and then pulse labeled with 25 μg/ml bAMF for 60 min at 37°C. Viable cells were then cell surface labeled at 4°C with Texas Red-streptavidin to reveal cell surface bAMF (A, C, E) and anti-fibronectin antibodies (B, D, F, H, J) or with anti-AMF-R antibodies (G, I). Fibronectin and AMF-R were subsequently revealed with the appropriate FITC-conjugated secondary antibodies. Bar = 20 μM.


A
B
C
D
E
G
H
I
J
K

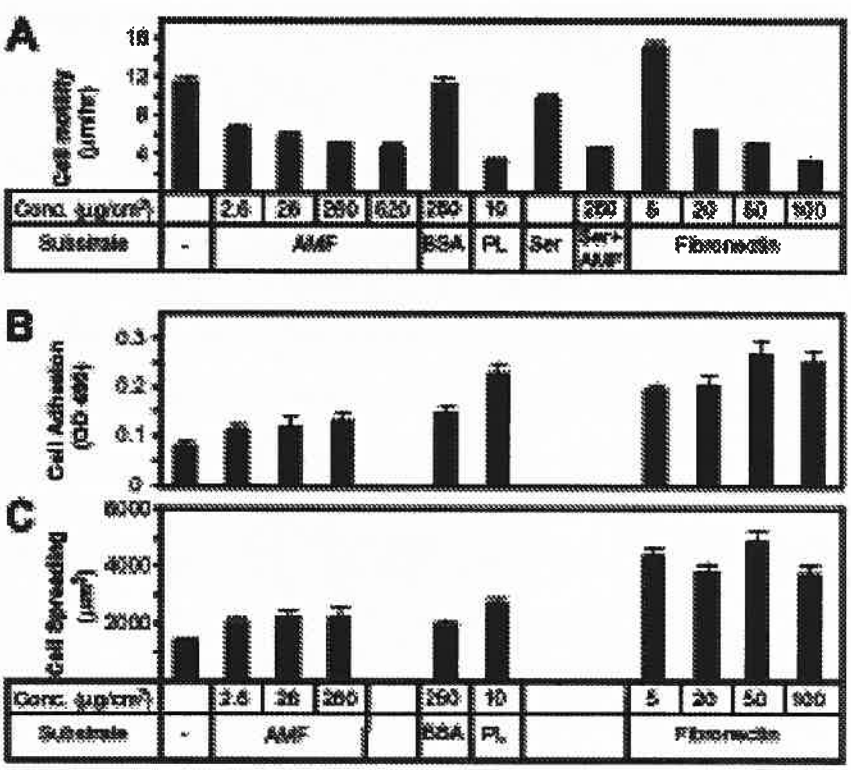


Figure 10: Substrate attached AMF reduces motility but does not affect substrate adhesion of NIH-3T3 fibroblasts. A) Cell motility of NIH-3T3 cells plated on plastic dishes coated with the indicated concentrations of AMF, BSA, polylysine, fibronectin or with AMF after precoating with serum containing medium was measured by videomicroscopy over 13 hours. Motility is expressed in μm/hr and each value represents the average of at least 30 cells measured in at least 3 different video sessions. B) Adhesion of NIH-3T3 cells to microtitre plate wells coated with the indicated concentrations of AMF, fibronectin, polylysine, or BSA was measured following a 30 minute incubation at 37°C. C) Cell spreading of NIH-3T3 cells plated on plastic dishes coated with the indicated concentrations of AMF, BSA, polylysine, or fibronectin was determined from videomicroscopy images obtained one hour after cell plating by measuring the the surface area of the cells.

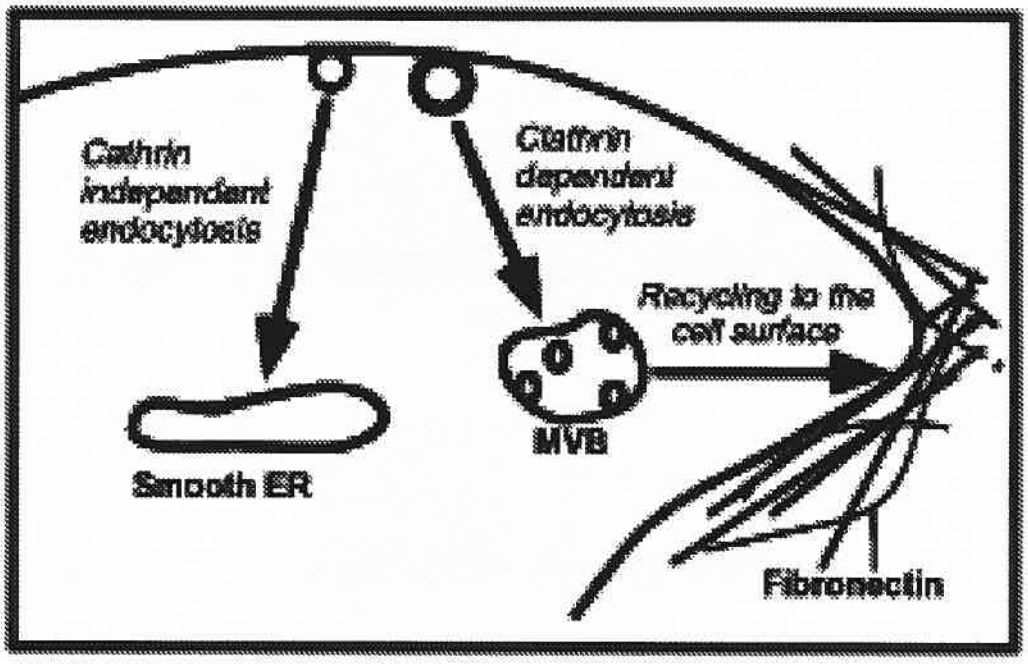


Figure 11: Dual pathways of AMF/AMF-R internalization.

AMF-AMF-R complex is internalized by a clathrin-independent pathway mediated by caveolae or caveolae-like structures to smooth ER AMF-R tubules and by a clathrin-dependent pathway to multivesicular bodies which mediates its recycling to cell surface fibronectin fibrils. The latter is implicated in cell movement.

ARTICLE 3

Caveolin-1 is a negative regulator of caveolae-mediated endocytosis to the endoplasmic reticulum

Le, P.U., G. Guay, Y. Altschuler, and I.R. Nabi, (2002).

J. Biol. Chem. 277:3371-9

CONTRIBUTION DES AUTEURS

J'ai établi tous les protocoles et j'ai effectué toutes les expériences qui ont conduit à la publication de cet article. J'ai également participé activement à la rédaction de ce papier.

Ginette Guay m'a beaucoup aidé pour les techniques de microscopie électronique et aux analyses quantitatives.

Yoram Altschuler a fournis les adénovirus qui ont été utilisés dans cet article.

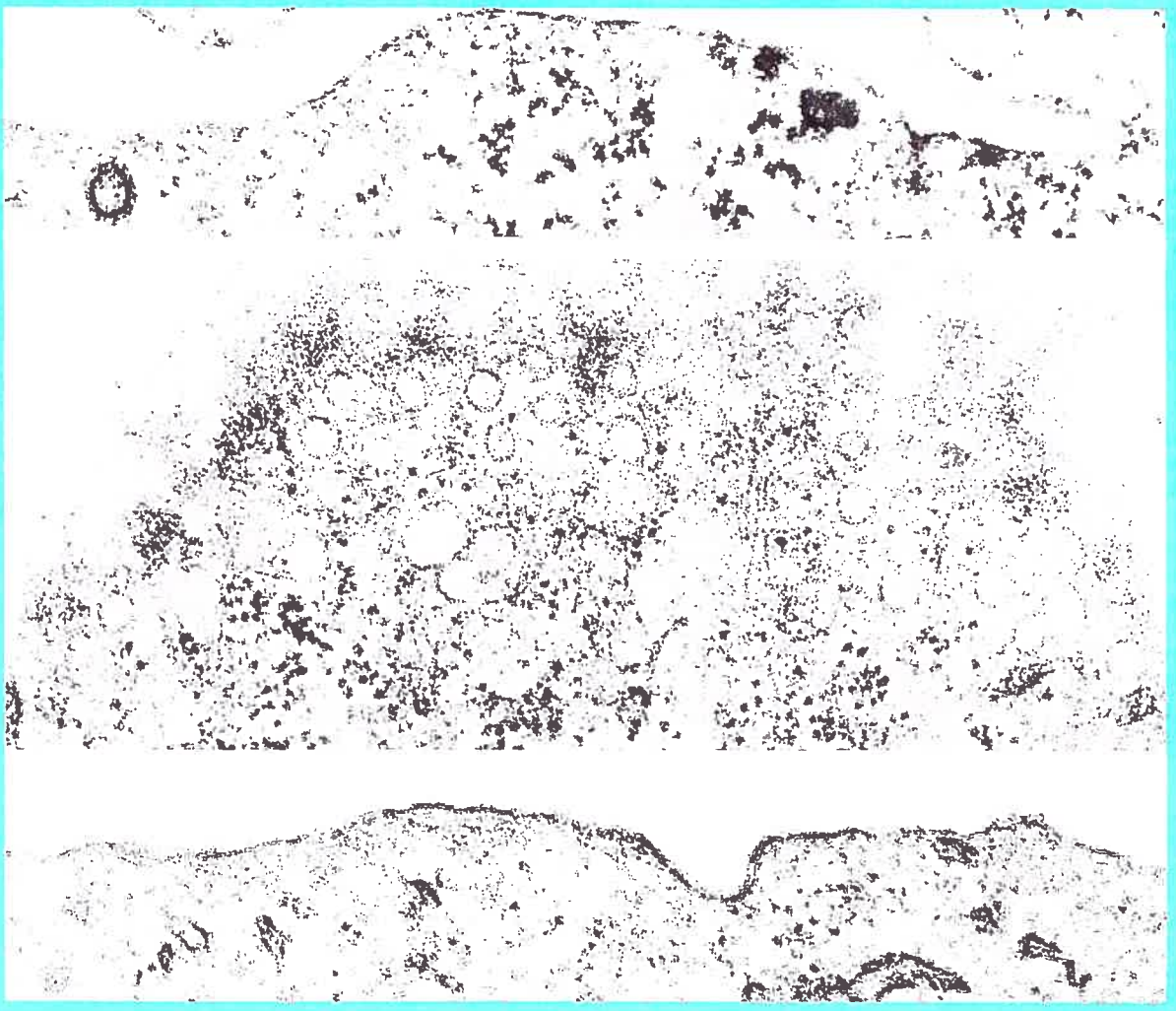
Ivan R. Nabi a principalement participé à la rédaction de l'article.

ISSN 0021-9258 (print)
ISSN 1083-351x (electronic)
JBCHA3 277(5) 3061-3800 (2002)

The Online Version of This Issue Contains Supplemental Material

The Journal of Biological Chemistry

FEBRUARY 1, 2002 VOLUME 277 NUMBER 5



PUBLISHED BY THE AMERICAN SOCIETY FOR
BIOCHEMISTRY AND MOLECULAR BIOLOGY

Founded by Christian A Herter and Sustained in Part by the Christian A Herter Memorial Fund

Caveolin-1 is a negative regulator of caveolae-mediated endocytosis to the endoplasmic reticulum

Phuong U. Le*, Ginette Guay*, Yoram Altschuler‡ and Ivan R. Nabi*

*Department of Pathology and Cell Biology, Université de Montréal, Montreal, Quebec, Canada H3C 3J7; ‡Department of Pharmacology, Hebrew University of Jerusalem, Israel.

Corresponding author:
Dr. Ivan R. Nabi
Département de pathologie et biologie cellulaire
Université de Montréal
C. P. 6128, succursale A
Montréal, Québec
Canada H3C 3J7
Courier address:
Département de pathologie et biologie cellulaire
Université de Montréal, Pavillion principal N-812
2900 Blvd. Edouard Montpetit
Montréal, Québec
Canada H3T 1J4

Running title: Caveolae-mediated endocytosis

SUMMARY

Caveolae are flask-shaped invaginations at the plasma membrane that constitute a subclass of detergent-resistant membrane domains enriched in cholesterol and sphingolipids and that express caveolin, a caveolar coat protein. Autocrine motility factor receptor (AMF-R) is stably localized to caveolae and the cholesterol extracting reagent, methyl-ß-cyclodextrin, inhibits its internalization to the endoplasmic reticulum implicating caveolae in this distinct receptor-mediated endocytic pathway. Curiously, the rate of methyl-ß-cyclodextrin-sensitive endocytosis of AMF-R to the endoplasmic reticulum is increased in ras and abl transformed NIH-3T3 cells that express significantly reduced levels of caveolin and few caveolae. Overexpression of the dynamin K44A dominant negative mutant via an adenovirus expression system induces caveolar invaginations sensitive to methyl-ß-cyclodextrin extraction in the transformed cells without increasing caveolin expression. Dynamin K44A expression further inhibits AMF-R mediated endocytosis to the endoplasmic reticulum in untransformed and transformed NIH-3T3 cells. Adenoviral expression of caveolin-1 also induces caveolae in the transformed NIH-3T3 cells and reduces AMF-R mediated endocytosis to the endoplasmic reticulum to levels observed in untransformed NIH-3T3 cells. Cholesterol-rich detergent-resistant membrane domains or glycolipid rafts therefore invaginate independently of caveolin-1 expression to form endocytosis-competent caveolar vesicles via rapid dynamin-dependent detachment from the plasma membrane. Caveolin-1 stabilizes the plasma membrane association of caveolae and thereby acts as a negative

regulator of caveolae-mediated endocytosis of AMF-R to the endoplasmic reticulum.

Abbreviations used: AMF: autocrine motility factor; AMF-R: Autocrine motility factor receptor; bAMF: biotinylated autocrine motility factor; ER: endoplasmic reticulum; mßCD: methyl-ß-cyclodextrin; dynK44A: dynamin-1 K44A mutant; tTA: tetracycline-regulatable chimeric transcription activator.

INTRODUCTION

Endocytosis via clathrin-coated vesicles represents the best characterized endocytic pathway however other clathrin-independent endocytic mechanisms also exist (1-4). The large GTPase dynamin has been shown to regulate the fission of clathrin-coated pits and expression of the dynamin K44A (dynK44A) mutant inhibits clathrin-mediated endocytosis (5-7). The dynK44A mutant does not affect fluid phase endocytosis or the clathrin-independent endocytic pathway defined by ricin endocytosis indicating that non-clathrin coated cell surface invaginations can detach from the plasma membrane in the apparent absence of dynamin-mediated membrane fission (8-11). However, introduction of inhibitory antibodies to dynamin into hepatocytes resulted in the accumulation of both clathrin-coated vesicles and smooth caveolar invaginations and inhibited the endocytosis of cholera toxin (12). In endothelial cells, caveolae budding from isolated membranes was shown to be dynamin dependent and caveolae shown to contain the molecular machinery necessary for vesicle budding (13,14). Regulation of caveolae budding by dynamin identifies caveolae as endocytosis-competent cell surface invaginations.

Caveolae or smooth plasmalemmal vesicles were first identified in endothelial cells and are morphologically identifiable as smooth flask shaped invaginations of the plasma membrane (15-18). Caveolae are rich in cholesterol and sphingolipids, disrupted by cholesterol extracting agents, and insoluble in Triton X-100 and are therefore considered to form a subclass of cholesterol-rich detergent-resistant membrane domains or glycolipid rafts (19-22). The caveolins (caveolin-1, -2 and –3) are cholesterol binding proteins that form a spiral coat on

the cytoplasmic surface of caveolar invaginations and represent caveolae markers (23-25). Caveolar invaginations are not present in cells that express little or no caveolin and the reintroduction of caveolin-1 into such cells has been shown to induce the formation of caveolae implicating caveolin in the invagination of glycolipid raft microdomains (26-28). Caveolin-1 expression is inversely proportional to cell transformation and caveolin-1 has been characterized as a tumor suppressor gene (27,29-31).

Caveolae have long been proposed to be involved in transcytosis across the endothelial cell (18,32-34). Caveolae or raft mediated endocytosis has been reported for cholera toxin bound GM1 ganglioside, sphingolipids, GPI-anchored proteins, SV40, and bacteria, as well as the endothelin, growth hormone, IL2 and autocrine motility factor (AMF) receptors (35-46). Autocrine motility factor receptor (AMF-R) is a seven transmembrane domain receptor localized at steady state to caveolae and the smooth endoplasmic reticulum (ER) that follows an endocytic pathway sensitive to cholesterol extraction with methyl-ß-cyclodextrin (mßCD) via caveolae to the smooth ER (43,44,47-49). Using AMF as a marker for this caveolae-mediated endocytic pathway, we show that caveolar invaginations and caveolar vesicles mediate AMF-R endocytosis in ras and abl transformed NIH-3T3 cells that express little caveolin and few caveolae. Adenoviral expression of the dominant negative dynK44A mutant or of caveolin-1 has allowed us to demonstrate that: 1) even when caveolin levels are significantly reduced or absent, caveolae form and rapidly bud from the plasma membrane to form caveolar vesicles that target the ER; and 2) caveolin-1

regulates this endocytic pathway by stabilizing caveolae expression at the plasma membrane thereby slowing down the internalization of caveolar vesicles.

EXPERIMENTAL PROCEDURES

Antibodies, reagents and cells

Monoclonal rat antibody against AMF-R was used in the form of concentrated hybridoma supernatant (50). Rabbit anti-caveolin antibody was purchased from Transduction Laboratories (Mississauga, ON), mouse anti-c-myc from Santa Cruz Biotechnology (Santa Cruz, CA), and mouse anti-hemagglutinin (HA) was a gift from Luc Desgroseillers (Department of Biochemistry, Université de Montréal). HRP, FITC and gold-conjugated secondary antibodies from Jackson Immunoresearch Laboratories (West Grove, PA). Rabbit phosphohexose isomerase (referred to as AMF) was purchased from Sigma (Oakville, ON) and biotinylated with NHS-LC-biotin (Pierce, Rockford, IL) according to the manufacturer's instructions. Nanogold-streptavidin and the HQ silver™ Enhancement Kit were purchased from Nanoprobes, Inc. (Stony Brook, NY). MßCD, poly-L-lysine, propidium iodide were purchased from Sigma.

An NIH-3T3 fibroblast clone (43) and H-Ras and v-abl transformed NIH-3T3 cells (29) were grown in complete medium consisting of DME supplemented with 10% calf serum, non-essential amino acids, vitamins, glutamine, and a penicillin-streptomycin antibiotic mixture (Canadian Life Technologies, Burlington, ON). Treatment of cells with 5 mM mßCD was performed as previously described (44).

Viral infection

Recombinant adenoviruses expressing the tetracycline-regulatable chimeric transcription activator (tTA), HA-tagged and dynK44A, and myc-tagged caveolin-1 under the control of the tetracycline-regulated promoter were as previously described (51-53). To enhance infection rates, viral stocks of the tTA and dynK44A or caveolin-1 adenoviruses were diluted in 320 µl sterile PBS and pre-incubated with 72 µl of 1 µg/ml polylysine for 30 minutes at room temperature. Infection with only the tTA adenovirus was used as a control. 2.5 X 10^5 untransformed, ras-transformed or abl-transformed NIH-3T3 cells were plated on 10 cm dishes for 10 hours and rinsed once with PBS before addition of the adenovirus polylysine mixture in 3 ml serum-free media for one hour at 37°C. After removal of the adenovirus mixture, the cells were rinsed twice with serum free media and then incubated for 36 hours in regular culture media. For the EM studies, infection rates of the three cell lines were determined by immunofluorescence labeling with anti-HA or anti-myc antibodies and viral titers were used that resulted in greater than 75% infection rates. The rate of infection was determined in parallel for each experiment and infection with only the tTA adenovirus was used as a control. Where indicated, cells coinfected with tTA and dynK44A adenoviruses were then treated with 5 mM mßCD for 90 minutes prior to fixation.

Electron microscopy

All three cell types were pulsed with bAMF for the indicated times, processed for electron microscopy and bAMF revealed with nanogold-

streptavidin followed by silver enhancement as previously described (44). For the double labeling with anti-AMF-R or polyclonal anti-caveolin antibodies, bAMF was first revealed with streptavidin-nanogold and silver amplification and then the sections were labeled with the primary antibodies following by the appropriate gold-conjugated secondary antibodies. The sections were contrasted with uranyl acetate and lead citrate and examined in a Zeiss CEM902 electron microscope. In the absence of nanogold-streptavidin labeling, silver particles due to non-specific silver enhancement were not observed.

For the quantification of internalized bAMF, the number of silver particles localized to ER, endosomes, and mitochondria were counted and the surface area of the indicated organelles measured using a Sigma Scan measurement system. ER labeling included both ribosome studded rough ER profiles as well as morphologically identified smooth ER (43,47-49). To ensure that the smooth membranous organelles were not early endosomes, only smooth membrane bound structures wider than 75 nm and longer than 200 nm were considered to be ER tubules. Similarly, smooth caveolar invaginations and clathrin coated vesicles within 100 nm of the plasma membrane were counted per unit membrane (43). The average and standard error from 36 images obtained from two separate experiments are presented. Alternatively, bAMF expression at the plasma membrane, in endosomes and in the ER, and caveolin expression at the plasma membrane (including membrane invaginations) and in morphologically identifiable smooth caveolar invaginations were quantified from cells incubated with bAMF and labeled with streptavidin nanogold and silver amplification followed by anti-caveolin antibodies and 12nm gold-conjugated anti-rabbit

secondary antibodies by postembedding immunoelectron microscopy. For each experiment, 25 intact cell profiles were counted.

FACS Analysis

Cells were detached from the dish with EDTA, resuspended in bicarbonate-free medium supplemented with 25 mM Hepes and 5% calf serum. The cells were then incubated with anti-AMF-R antibodies at 4°C for 1 hour, washed three times with cold media, incubated with FITC-conjugated anti-rat IgM at 4°C for 1 hour, washed three more times with cold media and then twice with PBS. The cells were then incubated with 0.5 µg/ml with propidium iodide for 10 minutes at 4°C. Cell associated fluorescence intensity was analyzed on a Beckman FACScan. Cells stained for propidium iodide were discarded from the analysis and cell surface AMF-R expression determined only on intact cells.

Immunoblot

Cells cultured at approximately 70% confluency were scraped, lysed and sonicated in lysis buffer containing 1% SDS, 5 mM EDTA and protease inhibitors. Protein content was assayed using the BCA protein assay (Pierce, Rockford, Illinois) and 40 µg of protein were separated by SDS-PAGE and blotted onto nitrocellulose paper. The blots were blocked with 5% milk in PBS-CM, incubated with rabbit anti-caveolin antibody together with mouse anti-HA and then with HRP-conjugated anti-rabbit and anti-mouse secondary antibodies. The labeled bands were revealed by chemiluminescence and exposed to preflashed Kodak XRP-1 film.

RESULTS

Caveolae-mediated endocytosis of AMF-R to the ER

In NIH-3T3 cells, AMF-R is localized to smooth caveolar invaginations and is endocytosed via a mßCD sensitive caveolae-mediated pathway to the smooth ER tubules defined by AMF-R expression (43,44). In order to specifically assess the specific role of caveolae in this endocytic route, endocytosis of biotinylated AMF (bAMF) was followed in ras and abl transformed NIH-3T3 cells that exhibit significantly reduced expression of caveolae and caveolin (29). Following a 60 minute bAMF pulse at 37°C, endocytosed bAMF detected by nanogold labeling and silver amplification was localized to smooth and rough ER tubules of NIH-3T3 and ras and abl transformed NIH-3T3 cells (Figure 1). Endocytosis of bAMF to multivesicular bodies (MVBs) was also detected in all three cell lines (Figure 1) as previously reported in NIH-3T3 cells (44). Double labeling of the cells for bAMF (nanogold and silver amplification) and for AMF-R (12 nm gold) by electron microscopy confirmed that bAMF is delivered to AMF-R positive ER tubules in all three cell types (Figure 2). AMF-R labeling of rough ER tubules appears to be qualitatively increased in the ras and abl transformed NIH-3T3 cells relative to untransformed NIH-3T3 cells. Previous quantitative studies have shown that the predominant distribution of AMF-R at steady state is to smooth ER tubules in MDCK, NIH-3T3 and HeLa cells, although significant labeling of rough ER tubules was observed in MDCK cells (43,47). For the purpose of this study the rough and smooth ER were not morphologically distinguished.

Quantification of bAMF labeling of the ER, endosomes and mitochondria (see Materials and Methods for details) showed increased bAMF internalization to the ER in ras and abl transformed cells compared to control NIH-3T3 cells (Figure 3 A). Clathrin-dependent endocytosis to endosomal structures (44) was detected at similar levels between the three cell types. Non-specific labeling of mitochondria and control labeling performed in the absence of endocytosed bAMF are also presented. The significantly reduced expression of caveolae and caveolin in the transformed NIH-3T3 cell lines does not therefore prevent endocytosis of bAMF to the ER.

Kinetic analysis of bAMF endocytosis to the ER in the three cell lines showed that the rate of accumulation of bAMF in the ER was equivalent in the ras and abl transformed NIH-3T3 cells and approximately twofold greater than that in untransformed NIH-3T3 cells (Figure 3 B). Over the two hour time course of the experiment, delivery of bAMF to the ER was maintained and not saturable in the three cell lines. FACS analysis showed that cell surface expression of AMF-R is reduced following transformation of NIH-3T3 cells (Figure 3 C) although total AMF-R expression as determined by immunoblot was equivalent or increased in ras and abl transformed NIH-3T3 cells, respectively, compared to untransformed NIH-3T3 cells (data not shown). The increased rate of delivery of bAMF to the ER is therefore not a consequence of increased receptor expression at the plasma membrane but rather due to rapid receptor recycling.

Incubation of NIH-3T3 cells with 5 mM mßCD selectively blocks bAMF endocytosis to the ER but not the clathrin-dependent endocytosis of bAMF to multivesicular endosomes (44). As can be seen in Figure 3 A, mßCD also

blocks bAMF delivery to the ER in both untransformed and ras and abl transformed NIH-3T3 cells without significantly affecting bAMF endocytosis to endosomes. High concentrations (10 mM) of mßCD have been shown to block clathrin-dependent endocytosis (54,55) however the lack of an effect on the clathrin-dependent endocytosis of bAMF to endosomes serves as an internal control demonstrating that at the 5 mM concentration used, mßCD is selectively inhibiting the caveolae-like pathway of bAMF to the ER. The ability of mßCD to inhibit bAMF endocytosis to the ER in ras and abl transformed NIH-3T3 cells confirms the similar nature of this pathway in the three cell lines.

The caveolar distribution of AMF-R is based on the EM localization of AMF-R to smooth plasmalemmal invaginations and its partial colocalization with caveolin by immunofluorescence labeling (43). To ensure that the smooth invaginations to which AMF-R is localized are indeed caveolin positive and therefore correspond to accepted definitions of caveolae, NIH-3T3 cells were double labeled by postembedding EM for bAMF (nanogold and silver amplification) and caveolin (12 nm gold particles) (Figure 4A) or for AMF-R (12 nm gold particles) and caveolin (18 nm gold particles) (Figure 4B). Both bAMF and AMF-R positive invaginations are labeled for caveolin.

Adenoviral expression of dynK44A induces caveolae and inhibits AMF endocytosis

Infection of NIH-3T3 cells with an adenovirus expressing the dynK44A mutant enhanced our ability to identify double labeled caveolae. NIH-3T3 cells expressing this mutant show numerous caveolin-positive caveolae at the plasma membrane (Figure 4 C-H) including those exhibiting the typical long neck

associated with dynamin inhibition (Figure 4 G) (12). Caveolae double labeled for caveolin and either bAMF (Figure 4 C, E) or AMF-R (Figure 4 D, F, H) are readily detected. bAMF is therefore localized with its receptor to caveolae in NIH-3T3 cells.

Ras and abl transformed NIH-3T3 cells exhibit significantly fewer caveolae relative to NIH-3T3 cells (Figure 5 A, B), as reported previously (29), and introduction of the dynK44A mutant by adenoviral infection (51,52) into ras and abl transformed NIH-3T3 cells induced the expression of numerous smooth invaginations morphologically similar to caveolae (Figure 5 C-I). Treatment of dynK44A infected ras and abl transformed NIH-3T3 cells with mßCD prior to fixation resulted in the complete absence of smooth caveolar invaginations (Figure 5 K, L), as observed for uninfected cells (Figure 5 A, B). The caveolar invaginations induced by dynK44A are therefore sensitive to cholesterol depletion and represent a cholesterol-rich membrane domain or class of glycolipid rafts. Quantification of the expression of caveolae and clathrin coated pits in uninfected and dynK44A infected cells demonstrated the significantly increased expression per μm membrane of morphological caveolae but not of clathrin coated pits in all three cell types (Figure 6).

Adenoviral expression of dynK44A blocked both the clathrin-dependent endocytosis of bAMF to endosomes and the caveolae-like pathway to the smooth ER (Figure 7 A). Expression of the tTA adenovirus alone did not influence either of the AMF endocytic pathways indicating that inhibition of AMF endocytosis is specifically due to expression of the dynK44A mutant and not to adenoviral infection (Figure 7 B). Dynamin-mediated budding of caveolar

vesicles from the plasma membrane therefore regulates AMF-R endocytosis to the ER.

In order to ensure that dynK44A expression is not inducing caveolin expression and thereby affecting the expression of caveolae, we quantified plasma membrane associated caveolin labeling by postembedding immunoelectron microscopy of whole cell profiles. As presented per μm membrane in Figure 6, the number of caveolar invaginations per cell is dramatically reduced in ras and abl transformed cells and dynK44A expression induces the stable expression of a large number of smooth caveolar invaginations (Figure 8 A). Caveolin labeling associated with the plasma membrane including caveolae (Figure 8 B) or specifically with caveolae (Figure 8 C) is significantly reduced in ras and abl transformed NIH-3T3 cells relative to untransformed NIH-3T3 cells. Expression of dynK44A does not affect total plasma membrane associated caveolin expression (Figure 8 B) indicating that increased expression of caveolin or its increased recruitment to the plasma membrane is not responsible for the dynK44A mediated induction of smooth caveolar invaginations. A slight increase in caveolin labeling of caveolar invaginations is observed in all three cell lines (Figure 8 C) but is minimal relative to the increased number of caveolae expressed (Figure 8 A). Immunoblot analysis reveals that caveolin expression in the ras and abl transformants remains significantly below that in NIH-3T3 cells even after adenoviral expression of HA-tagged dynK44A (Figure 9). Expression of dynK44A has not therefore induced the formation of caveolar invaginations by increasing caveolin expression levels.

Adenoviral expression of caveolin-1 negatively regulates AMF endocytosis to the ER

Infection of the three cell types with tTA and caveolin-1 adenoviruses induces increased levels of caveolin-1 expression significantly above those in uninfected NIH-3T3 cells (Figure 9). As previously reported (27), the reintroduction of caveolin-1 into ras and abl transformed NIH-3T3 cells induces numerous caveolae at the plasma membrane (Figure 10) that are morphologically indistinguishable from the caveolae induced by dynK44A infection (Figure 5). Quantitatively, a dramatic increase in caveolae expression and in anti-caveolin labeling at both the plasma membrane and in caveolae was observed in ras and abl infected cells such that caveolae and caveolin levels were equivalent to or greater than those of uninfected NIH-3T3 cells (Figure 11 A, B, C). Infection of NIH-3T3 cells with the caveolin-1 adenovirus induced lesser (1.5-2 fold) increases in the number of caveolae and in caveolin expression at the cell surface (Figure 11 A, B, C). Caveolin-1 overexpression in the transformed cells reduced bAMF endocytosis to the ER to levels comparable to uninfected NIH-3T3 cells but did not affect bAMF internalization to endosomes; the increased expression of caveolin-1 in NIH-3T3 cells also selectively decreased bAMF endocytosis to the ER (Figure 11 D, E). Of particular interest, the reintroduction of caveolin-1 into ras and abl transformed NIH-3T3 cells was associated with the accumulation of bAMF within cell surface caveolae (Figure 11 F). Overexpression of caveolin-1 therefore reduces caveolae-mediated internalization of bAMF identifying caveolin-1 as a negative regulator of caveolae-mediated endocytosis.

DISCUSSION

Caveolae mediated endocytosis to the ER

The AMF/AMF-R endocytic pathway to the ER represents the first identified receptor-mediated endocytic pathway that delivers its ligand to an ER subdomain via caveolae. Inhibition of AMF endocytosis to the ER by both mßCD and dynK44A distinguishes this pathway from the clathrin-independent pathway (9,10,54) and defines at least two distinct non-clathrin endocytic pathways. SV40 is also internalized via cell surface caveolae to the smooth ER (41,46,56-58). Internalization of SV40 to the ER is significantly slower (4-6 hours) (41) than that observed for AMF-R and identity between the two pathways remains to be established. If identical, the fact that a cellular receptor, AMF-R, is delivered via caveolae to the ER suggests that SV40 has not induced a novel endocytic pathway but has rather co-opted a preexisting one.

Caveolin is not necessary for caveolar invagination and budding of caveolar vesicles

The caveolae-mediated endocytic pathway of AMF-R to the ER is still present in ras and abl transformed NIH-3T3 cells that exhibit reduced levels of caveolin and caveolae. Overexpression in these cells of the dynK44A dominant negative mutant using an adenoviral expression system induces the expression of morphologically identifiable caveolae and inhibits AMF-R endocytosis to the ER confirming previous reports that dynamin regulates the budding and endocytic function of caveolae (12,13). The smooth caveolar invaginations formed in dynK44A infected ras and abl transformed NIH-3T3 cells are not enriched for caveolin yet are still sensitive to cholesterol depletion with mßCD.

In cells that express limited amounts of caveolin, cholesterol-rich detergent-resistant membrane domains or glycolipid rafts invaginate to form caveolae that rapidly give rise to endocytosis-competent vesicles such that caveolae are visible only when budding is inhibited. Caveolin is not essential for caveolae invagination or endocytosis and, indeed, the endocytic potential of cholesterol-rich detergent-resistant membrane domains or glycolipid rafts in the absence of caveolin is quite significant. Glycolipid rafts are therefore dynamic endocytic structures (59) that upon invagination and budding from the plasma membrane are equivalent, if only transiently, to the morphological definition of caveolae. The absence of caveolae at the plasma membrane does not preclude the presence of a caveolar endocytic pathway.

The induction of caveolae by caveolin expression in caveolin-minus cells, as described here and in other reports (26-28), is due to the stabilization of caveolae by caveolin at the plasma membrane, permitting their visualization by electron microscopy of fixed samples, as previously suggested (43). Caveolin association with rafts may modify their functional properties by regulating the protein and lipid composition of individual plasma membrane microdomains (19,21). Distinct dominant-negative caveolin mutants differentially affect SV40 endocytosis and ras signaling and suggests that caveolin may act to regulate caveolae function and endocytosis by controlling the cholesterol content of glycolipid rafts and perhaps caveolar vesicles (58).

Our study therefore demonstrates that morphological flask-shaped caveolae form independently of caveolin-1 expression. Indeed, the term caveolae was invoked long before the identification of caveolin (15).

Nevertheless, since few caveolae are visualized at the plasma membrane in the absence of caveolin, stably expressed caveolae are necessarily caveolin-associated and caveolin is therefore a reliable marker for caveolae expression. The caveolin-1 knockout mouse is viable and the phenotype relatively minor suggesting that if caveolae function is essential for development and survival of the organism, it is not dependent on caveolin-1 expression (60,61). Furthermore, while caveolae expression was dramatically reduced in the caveolin-1 knockout mice, a few caveolar invaginations were still identified (61).

Caveolin is a negative regulator of caveolae internalization

Similar to the AMF endocytosis to the ER reported here in ras and abl transformed NIH-3T3 cells, prior studies have also reported the internalization of cholera toxin, GPI anchored proteins or the interleukin 2 receptor via non-clathrin cholesterol-dependent pathways in cells that do not express caveolin (42,45,62). The significant overexpression of caveolin-1 obtained using adenoviral infection significantly reduced but did not completely inhibit AMF internalization (Figures 11D) indicating that caveolin-1 stabilization of caveolae at the plasma membrane slows but does not prevent caveolae-mediated endocytosis.

Caveolae-mediated endocytosis in endothelial cells that express significant amounts of caveolin-1 is well documented (63). Although caveolin-1 knockout mice did not exhibit altered serum albumin levels (61), lung endothelial cells of caveolin-1 knockout mice exhibit reduced albumin uptake (64) and reintroduction of caveolin-1 into caveolin-1 knockout fibroblasts induced albumin internalization (60). Internalization of albumin by gp60 or albondin requires gp60

activation and interaction with caveolin-1 and albumin endocytosis was disrupted by caveolin-1 overexpression which resulted in the sequestration of Gαi preventing gp60 activation (34). Caveolin-1 expression and association with caveolar domains may regulate not only their rate of internalization but also select the cargo that follows this endocytic route.

It is possible that all glycolipid raft domains, currently defined biochemically, can invaginate to form caveolar vesicles. However, it is more likely that different classes of rafts exist with differential abilities to invaginate and bud from the plasma membrane and to form functionally distinct caveolar vesicles. For instance, caveolae and raft domains mediate both endocytosis to the ER (41,43,44,46) and to endosomes and the Golgi (35-37,59,65,66) and in endothelial cells, distinct caveolar vesicle populations have been shown to mediate transcytosis of albumin and insulin (67).

Caveolin-1 is shown here to be a negative regulator of caveolar endocytosis that acts to slow detachment of caveolar vesicles from the plasma membrane. Reduced caveolin-1 expression is associated with different forms of cancer in vivo and decreased caveolin-1 expression in vitro is associated with cell transformation and tumorigenicity identifying caveolin-1 as a tumor suppressor gene (27,29,30,53,68-71). The increased rate of internalization of AMF-R to the ER in transformed NIH-3T3 cells corresponds to decreased surface expression of AMF-R suggesting that in cells lacking caveolin-1, AMF-R is rapidly transiting the plasma membrane. Similarly, FACS analysis of B16 melanoma and K1735 fibrosarcoma metastatic variants reported decreased cell surface AMF-R expression in the high metastatic variants (72). It is therefore

conceivable that decreased expression of caveolin-1 results in the destabilization of AMF-R cell surface expression and the deregulation of AMF-R traffic. The well-characterized association of AMF-R expression with tumor malignancy (73-79) implicates this caveolae-mediated endocytic pathway in AMF-R function in tumor cell motility and metastasis.

ACKNOWLEDGEMENTS

We thank Michael Lisanti and Philippe Frank for kindly providing the ras and abl transformed NIH-3T3 cells and caveolin-1 adenovirus as well as for their helpful suggestions. We thankfully acknowledge the precious help of Anne Guénette for the EM quantification and Jean Léveillé for the preparation of the figures. This study was supported by a grant from the Canadian Institutes for Health Research (CIHR). I. R. Nabi is the recipient of a CIHR Investigator award and P. U. Le is the recipient of a Graduate Student Award from the Cancer Research Society Inc.

REFERENCES

1. Lamaze, C., and Schmid, S. L. (1995) *Curr. Opin. Cell Biol.* **7**, 573-580
2. Mellman, I. (1996) *Annu. Rev. Cell Dev. Biol.* **12**, 575-626
3. Sandvig, K., and van Deurs, B. (1999) *FEBS Lett.* **452**, 67-70
4. Nichols, B. J., and Lippincott-Schwartz, J. (2001) *Trends Cell Biol.* **11**, 406-412.
5. Herskovits, J. S., Burgess, C. C., Obar, R. A., and Vallee, R. B. (1993) *J. Cell Biol.* **122**, 565-578
6. van der Bliek, A. M., Redelmeier, T. E., Damke, H., Tisdale, E. J., Meyerowitz, E. M., and Schmid, S. L. (1993) *J. Cell Biol.* **122**, 553-563
7. Damke, H., Baba, T., Warnock, D. E., and Schmid, S. L. (1994) *J. Cell Biol.* **127**, 915-934
8. Damke, H., Baba, T., van der Bliek, A. M., and Schmid, S. L. (1995) *J. Cell Biol.* **131**, 69-80
9. Simpson, J. C., Smith, D. C., Roberts, L. M., and Lord, J. M. (1998) *Exp. Cell Res.* **239**, 293-300
10. Llorente, A., Rapak, A., Schmid, S. L., van Deurs, B., and Sandvig, K. (1998) *J. Cell Biol.* **140**, 553-563
11. Contamin, S., Galmiche, A., Doye, A., Flatau, G., Benmerah, A., and Boquet, P. (2000) *Mol. Biol. Cell* **11**, 1775-1787
12. Henley, J. R., Krueger, E. W., Oswald, B. J., and McNiven, M. A. (1998) *J. Cell Biol.* **141**, 85-99

13. Oh, P., McIntosh, D. P., and Schnitzer, J. E. (1998) *J. Cell Biol.* **141**, 101-114

14. Schnitzer, J. E., Liu, J., and Oh, P. (1995) *J. Biol. Chem.* **270**, 14399-14404

15. Yamada, E. (1955) *J. Biophys. Biochem. Cytol.* **1**, 445-448

16. Palade, G. E. (1953) *J. Appl. Phys.* **24**, 1424

17. Palade, G. E. (1958) *Anat. Rec.* **130**, 467-468

18. Bruns, R. R., and Palade, G. E. (1968) *J. Cell Biol.* **37**, 244-276.

19. Harder, T., and Simons, K. (1997) *Curr. Opin. Cell Biol.* **9**, 534-542

20. Anderson, R. G. (1998) *Annu. Rev. Biochem.* **67**, 199-225

21. Smart, E. J., Graf, G. A., McNiven, M. A., Sessa, W. C., Engelman, J. A., Scherer, P. E., Okamoto, T., and Lisanti, M. P. (1999) *Mol. Cell Biol.* **19**, 7289-7304.

22. Galbiati, F., Razani, B., and Lisanti, M. P. (2001) *Cell* **106**, 403-411.

23. Rothberg, K. G., Heuser, J. E., Donzell, W. C., Ying, Y.-S., Glenney, J. R., and Anderson, R. G. W. (1992) *Cell* **68**, 673-682

24. Murata, M., Peranen, J., Schreiner, R., Wieland, F., Kurzchalia, T. V., and Simons, K. (1995) *Proc. Natl. Acad. Sci. U.S.A.* **92**, 10339-10343

25. Li, S., Song, K. S., and Lisanti, M. P. (1996) *J. Biol. Chem.* **271**, 568-573

26. Fra, A. M., Williamson, E., Simons, K., and Parton, R. G. (1995) *Proc. Natl. Acad. Sci. U.S.A.* **92**, 8655-8659

27. Engelman, J. A., Wykoff, C. C., Yasuhara, S., Song, K. S., Okamoto, T., and Lisanti, M. P. (1997) *J. Biol. Chem.* **272**, 16374-16381

28. Smart, E. J., Ying, Y., Donzell, W. C., and Anderson, R. G. (1996) *J. Biol. Chem.* **271**, 29427-29435

29. Koleske, A. J., Baltimore, D., and Lisanti, M. P. (1995) *Proc. Natl. Acad. Sci. U.S.A.* **92**, 1381-1385

30. Galbiati, F., Volonte, D., Engelman, J. A., Watanabe, G., Burk, R., Pestell, R. G., and Lisanti, M. P. (1998) *EMBO J.* **17**, 6633-6648

31. Razani, B., Schlegel, A., Liu, J., and Lisanti, M. P. (2001) *Biochem Soc Trans* **29**, 494-499.

32. Ghitescu, L., Fixman, A., Simionescu, M., and Simionescu, N. (1986) *J. Cell Biol.* **102**, 1304-1311

33. Schnitzer, J. E., Oh, P., Pinney, E., and Allard, J. (1994) *J. Cell Biol.* **127**, 1217-1232

34. Minshall, R. D., Tiruppathi, C., Vogel, S. M., Niles, W. D., Gilchrist, A., Hamm, H. E., and Malik, A. B. (2000) *J. Cell Biol.* **150**, 1057-1070

35. Montesano, R., Roth, J., Robert, A., and Orci, L. (1982) *Nature (Lond.)* **296**, 651-653

36. Tran, D., Carpentier, J. L., Sawano, F., Gorden, P., and Orci, L. (1987) *Proc. Natl. Acad. Sci. U.S.A.* **84**, 7957-7961.

37. Parton, R. G., Joggerst, B., and Simons, K. (1994) *J. Cell Biol.* **127**, 1199-1215

38. Lencer, W. I., Hirst, T. R., and Holmes, R. K. (1999) *Biochim Biophys Acta* **1450**, 177-190.

39. Okamoto, Y., Ninomiya, H., Miwa, S., and Masaki, T. (2000) *J. Biol. Chem.* **275**, 6439-6446

40. Shin, J. S., Gao, Z., and Abraham, S. N. (2000) *Science* **289**, 785-788

41. Pelkmans, L., Kartenbeck, J., and Helenius, A. (2001) *Nat. Cell Biol.* **3**, 473-483.

42. Lamaze, C., Dujeancourt, A., Baba, T., Lo, C. G., Benmerah, A., and Dautry-Varsat, A. (2001) *Mol. Cell* **7**, 661-671.

43. Benlimame, N., Le, P. U., and Nabi, I. R. (1998) *Mol. Biol. Cell* **9**, 1773-1786

44. Le, P. U., Benlimame, N., Lagana, A., Raz, A., and Nabi, I. R. (2000) *J. Cell Sci.* **113**, 3227-3240

45. Orlandi, P. A., and Fishman, P. H. (1998) *J. Cell Biol.* **141**, 905-915

46. Kartenbeck, J., Stukenbrok, H., and Helenius, A. (1989) *J. Cell Biol.* **109**, 2721-2729

47. Benlimame, N., Simard, D., and Nabi, I. R. (1995) *J. Cell Biol.* **129**, 459-471

48. Wang, H.-J., Benlimame, N., and Nabi, I. R. (1997) *J. Cell Sci.* **110**, 3043-3053

49. Wang, H.-J., Guay, G., Pogan, L., Sauve, R., and Nabi, I. R. (2000) *J. Cell Biol.* **150**, 1489-1498

50. Nabi, I. R., Watanabe, H., and Raz, A. (1990) *Cancer Res.* **50**, 409-414

51. Altschuler, Y., Barbas, S. M., Terlecky, L. J., Tang, K., Hardy, S., Mostov, K. E., and Schmid, S. L. (1998) *J. Cell Biol.* **143**, 1871-1881

52. Altschuler, Y., Liu, S., Katz, L., Tang, K., Hardy, S., Brodsky, F., Apodaca, G., and Mostov, K. (1999) *J. Cell Biol.* **147**, 7-12.

53. Zhang, W., Razani, B., Altschuler, Y., Bouzahzah, B., Mostov, K. E., Pestell, R. G., and Lisanti, M. P. (2000) *J. Biol. Chem.* **275**, 20717-20725

54. Rodal, S. K., Skretting, G., Garred, Ø., Vilhardt, F., van Deurs, B., and Sandvig, K. (1999) *Mol. Biol. Cell* **10**, 961-974

55. Subtil, A., Gaidarov, I., Kobylarz, K., Lampson, M. A., Keen, J. H., and McGraw, T. E. (1999) *Proc. Natl. Acad. Sci. U.S.A.* **96**, 6775-6780

56. Anderson, H. A., Chen, Y., and Norkin, L. C. (1996) *Mol. Biol. Cell* **7**, 1825-1834

57. Stang, E., Kartenbeck, J., and Parton, R. G. (1997) *Mol. Biol. Cell* **8**, 47-57

58. Roy, S., Luetterforst, R., Harding, A., Apolloni, A., Etheridge, M., Stang, E., Rolls, B., Hancock, J. F., and Parton, R. G. (1999) *Nat. Cell Biol.* **1**, 98-105.

59. Nichols, B. J., Kenworthy, A. K., Polishchuk, R. S., Lodge, R., Roberts, T. H., Hirschberg, K., Phair, R. D., and Lippincott-Schwartz, J. (2001) *J. Cell Biol.* **153**, 529-541.

60. Razani, B., Engelman, J. A., Wang, X. B., Schubert, W., Zhang, X. L., Marks, C. B., Macaluso, F., Russell, R. G., Li, M., Pestell, R. G., Di Vizio, D., Hou, H., Jr., Kneitz, B., Lagaud, G., Christ, G. J., Edelmann, W., and Lisanti, M. P. (2001) *J. Biol. Chem.* **276**, 38121-38138.

61. Drab, M., Verkade, P., Elger, M., Kasper, M., Lohn, M., Lauterbach, B., Menne, J., Lindschau, C., Mende, F., Luft, F. C., Schedl, A., Haller, H., and Kurzchalia, T. V. (2001) *Science* **293**, 2449-2452.

62. Verkade, P., Harder, T., Lafont, F., and Simons, K. (1999) *J. Cell Biol.* **148**, 727-739

63. Mineo, C., and Anderson, R. G. (2001) *Histochem Cell Biol* **116**, 109-118.

64. Schubert, W., Frank, P. G., Chow, C. W., and Lisanti, M. P. (2001) *J. Biol. Chem.* published online October 31.

65. Puri, V., Watanabe, R., Singh, R. D., Dominguez, M., Brown, J. C., Wheatley, C. L., Marks, D. L., and Pagano, R. E. (2001) *J. Cell Biol.* **154**, 535-547.

66. Gines, S., Ciruela, F., Burgueno, J., Casado, V., Canela, E. I., Mallol, J., Lluis, C., and Franco, R. (2001) *Mol. Pharmacol.* **59**, 1314-1323.

67. Bendayan, M., and Rasio, E. A. (1996) *J. Cell Sci.* **109**, 1857-1864

68. Lee, S. W., Reimer, C. L., Oh, P., Campbell, D. B., and Schnitzer, J. E. (1998) *Oncogene* **16**, 1391-1397

69. Racine, C., Belanger, M., Hirabayashi, H., Boucher, M., Chakir, J., and Couet, J. (1999) *Biochem. Biophys. Res. Commun.* **255**, 580-586

70. Bender, F. C., Reymond, M. A., Bron, C., and Quest, A. F. (2000) *Cancer Res.* **60**, 5870-5878

71. Razani, B., Altschuler, Y., Zhu, L., Pestell, R. G., Mostov, K. E., and Lisanti, M. P. (2000) *Biochem.* **39**, 13916-13924

72. Watanabe, H., Nabi, I. R., and Raz, A. (1991) *Cancer Res.* **51**, 2699-2705

73. Nakamori, S., Watanabe, H., Kameyama, M., Imaoka, S., Furukawa, H., Ishikawa, O., Sasaki, Y., Kabuto, T., and Raz, A. (1994) *Cancer* **74**, 1855-1862

74. Otto, T., Birchmeier, W., Schmidt, U., Hinke, A., Schipper, J., Rübben, H., and Raz, A. (1994) *Cancer Res.* **54**, 3120-3123

75. Maruyama, K., Watanabe, H., Hitoshi, S., Takayama, T., Gofuku, J., Yano, H., Inoue, M., Tamura, S., Raz, A., and Monden, M. (1995) *Int. J. Cancer* **64**, 316-321

76. Hirono, Y., Fushida, S., Yonemura, Y., Yamamoto, H., Watanabe, H., and Raz, A. (1996) *Br. J. Cancer* **74**, 2003-2007

77. Nagai, Y., Ishikawa, O., Miyachi, Y., and Watanabe, H. (1996) *Dermatology* **192**, 8-11

78. Takanami, I., Takeuchi, K., Naruke, M., Kodaira, S., Tanaka, F., Watanabe, H., and Raz, A. (1998) *Tumour Biol* **19**, 384-389

79. Taniguchi, K., Yonemura, Y., Nojima, N., Hirono, Y., Fushida, S., Fujimura, T., Miwa, K., Endo, Y., Yamamoto, H., and Watanabe, H. (1998) *Cancer* **82**, 2112-2122

Figure 1. AMF is internalized to the ER in ras and abl transformed NIH-3T3 cells. NIH-3T3 (A), NIH-ras (B) and NIH-abl (C) cells were pulse labeled with biotinylated AMF (bAMF) for 60 minutes at 37°C. Postembedding labeling with streptavidin-nanogold and silver amplification revealed bAMF localization to ER tubules as well as to MVBs. Arrowheads indicate ER tubules and arrows MVBs. Bar = 0.2 μm.

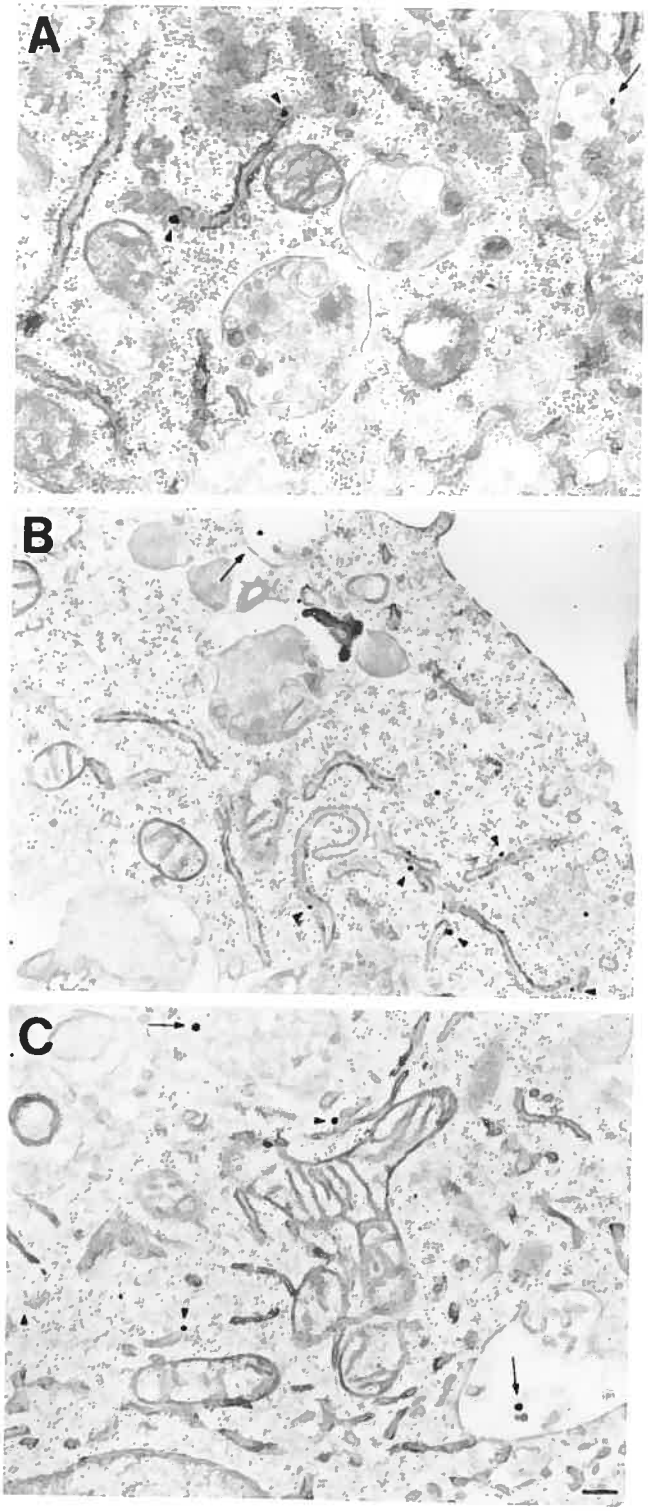
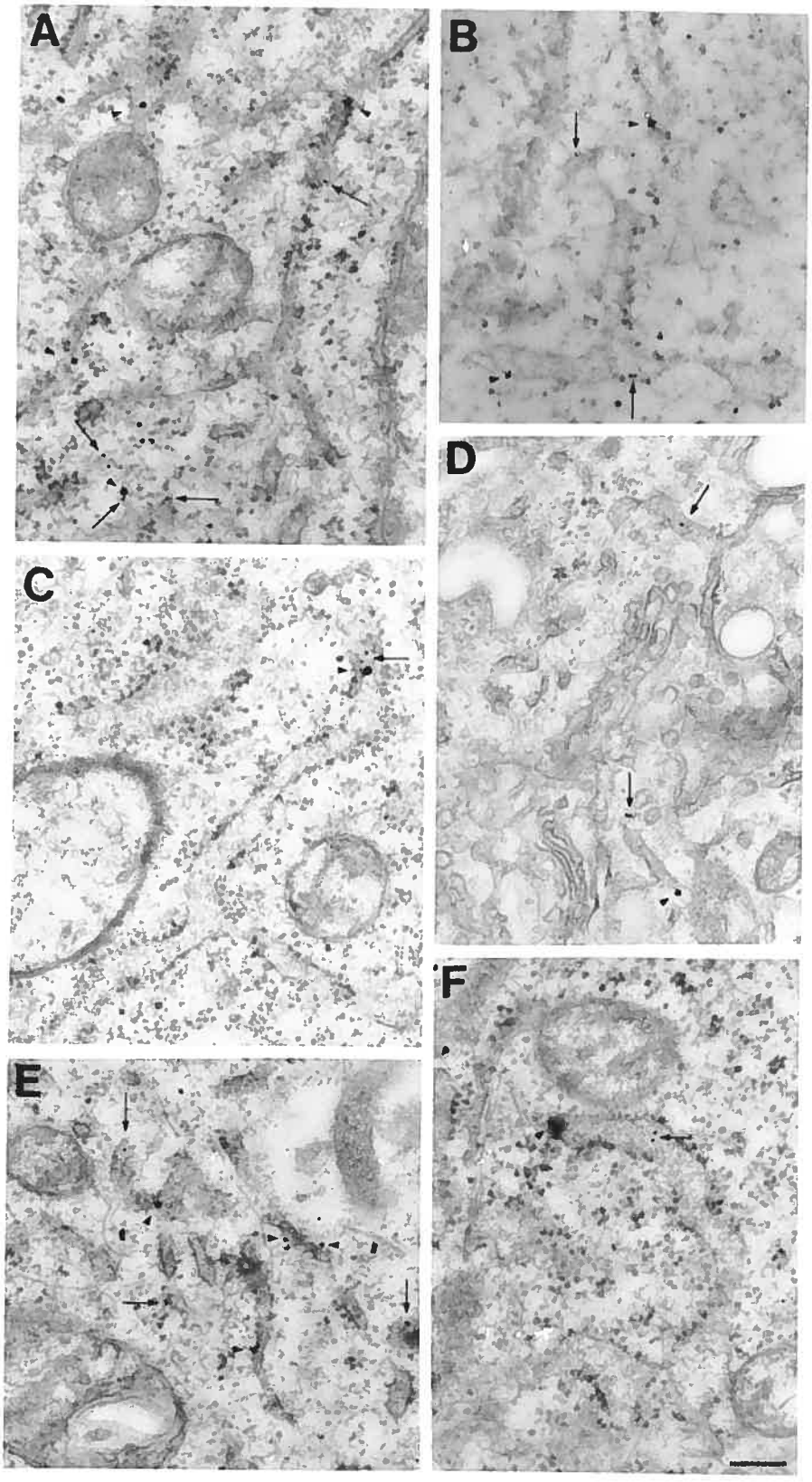

A
B
C

Figure 2. Endocytosed bAMF colocalizes with AMF-R in ER tubules. NIH-3T3 cells (A, B) as well as ras (C, D) and abl (E, F) transformed NIH-3T3 cells were pulse labeled with bAMF for 60 minutes at 37°C. bAMF was first revealed by streptavidin nanogold and silver amplification following by the labeling of AMF-R tubules with anti-AMF-R antibodies and 12 nm gold conjugated anti-rat IgM secondary antibodies. Arrowheads indicate bAMF labeling and arrows AMF-R labeling. Bar = 0.2 μm.


A
B
C
D
E
F

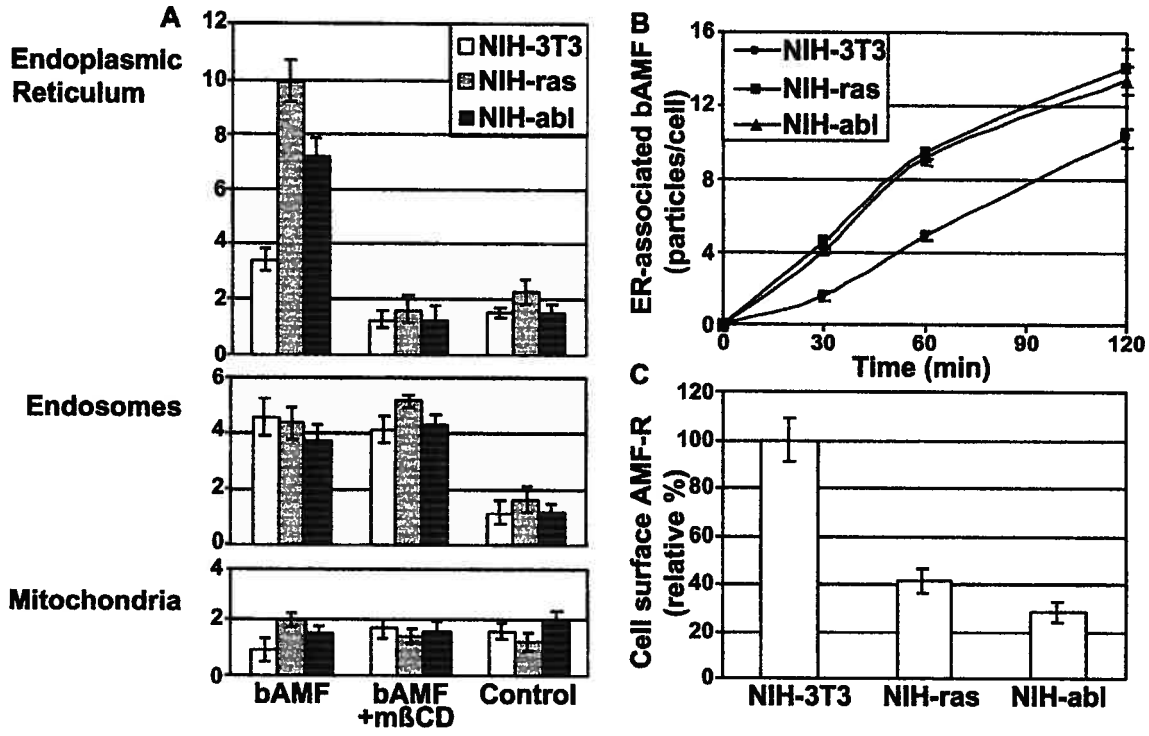


Fig. 3. Increased rate of AMF-R endocytosis to the ER in *ras*- and *abl*-transformed cells. *A*, NIH-3T3 (*white bars*) or *ras* (*gray bars*)- and *abl* (*black bars*)-transformed NIH-3T3 cells were pulsed with bAMF for 60 min at 37 °C in the absence or presence of mβCD. bAMF was revealed by postembedding labeling with nanogold-streptavidin and silver amplification. The numerical density of silver particles associated with the ER, endosomes, and mitochondria was determined from 36 images (_12,000) from two different experiments for each condition. Control values in the absence of a bAMF pulse are presented. The results are presented as a ratio of the number of silver particles to the surface area of each organelle (±S.E.). The data shows that bAMF targeting to the ER is selectively inhibited by mβCD. *B*, quantitative analysis of AMF delivery to the ER after 30-, 60-, and 120-min incubation with bAMF at 37 °C in NIH-3T3 (*circles*), NIH-*ras* (*squares*), and NIH-*abl* (*triangles*) cells. ER-associated silver particles were counted from 25 intact cell profiles, and the data represent the average per cell profile. *C*, FACS analysis of AMF-R cell surface expression in untransformed and *ras*- and *abl*-transformed NIH-3T3 cells shows that AMF-R cell surface expression is decreased following cell transformation. The data are presented as relative fluorescence intensity in percentage compared with NIH-3T3 cells.

Figure 4. bAMF and AMF-R are localized to caveolin-positive caveolae. NIH-3T3 (A, B) or NIH-3T3 cells infected with dynK44A (C-H) were pulse labeled with bAMF for 60 minutes at 37°C. Postembedding labeling with nanogold-streptavidin and silver amplification first revealed the localization of bAMF to caveolae and then caveolin distribution was determined using polyclonal anti-caveolin antibodies and 12 nm gold conjugated anti-rabbit secondary antibodies (A, C, E, and G). Double labeling for AMF-R and caveolin was performed by first adding anti-AMF-R and anti-caveolin primary antibodies following by 12 nm gold conjugated anti-rat IgM and 18 nm gold conjugated anti-rabbit secondary antibodies respectively (B, D, F and H). In A, C, E, and G, the arrows indicate caveolin and the arrowheads bAMF. In B, D, F, and H, the arrows indicate AMF-R and the arrowheads caveolin. Bars = 0.2 μm.

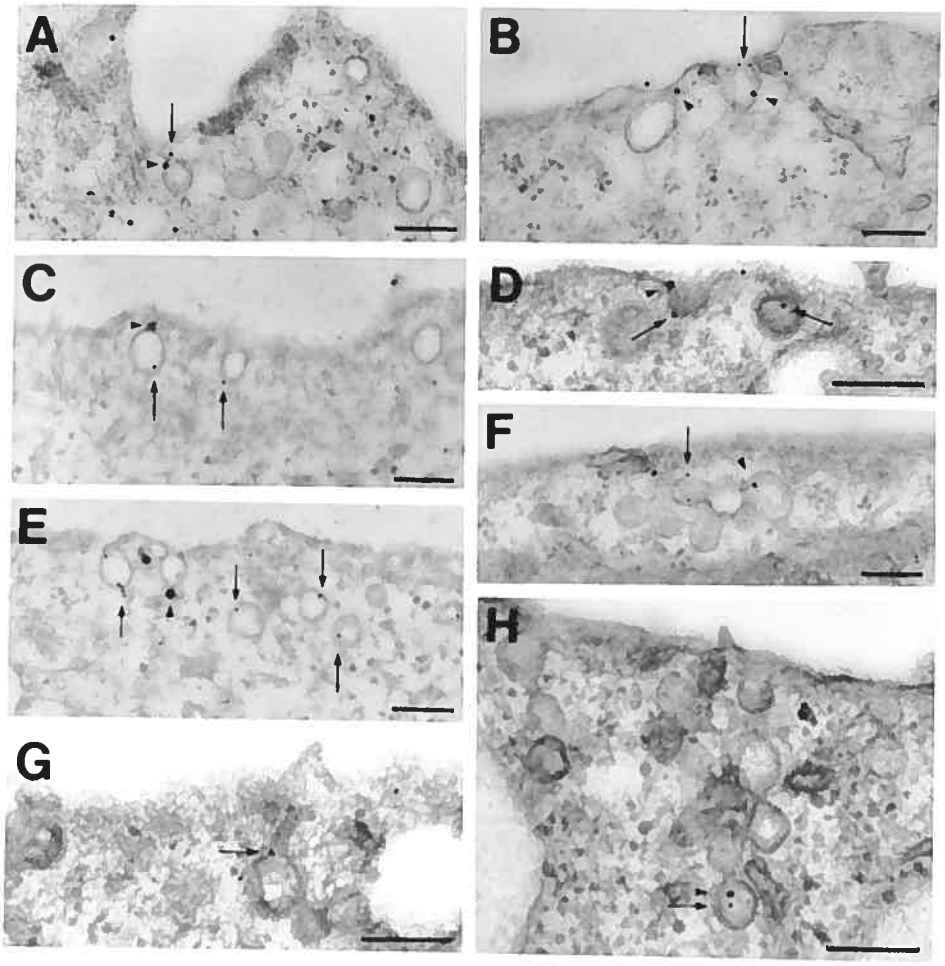
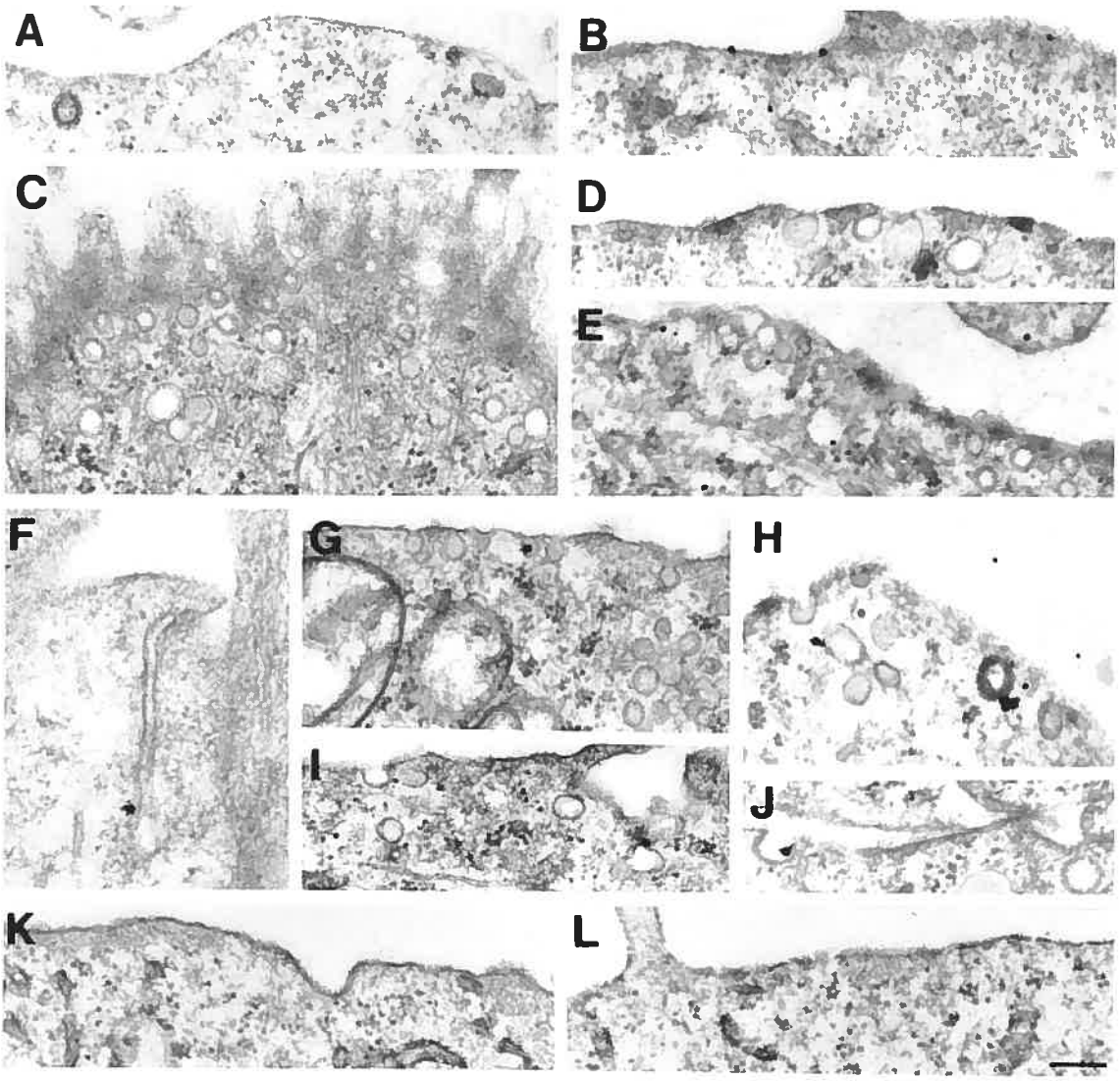

A
B
C
D
F
E
H
G

Figure 5. Expression of dynK44A induces caveolae in ras and abl transformed NIH-3T3 cells. Ras (A, C, F, G, I, K) and abl (B, D, E, H, J, L) transformed NIH-3T3 cells, either uninfected (A, B) or expressing dynK44A via adenoviral infection (C-L) were pulse labeled with bAMF for 60 minutes at 37°C. Plasma membrane profiles show the dramatically increased expression of smooth caveolar invaginations in the dynK44A infected cells (C-I) relative to uninfected cells (A, B). DynK44A infected ras and abl transformed NIH-3T3 cells pretreated with 5 mM mßCD for 90 minutes prior to fixation exhibited no caveolar invaginations (K, L). Postembedding labeling with streptavidin-nanogold and silver amplification revealed bAMF localization to caveolar invaginations (E, F, G, H) and to clathrin coated vesicles (H, J). Bar = 0.2 μm.


A
B
C
D
E
F
G
H
I
J
K
L

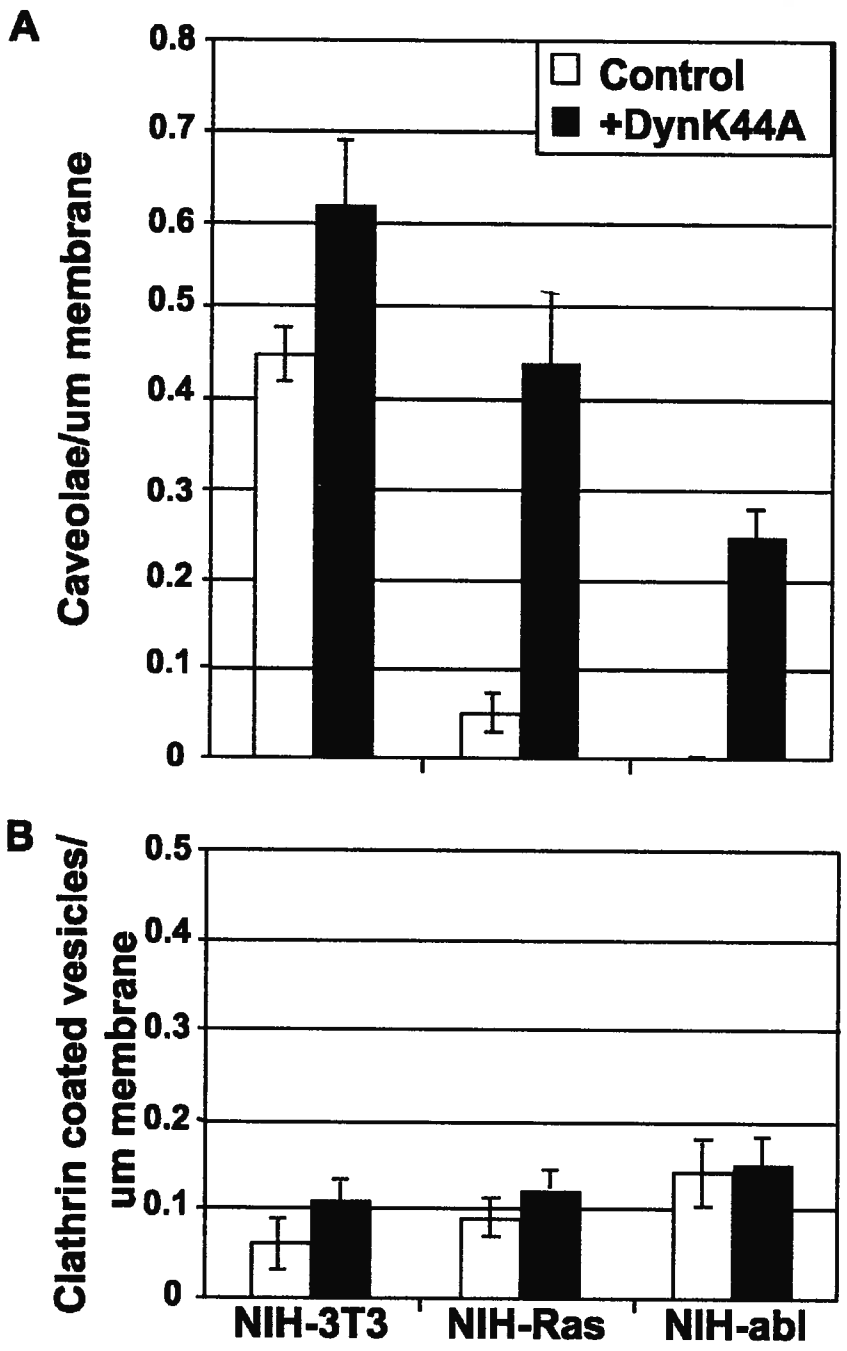


Fig. 6. Quantitative analysis of caveolae and clathrin-coated vesicles at the plasma membrane following dynK44A infection. The number of morphologically identifiable caveolae (*A*) and clathrin-coated vesicles (*B*) per micron of plasma membrane length was determined for untransformed and *ras*- and *abl*-transformed NIH-3T3 cells either uninfected (*white bars*) or expressing dynK44A via adenoviral infection (*black bars*). The increase in the number of caveolae expressed in dynK44A-infected NIH-3T3 cells was significant ($p < 10^{-3}$).

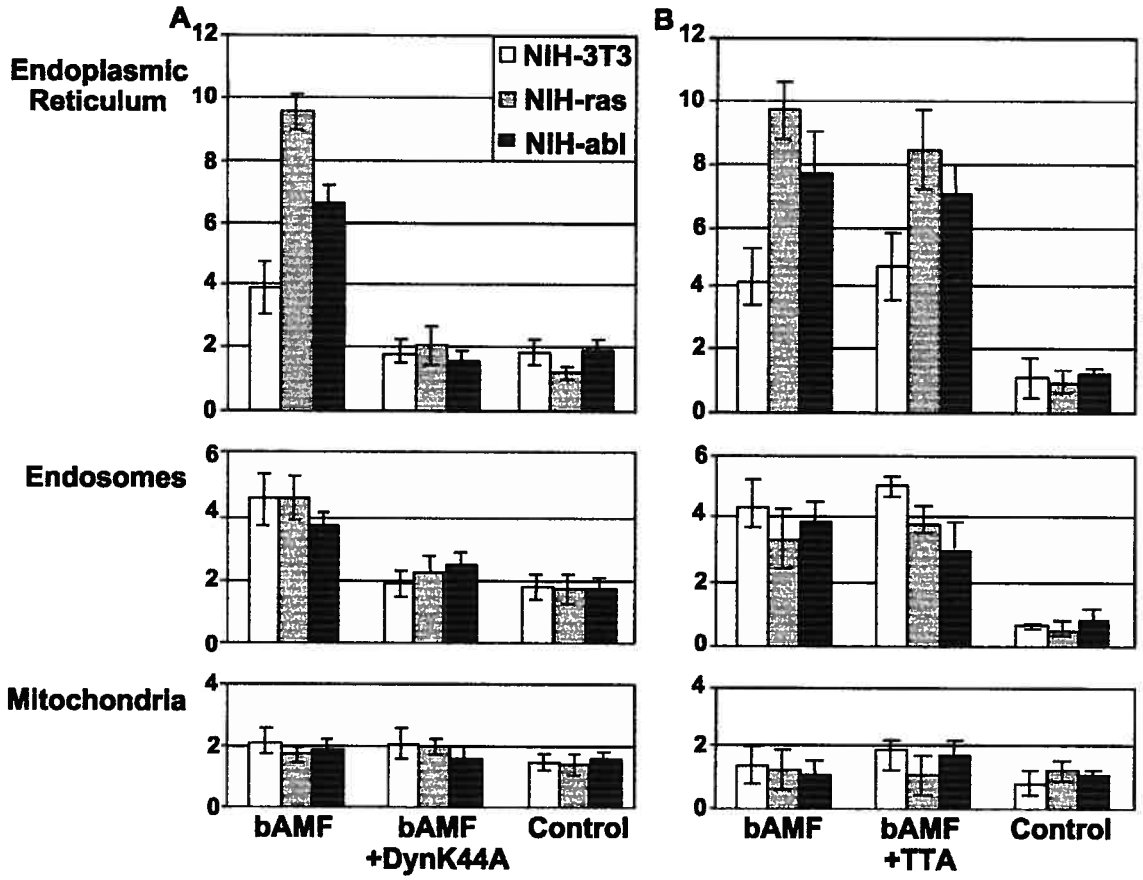


Fig. 7. DynK44A blocks endocytosis of biotinylated AMF to both the ER and endosomes. Untransformed NIH-3T3 (*white bars*) and *ras* (*gray bars*)- or *abl* (*black bars*)-transformed NIH-3T3 cells were infected with the tTA and dynK44A adenoviruses and then pulsed with bAMF for 60 min at 37 °C (*A*). bAMF was revealed by postembedding labeling with nanogold-streptavidin and silver amplification. The numerical density of silver particles and the surface area of the ER, endosomes, and mitochondria were determined from 36 images (_12,000) from two different experiments for each condition. Control labeling was measured in the absence of added bAMF. Infection with the tTA adenovirus alone did not affect the internalization of bAMF (*B*). The results are presented as the ratio of the number of silver particles to the surface area of each organelle (±S.E.).

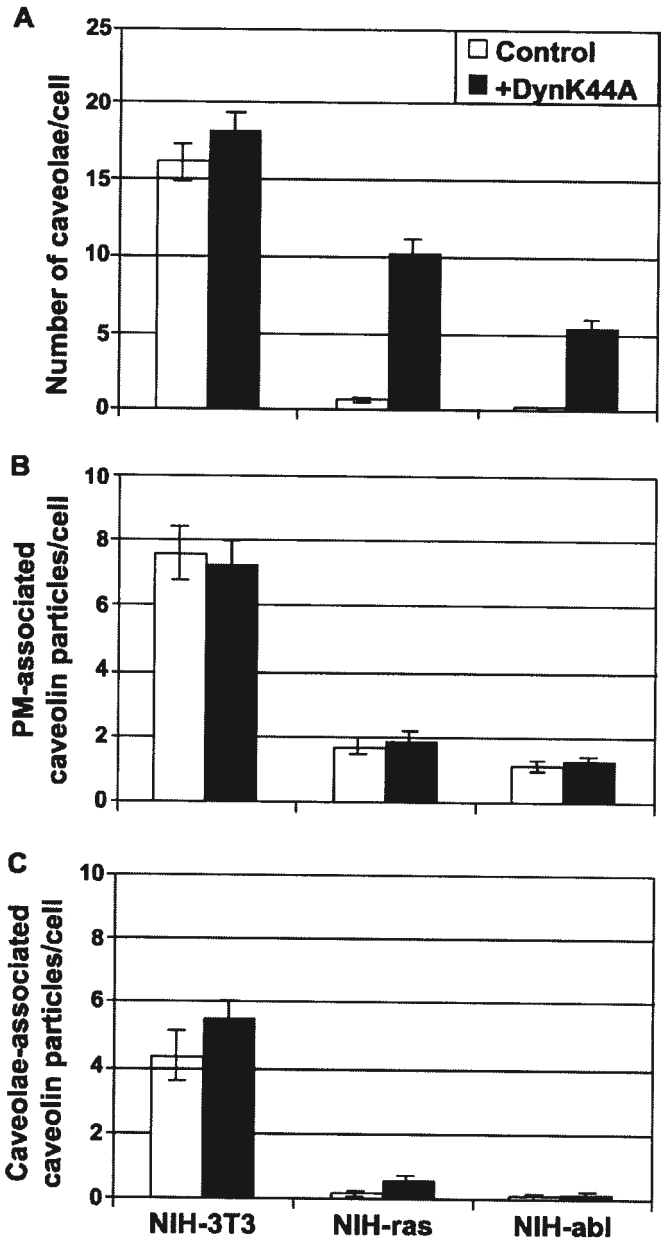


Fig. 8. Expression of caveolin at the plasma membrane is not increased following dynK44A infection. Quantification of the number of morphologically identifiable caveolae (*A*), caveolin labeling at the plasma membrane (including smooth caveolar invaginations) (*B*), and caveolin labeling specific to smooth caveolar invaginations (*C*) was determined for 50 complete cell profiles from anti-caveolin-labeled EM grids for untransformed and *ras*- and *abl*-transformed NIH-3T3 cells either uninfected (*white bars*) or expressing dynK44A via adenoviral infection (*black bars*). The data were obtained from two distinct experiments and represent the average per cell profile.

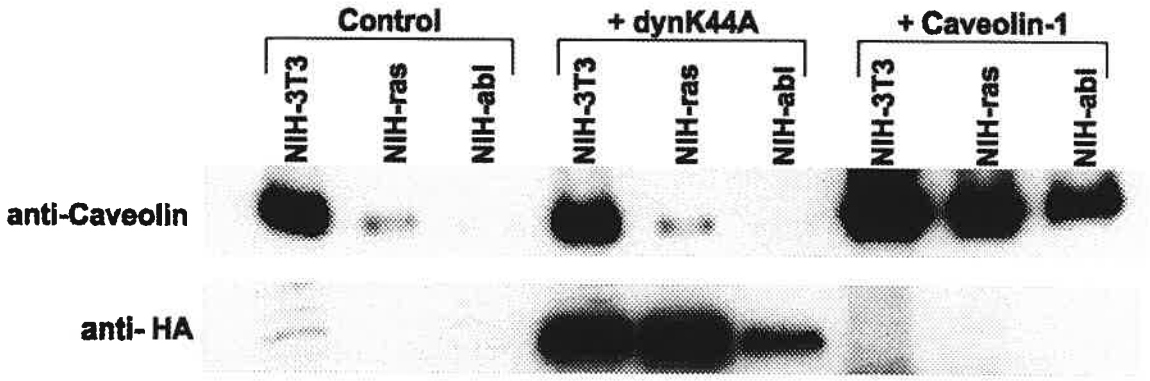
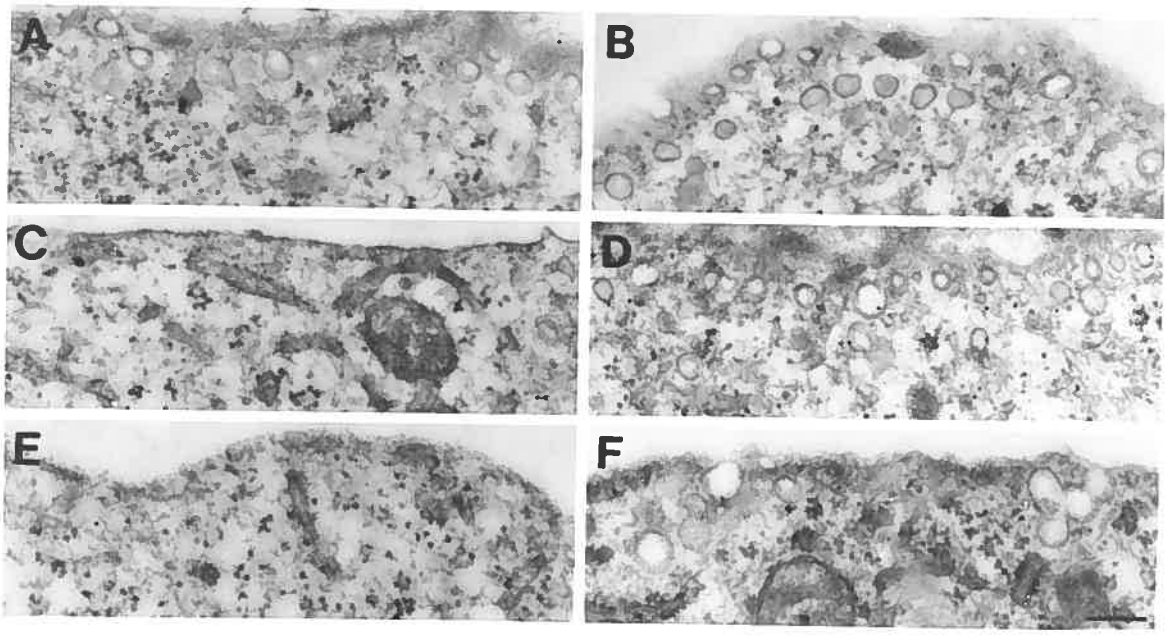


Fig. 9. Caveolin expression levels following dynK44A and caveolin-1 adenovirus infection. Cell lysates from NIH-3T3, and *ras*- and *abl*-transformed NIH-3T3 cells were separated by SDS-PAGE, and the blots were probed with antibodies to either caveolin or the HA tag of dynK44A, as indicated. Caveolin expression levels are significantly reduced in *ras*- and *abl*-transformed NIH-3T3 relative to untransformed NIH-3T3 cells and are not affected in dynK44A-expressing cells. However, in caveolin-1-infected cells, caveolin expression is significantly increased in the three cell lines.

Figure 10. Expression of caveolin-1 induces the formation of caveolae in ras and abl transformed NIH-3T3 cells. NIH-3T3 (A, B), ras (C, D) and abl (E, F) transformed NIH-3T3 cells, either uninfected (A, C, E) or expressing caveolin-1 via adenoviral infection (B, D, F) were pulse labeled with bAMF for 60 minutes at 37°C. Postembedding labeling with nanogold-streptavidin and silver amplification first revealed the localization of bAMF to caveolae and then caveolin distribution was determined using anti-caveolin polyclonal antibodies and 12 nm gold conjugated anti-rabbit secondary antibodies. Plasma membrane profiles of ras and abl transformed NIH-3T3 cells show the dramatically increased expression of caveolae in the caveolin-1 infected cells (D, F) relative to uninfected cells (C, E). Bar = 0.2 μm.


A
B
C
D
E
F

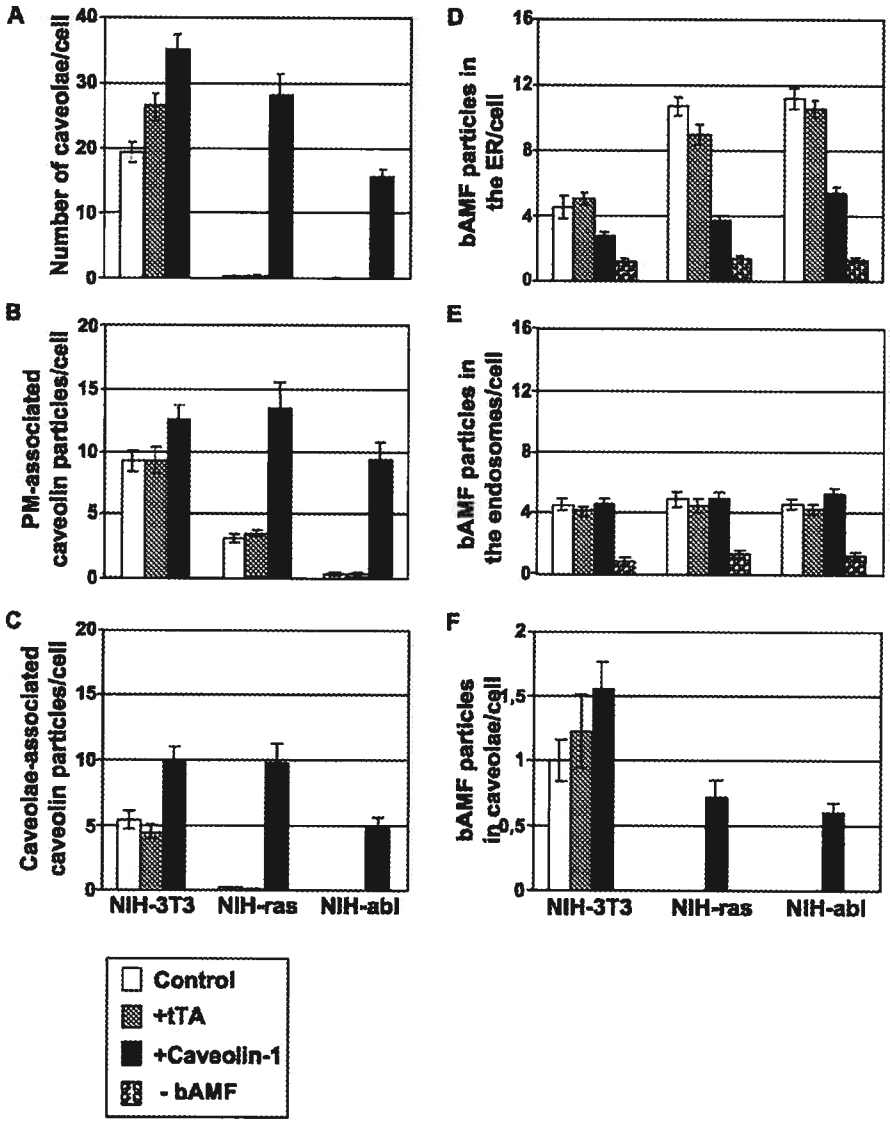


Fig. 11. Caveolin-1 expression induces caveolae formation and down-regulates bAMF endocytosis to the ER. Untransformed and *ras*- or *abl*-transformed NIH-3T3 cells were either not infected (*white bars*) or infected with the tTA (*gray bars*) or with the tTA and caveolin-1 adenoviruses (*black bars*) and then pulsed with bAMF for 60 min at 37 °C. Control cells were not pulsed with bAMF (*dotted bars*). Postembedding labeling of bAMF was first revealed with nanogold-streptavidin and silver amplification, and then caveolin was revealed using anti-caveolin polyclonal antibodies and 12-nm gold-conjugated anti-rabbit secondary antibodies. Quantification of the number of caveolae at the plasma membrane (*A*), caveolin labeling at the plasma membrane (including caveolae) (*B*), caveolae-associated caveolin labeling (*C*), bAMF endocytosis to the ER (*D*) or to endosomes (*E*), and bAMF labeling of caveolae (*F*) was determined for 50 complete cell profiles obtained from two distinct experiments. The data presented represents the average per cell profile (±S.E.).

ARTICLE 4

Distinct caveolae-mediated endocytic pawthays targetting the Golgi apparatus and the endoplasmic reticulum

Le, P.U.and.I.R.Nabi., (2003).

J Cell Sci,. 116(Pt 6): p. 1059-71.

DISTINCT CAVEOLAE-MEDIATED ENDOCYTIC PATHWAYS TARGET THE GOLGI APPARATUS AND THE ENDOPLASMIC RETICULUM

Phuong U. Le and Ivan R. Nabi

Department of Pathology and Cell Biology, Université de Montréal, Montréal, Québec, Canada

Short title: Distinct caveolae-mediated endocytic pathways

Key words: endocytosis; caveolae; glycolipid rafts; cholera toxin; endoplasmic reticulum; autocrine motility factor

Corresponding author:
Dr. Ivan R. Nabi
Département de pathologie et biologie cellulaire
2459
Université de Montréal
[redacted]
C. P. 6128, succursale A
Montréal, Québec
Canada H3C 3J7
Courier address:
Département de pathologie et biologie cellulaire
Université de Montréal, Pavillion principal N-812
2900 Blvd. Edouard Montpetit
Montréal, Québec
Canada H3T1J4

SUMMARY

Internalization of autocrine motility factor (AMF) to the endoplasmic reticulum is sensitive to the cholesterol extracting reagent methyl-ß-cyclodextrin, inhibited by the dynamin-1 K44A mutant, and negatively regulated by caveolin-1 defining a caveolae-mediated endocytic pathway (Le et al, J. Biol. Chem. 277:3371-9, 2002). Similarly, we show here that endocytosis of cholera toxin (CTX) in NIH-3T3 fibroblasts is inhibited by adenoviral expression of the dynamin-1 K44A mutant but only partially by expression of the clathrin hub. Treatment with methyl-ß-cyclodextrin and overexpression of caveolin-1 but not the clathrin hub selectively diminishes CTX endocytosis to the Golgi apparatus but not to endosomes. CTX is therefore targeted via a caveolin-1 regulated caveolae-mediated pathway to the Golgi. Disruption of Golgi, caveosome or endosome mediated trafficking with brefeldin A, nocodazole or a 20°C temperature block, respectively, inhibit CTX endocytosis to the Golgi but do not affect AMF delivery to the endoplasmic reticulum. Following an incubation of only five minutes in the presence of the clathrin hub, AMF and CTX are not cointernalized and AMF is delivered to the AMF-R positive smooth ER. The internalization of both ligands is nevertheless sensitive to the tyrosine kinase inhibitor genistein, confirming that they are both internalized via caveolae/raft pathways. Two distinct caveolae-mediated endocytic pathways therefore exist including a novel direct pathway to the ER from the plasma membrane.

INTRODUCTION

Caveolae are smooth invaginations of the plasma membrane first described in endothelial cells (Palade, 1953; Yamada, 1955). They represent a subdomain of glycolipid rafts, cholesterol- and sphingolipid-rich membrane domains that are specifically associated with the cholesterol-binding caveolin proteins (Anderson, 1998; Galbiati et al., 2001; Kurzchalia and Parton, 1999). The endocytosis of cholera toxin (CTX) bound GM1 ganglioside and SV40 to the Golgi and endoplasmic reticulum (ER) was originally attributed to caveolae based on morphological grounds (Kartenbeck et al., 1989; Montesano et al., 1982; Parton et al., 1994; Tran et al., 1987). The use of cholesterol-sensitivity, dynamin-dependence and clathrin-independence has expanded the repertory of caveolae/raft endocytic ligands to include sphingolipids, GPI-anchored proteins, the autocrine motility factor (AMF), endothelin, growth hormone, and IL2 receptors, as well as bacteria (Benlimame et al., 1998; Lamaze et al., 2001; Le et al., 2000; Le et al., 2002; Lobie et al., 1999; Nichols et al., 2001; Okamoto et al., 2000; Pelkmans et al., 2001; Puri et al., 2001; Shin et al., 2000; Sukumaran et al., 2002).

However, in hippocampal neurons, CTX bound to GM1 is found in detergent insoluble membranes at the cell surface but CTX endocytosis was blocked by inhibitors of clathrin-dependent endocytosis and not by filipin or mβCD (Shogomori and Futerman, 2001a). MβCD rather blocks the cholesterol-dependent delivery of CTX from endosomes to the Golgi apparatus (Shogomori and Futerman, 2001b), as previously reported for GPI-anchored folate receptor

(Mayor et al., 1998). These studies therefore describe a clathrin-dependent, endosome-mediated pathway for CTX to the Golgi. They further indicate that cholesterol-sensitivity is not necessarily an indicator of caveolae or raft-mediated internalization to the Golgi.

Both clathrin- and caveolae/raft-dependent endocytosis are dynamin dependent. The fact that expression of mutant dynK44A inhibited only 40-50% of CTX uptake in HeLa cells and that neither filipin nor caveolin-1 overexpression inhibited CTX internalization in confluent CaCo-2 cells led to the suggestion that CTX could also follow the dynamin-insensitive non-clathrin pathway. However, in human skin fibroblasts and in subconfluent CaCo-2 cells, cholesterol disrupting agents inhibit CTX internalization while agents that inhibit clathrin-dependent endocytosis, such as chlorpromazine and potassium depletion, do not affect CTX endocytosis. Depletion of membrane cholesterol prevents CTX entry into the cells, transport to the Golgi and induction of chloride secretion. Furthermore, inhibition of dynamin function by either dynK44A expression or microinjection of anti-dynamin antibodies has been shown to qualitatively inhibit CTX endocytosis. These data from multiple studies in multiple cell types argue strongly for a role of caveolae in the internalization of CTX. However, a single study in a single cell type demonstrating the clathrin-independent, dynamin-dependent, cholesterol-sensitive, caveolae-mediated endocytosis of CTX has yet to be performed.

CTX delivery to the Golgi apparatus in Cos-7 cells is filipin-sensitive but not blocked by inhibition of clathrin-dependent endocytosis with the eps15

mutant or of endosome function with a rab5 mutant identifying a cholesterol-sensitive non-endosomal pathway for CTX to the Golgi apparatus (Nichols et al., 2001). CTX is delivered instead to a caveolin-1-GFP positive, transferrin receptor (TfR) negative endocytic intermediate prior to delivery to the Golgi implicating caveolae in this pathway (Nichols, 2002). Similarly, SV40 had previously been shown to be internalized to the ER via a caveolin-1-GFP positive intermediate, named the caveosome (Pelkmans et al., 2001). SV40 delivery via caveolae to the caveosome is associated with tyrosine kinase activation, the breakdown of actin filaments and the recruitment of dynamin II while passage from the caveosome to the ER is blocked by treatment of the cells with nocodazole and is therefore microtubule-dependent (Pelkmans et al., 2001; Pelkmans et al., 2002). The recent demonstration that BFA and a 20°C temperature block as well as mutants of arf1 and sar1 and antibodies to ß-cop block both SV40 internalization and infectivity as well as the internalization of CTX suggests that both caveolar ligands follow a similar internalization route (Norkin et al., 2002; Richards et al., 2002). While a role for the Golgi apparatus in SV40 delivery to the ER remains to be established (Kartenbeck et al., 1989; Norkin et al., 2002; Pelkmans et al., 2001; Richards et al., 2002)\, SV40 and CTX would appear to follow a similar, if not identical, pathway via the caveosome to the ER.

We have previously defined the caveolae-mediated endocytosis of AMF to the ER based on its sensitivity to mßCD, its inhibition by the dynamin-1 K44A mutant (dynK44A) and its negative regulation by overexpression of caveolin-1

(Benlimame et al., 1998; Le et al., 2000; Le et al., 2002). AMF-R is localized to a smooth ER subdomain and we have no evidence from multiple EM studies that AMF-R is localized to the Golgi or that endocytosed AMF traverses the Golgi (Accola et al., 2002; Benlimame et al., 1998; Benlimame et al., 1995; Le et al., 2002; Wang et al., 1997; Wang et al., 2000). In order to compare the caveolae-mediated endocytosis of AMF and the retrograde pathway of CTX via the Golgi to the ER, we first undertook to define the endocytic pathway of CTX in NIH-3T3 fibroblasts. We show here that in NIH-3T3 cells, 80% of CTX uptake is blocked by adenoviral expression of the dynK44A mutant but that inhibition of clathrin-dependent endocytosis with the dominant-negative hub fragment of the clathrin heavy chain has only a limited effect on CTX endocytosis. MßCD treatment and overexpression of caveolin-1 selectively reduce CTX endocytosis to the Golgi apparatus but not to endosomes. In NIH-3T3 fibroblasts, the majority of CTX is therefore internalized via a caveolae-dependent mechanism to the Golgi with a minor part targeted via a clathrin-dependent mechanism to the endosome. Of particular interest is the fact that while brefeldin A (BFA), nocodazole or a 20°C temperature block inhibit CTX delivery to the Golgi apparatus, they do not affect AMF delivery to the ER. Furthermore, while genistein inhibits the caveolae/raft-mediated endocytosis of both CTX and AMF to the Golgi and smooth ER, respectively, after five minutes of endocytosis in the presence of the clathrin hub the two ligands do not cointernalize and AMF is targeted, apparently directly, to the AMF-R labeled smooth ER. The caveolae-mediated endocytic pathway of AMF is therefore distinct from that of CTX and SV40. Two caveolae-mediated

endocytic pathways therefore exist targeting either the Golgi or the ER from the plasma membrane.

MATERIALS AND METHODS

Antibodies, reagents and cells

Monoclonal rat antibody against AMF-R was used in the form of concentrated hybridoma supernatant (Nabi et al., 1990). Mouse anti-GM130, polyclonal anti-caveolin antibody was purchased from Transduction Laboratories (Mississauga, ON), mouse anti-TfR from Zymed Laboratories Inc. (San Francisco, CA), mouse anti-T7 tag from Novagen (Madison WI), and mouse anti-c-Myc from Santa Cruz Biotechnology (Santa Cruz, CA). Mouse anti-hemagglutinin (HA) was a gift from Luc Desgroseillers (Department of Biochemistry, Université de Montréal). Alexa 488, 568 and 647 conjugated secondary antibodies and anti-FITC antibody were purchased from Molecular Probes (Eugene, OR) and rhodamine-red-X anti-rat IgM and Texas Red anti-mouse secondary antibodies from Jackson Immunoresearch Laboratories (West Grove, PA). MßCD, BFA, nocodazole, genistein, poly-L-lysine, FITC-conjugated B-subunit of CTX, tetramethylrhodamine conjugated transferrin (Rh-Tf) and rabbit phosphohexose isomerase (referred to as AMF) were purchased from Sigma (Oakville, ON) and the Alexa 594 conjugated B-subunit of CTX was purchased from Molecular Probes. AMF was conjugated to fluorescein with the Fluorescein-EX protein labeling kit (Molecular Probes) according to the manufacturer's instructions.

An NIH-3T3 fibroblast clone (Benlimame et al., 1998) was grown in complete medium consisting of DME supplemented with 10% calf serum, non-

essential amino acids, vitamins, glutamine, and a penicillin-streptomycin antibiotic mixture (Invitrogen Canada Inc., Burlington, ON).

Viral infection

Recombinant adenoviruses expressing the tetracycline-regulatable chimeric transcription activator (tTA), HA-tagged wild-type dynamin-1, HA-tagged dynK44A mutant, T7-tagged clathrin hub and myc-tagged caveolin-1 under the control of the tetracycline-regulated promoter were as previously described (Altschuler et al., 1998; Altschuler et al., 1999; Le et al., 2002; Zhang et al., 2000). To enhance infection rates, viral stocks of the tTA and the indicated adenoviruses were diluted in 100 µl sterile PBS and pre-incubated with 18 µl of 1 µg/ml polylysine for 30 minutes at room temperature. 50,000 NIH-3T3 cells were plated on 35 mm dishes for 10 hours and rinsed once with PBS before the addition of the adenovirus/polylysine mixture in 0.7 ml serum-free media. The cells were then incubated with the adenoviruses for one hour at 37°C. After removal of the adenovirus mixture, the cells were rinsed twice with serum-free media and then incubated for 36 hours in regular culture media. For the immunofluorescence studies, viral titres were used such that 25-50% of the cells were infected, as indicated. For some experiments, the degree of infection with the clathrin hub adenovirus was assessed by labeling paraformaldehyde fixed cells on parallel coverslips with monoclonal anti-T7 tag antibodies to ensure that at least 90% of the cells expressed the clathrin hub.

Immunofluorescence

NIH-3T3 cells were incubated with 5 µg/ml FITC-CTX alone or coincubated with 5 µg/ml FITC-CTX and with 15 µg/ml Rh-Tf for 30 minutes at 37°C, washed five times with culture medium and fixed with 3% paraformaldehyde and permeabilized with 0.2% Triton X-100. Alternatively, cells were incubated with 50 µg/ml of AMF-FITC alone or coincubated with 5 µg/ml Alexa 594-CTX at 37°C for the indicated time, washed five times with culture medium and then fixed with precooled (-80) methanol/acetone (Le et al., 2000). The cells were then labeled for GM130, TfR, AMF-R, caveolin, T7, Myc or HA tags with appropriate primary antibodies and Alexa 568 or 647 conjugated secondary antibodies, as indicated. Cell surface FITC-CTX labeling was performed by incubating the cells at 4°C with 5 µg/ml FITC-CTX in bicarbonate-free DMEM-Hepes containing 0.5% BSA (cold DMEM) for 30 minutes (Benlimame et al., 1998). The cells were then rinsed 3 times with cold DMEM/Hepes/BSA, fixed with 3% paraformaldehyde and permeabilized with 0.2% Triton X-100 prior to labeling for the appropriate tags as described previously. Where indicated, cells were pretreated for 30 minutes at 37°C with 5 mM mßCD (Le et al., 2002), 10 µg/ml BFA, 10 µM nocodazole, or 100 µg/ml genistein and the drugs were maintained during incubation with the endocytic ligands. AMF-FITC endocytosis at 20°C was performed in bicarbonate free DMEM-hepes containing 0.5% BSA. Fluorescently labeled cells were visualized with a Leica TCS-SP1 confocal microscope using 63X or 100X Planapochromat objectives.

To quantify endocytosis of FITC-CTX and Rh-Tf, fluorescence intensity within the perinuclear region of the cell was quantified from confocal images with Northern Eclipse imaging software (Empix Imaging, Mississuaga, Ontario). Cell surface FITC-CTX was quantified by measuring the fluorescence intensity of the entire cell. Specific FITC-CTX internalization to the Golgi or endosomes and AMF-FITC internalization to smooth ER tubules were quantified using Northern Eclipse mask overlay software (Empix Imaging) as previously described (Wang et al., 2000). The intensity of FITC-CTX labeled pixels within a mask region defined by either the GM130 labeled Golgi or TfR labeled endosomes was determined relative to total cellular FITC-CTX labeling. Due to the complex pattern of endocytosed AMF, including multivesicular bodies and fibronectin fibrils in addition to the AMF-R labeled smooth ER (Le et al., 2000), the absolute intensity of AMF-FITC labeled pixels within a mask region defined by smooth ER AMF-R labeling was measured. Each measurement represents the quantification of at least 30 cells from three distinct experiments.

RESULTS

CTX is internalized via caveolae to the Golgi apparatus in NIH-3T3 cells

Following incubation at 37°C for 30 minutes, FITC-CTX is internalized by NIH-3T3 fibroblasts to the perinuclear region. Double labeling with antibodies against either the TfR or the Golgi marker GM130 shows that FITC-CTX is delivered to both recycling endosomes and the Golgi apparatus (Fig. 1A-F). Infection of cells with adenoviruses coding for the dynK44A mutant blocked the internalization of FITC-CTX and the clathrin-specific internalization of Rh-Tf (Fig. 1G-J). FITC-CTX is therefore predominantly internalized in NIH-3T3 fibroblasts via dynamin-dependent clathrin- or caveolae/raft-mediated pathways.

Quantitative analysis shows that dynK44A mutant expression significantly inhibits both FITC-CTX and Rh-Tf endocytosis to the perinuclear region (> 80%) while the dynamin wild type exhibits no effect (Fig. 2A). Clathrin hub expression inhibits >75% of Rh-Tf endocytosis and <25% of FITC-CTX endocytosis while caveolin-1 overexpression does not affect Rh-Tf endocytosis but inhibits >60% of the endocytosis of FITC-CTX. To ensure that infection of NIH-3T3 cells with the various adenoviruses did not affect FITC-CTX binding to cell surface GM1, cell surface binding of FITC-CTX at 4°C was quantified in non-infected and infected cells. As shown in Fig. 2 B, cells infected with the various adenoviruses did not present a reduction of cell surface FITC-CTX labeling (Fig. 2B) indicating that infection with the various adenoviruses did not affect accessibility of exogenous FITC-CTX to cell surface GM1. A significant increase ($p<0.05$) in cell surface labeling was observed following infection with dynK44A consistent with

its ability to inhibit both the caveolae- and clathrin-mediated endocytosis of FITC-CTX. In NIH-3T3 cells the majority of FITC-CTX is therefore internalized via a caveolin-dependent pathway and a minor portion via a clathrin-dependent pathway.

Caveolae-mediated endocytosis of CTX selectively targets the Golgi and not endosomes

Treatment of NIH-3T3 cells with 5 mM mßCD resulted in the significantly increased cell surface binding of FITC-CTX, evidently due to the release of sequestered GM1 from closed caveolae, but did not inhibit the endocytosis of FITC-CTX or Rh-Tf to endosomes (Fig. 3A-F). In confocal sections obtained with a reduced pinhole (0.6 Airy units) to exclude the increased cell surface FITC-CTX labeling from the image, internalized FITC-CTX exhibited extensive colocalization with Rh-Tf positive endosomes (Fig. 3G,H,K). Within the crowded perinuclear region, the GM130-labeled FITC-CTX-unlabeled Golgi could be clearly seen to intercalate between FITC-CTX and Rh-Tf positive endosomes (Fig. 3J,L). Golgi delivery of CTX is therefore significantly reduced in the presence of mßCD.

We subsequently infected cells with the caveolin-1 adenovirus and assessed FITC-CTX delivery to either the GM130 labeled Golgi or TfR labeled endosomes. In caveolin-1 overexpressing cells, identified with a polyclonal anti-caveolin antibody, delivery of FITC-CTX to the Golgi was significantly reduced (Fig. 4A-D). Perinuclear FITC-CTX was localized predominantly to TfR positive endosomes that were not labeled for caveolin while peripheral FITC-CTX

labeling colocalized extensively with caveolin, apparently at the cell surface (Fig. 4E-J). Using mask overlay image analysis software (Wang et al., 2000), we quantified the delivery of FITC-CTX to either GM130 positive Golgi or TfR positive endosomes in caveolin-1 infected and in uninfected cells. Caveolin-1 overexpression resulted in a significant reduction (~50%) in the delivery of FITC-CTX to the Golgi but did not affect delivery of FITC-CTX to endosomes (Fig. 4K, L). The fact that caveolin-1 overexpression selectively reduces CTX delivery to the Golgi but not transferrin-positive endosomes provides direct evidence for a caveolae-mediated endocytic pathway to the Golgi apparatus.

To confirm that CTX internalization to the Golgi is caveolae-mediated, FITC-CTX was added to NIH-3T3 cells infected with the clathrin hub adenovirus (Fig. 5). Five minutes after its addition, FITC-CTX colocalizes extensively with caveolin and after 30 minutes, FITC-CTX is associated predominantly with the Golgi apparatus. The fact that the clathrin hub only blocks a minor portion of CTX endocytosis (Fig. 2) together with its inability to inhibit CTX delivery to the Golgi demonstrates clearly that in NIH-3T3 cells, the majority of CTX is targeted via a clathrin-independent, caveolin-regulated pathway to the Golgi apparatus.

Caveolae mediate distinct endocytic pathways to the Golgi and ER

AMF-R is a marker for a mitochondria-associated smooth ER subdomain (Benlimame et al., 1998; Benlimame et al., 1995; Wang et al., 1997; Wang et al., 2000) and in NIH-3T3 cells, AMF-R is specifically localized to smooth and not rough ER tubules (Benlimame et al., 1998). We have previously reported, using quantitative electron microscopy, that caveolin-1 overexpression reduces the

caveolae-mediated endocytosis of AMF to the ER in NIH-3T3 fibroblasts as well as in ras and abl-transformed NIH-3T3 fibroblasts that express little caveolin-1 (Le et al., 2002). Quantification using a mask overlay assay revealed a 42.0±2.4% reduction in AMF-FITC delivery to the AMF-R labeled smooth ER in NIH-3T3 fibroblasts that overexpress Myc-tagged caveolin-1 (Fig. 6). These results are equivalent to those previously obtained by quantitative EM (Le et al., 2002). The tubular distribution of the AMF-R labeled smooth ER, a reflection of its interaction with mitochondria, can be disrupted by ilimaquinone or low cytosolic calcium (Wang et al., 1997; Wang et al., 2000). In caveolin-1 infected cells, the AMF-R labeled smooth ER exhibits a more diffuse distribution (Fig. 6B, arrow) suggesting that regulation of caveolae-mediated endocytosis to this ER subdomain may influence the extent of its association with mitochondria. The fact that overexpression of caveolin-1 reduces the internalization of AMF to the ER and of CTX to the Golgi demonstrates that they are both mediated by a similar caveolae-based endocytic mechanism.

Entry of CTX is sensitive to BFA (Donta et al., 1993; Lencer et al., 1993; Morinaga et al., 2001; Nambiar et al., 1993; Orlandi et al., 1993) and BFA has recently been shown to prevent delivery of CTX to the Golgi but not to endosomes (Richards et al., 2002). In cells treated with nocodazole, SV40 entry is blocked at the level of the caveosome and it is not delivered to the ER (Pelkmans et al., 2001). Furthermore, a 20°C temperature block and BFA treatment inhibit early entry steps of SV40 preventing its delivery to the ER (Norkin et al., 2002; Richards et al., 2002). As seen in Fig. 7, BFA treatment of

NIH-3T3 fibroblasts inhibits FITC-CTX delivery to the fragmented Golgi but not to endosomes (Fig. 7A-F). In contrast, BFA does not affect AMF-FITC delivery to the AMF-R labeled smooth ER (Fig. 7G-I). In the presence of nocodazole, FITC-CTX is not present in the Golgi apparatus although extensive colocalization is observed with caveolin and with TfR (Fig. 8A-H). In contrast, AMF-FITC is delivered efficiently to the smooth ER in the presence of nocodazole as well as at 20°C (Fig. 8I-N).

The ability of BFA, nocodazole and a 20°C block to inhibit the targeting of CTX to the Golgi but not of AMF to the ER indicates that caveolae internalized ligands can follow distinct intracellular targeting pathways. To determine whether the sorting of caveolae-internalized CTX and AMF occurred at the plasma membrane or intracellularly, clathrin hub infected NIH-3T3 cells were incubated with AMF-FITC and Alexa594-CTX for only 5 minutes (Fig. 9A-F). Essentially no colocalization between the two caveolar ligands could be detected and, interestingly, internalized AMF colocalized with AMF-R labeled smooth ER tubules. After 30 minutes, while CTX is colocalized predominantly to the Golgi apparatus, internalized AMF remains colocalized with the smooth ER and is excluded from the Golgi (Fig. 9G-J). AMF and CTX would therefore appear to be segregated at the plasma membrane into different caveolae-mediated internalization pathways. To reconfirm that caveolae truly mediate delivery of both CTX to the Golgi and AMF to the smooth ER, we treated the cells with genistein, a tyrosine kinase inhibitor that inhibits the caveolae-mediated endocytosis of SV40 and the transcytosis of albumin across the endothelial cell

(Pelkmans et al., 2002; Tiruppathi et al., 1997). As seen in Fig. 10, in the presence of genistein, CTX delivery to the Golgi but not to TfR positive endosomes was inhibited, confirming that CTX delivery to the Golgi is caveolae-mediated. Genistein also inhibited the delivery, detected after 5 minutes incubation, of AMF-FITC to the smooth ER (Fig. 10G-L).

DISCUSSION

Caveolae mediate CTX endocytosis to the Golgi apparatus

The ability of dynK44A expression to inhibit essentially all CTX endocytosis demonstrates that it is predominantly internalized via dynamin-dependent clathrin- and caveolae-mediated pathways in NIH-3T3 cells (Figs 1, 2). Adenoviral expression of the clathrin hub (Altschuler et al., 1999) only slightly reduces CTX uptake indicating that a minor proportion of CTX is entering NIH-3T3 cells via the clathrin-dependent pathway. The differential inhibition of CTX endocytosis by dynK44A and the clathrin hub demonstrates clearly that in NIH-3T3 fibroblasts, the majority of CTX is internalized via a caveolae/raft pathway. While we cannot exclude the possibility that some CTX is endocytosed via the dynamin-independent non-clathrin pathway (Torgersen et al., 2001), it is necessarily a relatively minor route for CTX entry in NIH-3T3 cells. Our ability to inhibit CTX endocytosis with the dynK44A mutant may be due to superior dynK44A expression levels obtained using adenoviral infection.

Treatment of NIH-3T3 cells with mßCD selectively inhibited CTX delivery to the Golgi but not to transferrin-positive endosomes (Fig. 3). While these

results could be explained by intracellular mßCD inhibition of CTX delivery to the Golgi from the endosome (Mayor et al., 1998; Shogomori and Futerman, 2001b), overexpression of caveolin-1 (Fig. 4) and treatment with genistein (Fig. 10), an inhibitor of caveolae-mediated endocytosis (Pelkmans et al., 2002; Tiruppathi et al., 1997), also selectively inhibit Golgi delivery but not endosomal delivery of CTX. Caveolin-1 overexpression affects neither the cell surface expression of GM1 nor the endocytosis of CTX to endosomes and is therefore not reducing Golgi delivery of CTX by reducing clathrin-mediated endocytosis to the endosome. These data provide direct evidence for the existence of a caveolae-mediated endocytic route to the Golgi and are consistent with and support previous studies describing the caveolae-mediated endocytosis of CTX to the Golgi (Nichols, 2002; Nichols et al., 2001). CTX can therefore be endocytosed via both caveolae- and clathrin-mediated pathways. Differential rates of clathrin- or caveolae-mediated endocytosis and differential affinities of GM1 for endocytosis-competent raft domains may influence the choice of endocytic pathway of CTX in different cell types.

Caveolin-1 and caveolae/raft mediated endocytosis

In cells expressing caveolin-1-GFP, a caveolin-positive endocytic intermediate, the caveosome, has been implicated in caveolae-mediated endocytosis, including that of both CTX and SV40 (Mundy et al., 2002; Nichols, 2002; Parton et al., 1994; Pelkmans et al., 2001). In our study, expression of perinuclear caveolin-positive, TfR-negative vesicular structures was observed, particularly after caveolin-1 overexpression, and some were positive for

internalized CTX. Cell-associated CTX exhibited significant colocalization with caveolin-1, primarily to peripheral cell surface regions but also to some perinuclear structures (Figs 4, 5). It was however difficult to ascertain whether these caveolin-positive structures are indeed intracellular endocytic vesicles and potentially equivalent to caveosomes.

The reduced endocytosis of CTX in caveolin-1 expressing cells corroborates our previous report of the negative regulation by caveolin-1 of the caveolae-mediated delivery of AMF to the ER (Le et al., 2002). As reported for the ER delivery of AMF in ras and abl transformed NIH-3T3 cells expressing little caveolin (Le et al., 2002), CTX is internalized via a cholesterol-sensitive raft pathway in CaCo-2 cells that do not express caveolin (Orlandi and Fishman, 1998). Furthermore, reduction of caveolin-1 levels using RNAi did not affect the internalization of CTX to the Golgi (Nichols, 2002). The fact that the caveolae-mediated endocytosis of AMF and CTX to the smooth ER and Golgi, respectively, occurs independently of caveolin-1 expression argues that caveolae- and raft-mediated endocytosis are essentially equivalent processes defined by cholesterol sensitivity, tyrosine kinase activation, dynamin-dependence and regulation by caveolin-1.

The role of caveolin-1 as a negative regulator of caveolae/raft-mediated endocytosis is consistent with recent reports describing the immobilization of caveolin-1 at the cell surface (Pelkmans et al., 2002; Thomsen et al., 2002). Caveolin-1 expression may serve to stabilize the mobility of raft domains in and out of the plane of the membrane. The role of caveolin-1 as a regulator and not

as an essential component of the caveolae/raft endocytic machinery does not question its importance in defining this endocytic pathway. Caveolin-1 remains a critical determinant of caveolae/raft mediated endocytosis as either a negative regulator of caveolae budding from the plasma membrane, a recruiter of cargo to endocytic caveolar domains, or as a component of caveolae-specific endosomal intermediates.

Existence of two distinct caveolae/raft-mediated pathways

BFA and a 20°C incubation, classic inhibitors of CTX delivery to the Golgi (Donta et al., 1993; Lencer et al., 1993; Morinaga et al., 2001; Nambiar et al., 1993; Nichols et al., 2001; Orlandi et al., 1993; Richards et al., 2002), also inhibit SV40 endocytosis (Norkin et al., 2002; Richards et al., 2002). We further show here that nocodazole treatment, shown to prevent SV40 endocytosis past the caveosome (Pelkmans et al., 2001), also prevents CTX delivery to the Golgi. SV40 targeting to the ER may follow a similar retrograde pathway of CTX via the caveosome and potentially the Golgi apparatus before delivery to the ER.

In contrast to CTX endocytosis to the Golgi and SV40 delivery to the ER, neither BFA, nocodazole or a 20°C incubation prevent the caveolae-mediated delivery of AMF to the ER (Figs 7, 8). Furthermore, following incubations of both 5 and 30 minutes in the presence of the clathrin hub, the vast majority of AMF and CTX did not colocalize (Fig. 9). AMF is therefore internalized via a caveolae-mediated endocytic route distinct from that of either CTX or SV40. The fact that AMF could be detected in AMF-R positive smooth ER tubules after only 5 minutes argues strongly that this pathway is a direct pathway to the ER. We

have no evidence for the involvement of a caveolin-positive vesicular intermediate in the endocytosis of AMF. Since nocodazole does not inhibit AMF delivery to the ER, any such endosomal intermediate would necessarily be distinct from the caveosome that mediates SV40 endocytosis (Pelkmans et al., 2001). Distinct endosomal populations are apparently targeted by clathrin, non-clathrin, and caveolar vesicles (Nichols, 2002; Pelkmans et al., 2001; Sabharanjak et al., 2002). The demonstration here that caveolae-derived vesicles can target different organelles shows that intracellular targeting in endocytosis is more complex than one vesicle-one endosome.

The functional significance of an alternate, apparently direct endocytic pathway to the ER remains to be determined. Such a pathway could potentially be involved in the recovery of Golgi or ER proteins mistargeted to the plasma membrane or in the targeting of proteins for degradation in the ER. Caveolin redistributes to the ER in the presence of cholesterol oxidase (Conrad et al., 1995) and cholesteryl ester is transported to the ER from the plasma membrane (Uittenbogaard et al., 2002), however these pathways appear to be non-vesicular and distinct from the endocytic pathway of AMF-R. AMF-R is localized to a mitochondria-associated subdomain of the smooth ER whose association with mitochondria is calcium-dependent (Wang et al., 2000). A direct endocytic pathway to this smooth ER subdomain may function to maintain its integrity and functionality. The IP3R is also localized to caveolae and smooth ER (Fujimoto et al., 1992; Ross et al., 1989; Sharp et al., 1992) implicating these two organelles and any interaction between them in the regulation of calcium homeostasis.

The existence of two caveolae-mediated endocytic pathways necessarily implies the existence of distinct endocytosis-competent caveolae populations at the cell surface (Maxfield, 2002). AMF and CTX may be either segregated in different caveolae/raft domains at the plasma membrane or their segregation may occur upon internalization of these domains. In endothelial cells of the rete mirabel, albumin and insulin were localized to distinct caveolae populations demonstrating the segregation of caveolar endocytic cargo (Bendayan and Rasio, 1996). The existence of distinct rafts and segregation of raft components has been demonstrated by the distribution of GM1 to the uropod and GM3 to the lamellipodia of migrating T lymphocytes (Gomez-Mouton et al., 2001). Furthermore, immunoisolation techniques have allowed the separation of caveolin-1 positive rafts from caveolin-1 negative rafts suggesting the existence of at least two, and potentially multiple, distinct classes of rafts at the plasma membrane (Badizadegan et al., 2000; Matveev and Smart, 2002; Riddell et al., 2001; Stan et al., 1997).

The composition of raft domains is necessarily a determinant of their endocytic potential. As shown here for CTX and previously for AMF (Le et al., 2002), caveolin-1 is a regulator of caveolae/raft endocytosis that may act to segregate endocytic and non-endocytic raft domains. However, the caveolin-1 independent internalization of CTX (Nichols, 2002; Orlandi and Fishman, 1998) , AMF (Le et al., 2002) and the IL2 receptor in lymphocytes (Lamaze et al., 2001), argues that regulation of caveolae/raft-mediated endocytosis is necessarily more complex than just caveolin. The ability of SV40 to induce a signaling cascade

that regulates its internalization demonstrates a role for ligand binding and receptor-mediated signal transduction in the induction of caveolae invagination and internalization (Pelkmans et al., 2002). While common denominators of caveolae/raft-mediated endocytosis include cholesterol-sensitivity, dynamin-mediated internalization, tyrosine kinase activation and regulation by caveolin-1, other factors may sort cargo to diverse plasma membrane caveolae/raft domains, segregate endocytic cargo within caveolae/raft domains and regulate the invagination and intracellular targeting of caveolae/raft derived caveolar vesicles.

Acknowledgments

We are particularly grateful to Dr. Yoram Altschuler (Hebrew University of Jerusalem) for providing the adenoviruses used in this study. This study was supported by a grant from the Canadian Institutes for Health Research. I. R. Nabi is an investigator of the Canadian Institutes for Health Research. P. U. Le was supported in part by a Graduate Student Award from the Cancer Research Society Inc.

Abbreviations list

AMF: autocrine motility factor; AMF-R: Autocrine motility factor receptor; CTX: cholera toxin; mßCD: methyl-ß-cyclodextrin; BFA: brefeldin A; dynK44A: dynamin-1 K44A mutant; ER: endoplasmic reticulum; Rh-Tf: rhodamine-transferrin; TfR: transferrin receptor; tTA: tetracycline-regulatable chimeric transcription activator.

References

Accola, M. A., Huang, B., Al Masri, A. and McNiven, M. A. (2002). The Antiviral Dynamin Family Member, MxA, Tubulates Lipids and Localizes to the Smooth Endoplasmic Reticulum. *J. Biol. Chem.* **277**, 21829-35.

Altschuler, Y., Barbas, S. M., Terlecky, L. J., Tang, K., Hardy, S., Mostov, K. E. and Schmid, S. L. (1998). Redundant and distinct functions for dynamin-1 and dynamin-2 isoforms. *J. Cell Biol.* **143**, 1871-81.

Altschuler, Y., Liu, S., Katz, L., Tang, K., Hardy, S., Brodsky, F., Apodaca, G. and Mostov, K. (1999). ADP-ribosylation factor 6 and endocytosis at the apical surface of Madin-Darby canine kidney cells. *J. Cell Biol.* **147**, 7-12.

Anderson, R. G. (1998). The caveolae membrane system. *Annu. Rev. Biochem.* **67**, 199-225.

Badizadegan, K., Dickinson, B. L., Wheeler, H. E., Blumberg, R. S., Holmes, R. K. and Lencer, W. I. (2000). Heterogeneity of detergent-insoluble membranes from human intestine containing caveolin-1 and ganglioside (GM1). *Am. J. Physiol. Gastrointest. Liver Physiol.* **278**, G895-904.

Bendayan, M. and Rasio, E. A. (1996). Transport of insulin and albumin by the microvascular endothelium of the rete mirabile. *J. Cell Sci.* **109**, 1857-1864.

Benlimame, N., Le, P. U. and Nabi, I. R. (1998). Localization of autocrine motility factor receptor to caveolae and clathrin-independent internalization of its ligand to smooth endoplasmic reticulum. *Mol. Biol. Cell* **9**, 1773-1786.

Benlimame, N., Simard, D. and Nabi, I. R. (1995). Autocrine motility factor receptor is a marker for a distinct tubular membrane organelle. *J. Cell Biol.* **129**, 459-471.

Conrad, P. A., Smart, E. J., Ying, Y. S., Anderson, R. G. and Bloom, G. S. (1995). Caveolin cycles between plasma membrane caveolae and the Golgi complex by microtubule-dependent and microtubule-independent steps. *J. Cell Biol.* **131**, 1421-33.

Damke, H., Baba, T., Warnock, D. E. and Schmid, S. L. (1994). Induction of mutant dynamin specifically blocks endocytic coated vesicle formation. *J. Cell Biol.* **127**, 915-34.

Donta, S. T., Beristain, S. and Tomicic, T. K. (1993). Inhibition of heat-labile cholera and Escherichia coli enterotoxins by brefeldin A. *Infect. Immun.* **61**, 3282-6.

Fujimoto, T., Nakade, S., Miyawaki, A., Mikoshiba, K. and Ogawa, K. (1992). Localization of 1,4,5-triphosphate receptor-like protein in plasmalemmal caveolae. *J. Cell Biol.* **119**, 1507-1513.

Galbiati, F., Razani, B. and Lisanti, M. P. (2001). Emerging themes in lipid rafts and caveolae. *Cell* **106**, 403-11.

Gomez-Mouton, C., Abad, J. L., Mira, E., Lacalle, R. A., Gallardo, E., Jimenez-Baranda, S., Illa, I., Bernad, A., Manes, S. and Martinez, A. C. (2001). Segregation of leading-edge and uropod components into specific lipid rafts during T cell polarization. *Proc. Natl. Acad. Sci. U.S.A.* **98**, 9642-7.

Henley, J. R., Krueger, E. W., Oswald, B. J. and McNiven, M. A. (1998). Dynamin-mediated internalization of caveolae. *J. Cell Biol.* **141**, 85-99.

Herskovits, J. S., Burgess, C. C., Obar, R. A. and Vallee, R. B. (1993). Effects of mutant rat dynamin on endocytosis. *J. Cell Biol.* **122**, 565-78.

Kartenbeck, J., Stukenbrok, H. and Helenius, A. (1989). Endocytosis of simian virus 40 into the endoplasmic reticulum. *J. Cell Biol.* **109**, 2721-2729.

Kurzchalia, T. V. and Parton, R. G. (1999). Membrane microdomains and caveolae. *Curr. Opin. Cell Biol.* **11**, 424-31.

Lamaze, C., Dujeancourt, A., Baba, T., Lo, C. G., Benmerah, A. and Dautry-Varsat, A. (2001). Interleukin 2 receptors and detergent-resistant membrane domains define a clathrin-independent endocytic pathway. *Mol. Cell* **7**, 661-71.

Le, P. U., Benlimame, N., Lagana, A., Raz, A. and Nabi, I. R. (2000). Clathrin-mediated endocytosis and recycling of autocrine motility factor receptor to fibronectin fibrils is a limiting factor for NIH-3T3 cell motility. *J. Cell Sci.* **113**, 3227-3240.

Le, P. U., Guay, G., Altschuler, Y. and Nabi, I. R. (2002). Caveolin-1 is a negative regulator of caveolae-mediated endocytosis to the endoplasmic reticulum. *J. Biol. Chem.* **277**, 3371-9.

Lencer, W. I., de Almeida, J. B., Moe, S., Stow, J. L., Ausiello, D. A. and Madara, J. L. (1993). Entry of cholera toxin into polarized human intestinal epithelial cells. Identification of an early brefeldin A sensitive event required for A1-peptide generation. *J. Clin. Invest.* **92**, 2941-51.

Lobie, P. E., Sadir, R., Graichen, R., Mertani, H. C. and Morel, G. (1999). Caveolar internalization of growth hormone. *Exp. Cell Res.* **246**, 47-55.

Matveev, S. V. and Smart, E. J. (2002). Heterologous desensitization of EGF receptors and PDGF receptors by sequestration in caveolae. *Am. J. Physiol. Cell Physiol.* **282**, C935-46.

Maxfield, F. R. (2002). Plasma membrane microdomains. *Curr. Opin. Cell Biol.* **14**, 483-487.

Mayor, S., Sabharanjak, S. and Maxfield, F. R. (1998). Cholesterol-dependent retention of GPI-anchored proteins in endosomes. *EMBO J.* **17**, 4626-38.

Montesano, R., Roth, J., Robert, A. and Orci, L. (1982). Non-coated membrane invaginations are involved in binding and internalization of cholera and tetanus toxins. *Nature (Lond.)* **296**, 651-653.

Morinaga, N., Kaihou, Y., Vitale, N., Moss, J. and Noda, M. (2001). Involvement of ADP-ribosylation factor 1 in cholera toxin-induced morphological changes of Chinese hamster ovary cells. *J. Biol. Chem.* **276**, 22838-43.

Mundy, D. I., Machleidt, T., Ying, Y. S., Anderson, R. G. and Bloom, G. S. (2002). Dual control of caveolar membrane traffic by microtubules and the actin cytoskeleton. *J. Cell Sci.* **115**, 4327-39.

Nabi, I. R., Watanabe, H. and Raz, A. (1990). Identification of B16-F1 melanoma autocrine motility-like factor receptor. *Cancer Res.* **50**, 409-414.

Nambiar, M. P., Oda, T., Chen, C., Kuwazuru, Y. and Wu, H. C. (1993). Involvement of the Golgi region in the intracellular trafficking of cholera toxin. *J. Cell Physiol.* **154**, 222-8.

Nichols, B. J. (2002). A distinct class of endosome mediates clathrin-independent endocytosis to the Golgi complex. *Nat. Cell Biol.* **15**, 15.

Nichols, B. J., Kenworthy, A. K., Polishchuk, R. S., Lodge, R., Roberts, T. H., Hirschberg, K., Phair, R. D. and Lippincott-Schwartz, J. (2001). Rapid cycling of lipid raft markers between the cell surface and Golgi complex. *J. Cell Biol.* **153**, 529-41.

Norkin, L. C., Anderson, H. A., Wolfrom, S. A. and Oppenheim, A. (2002). Caveolar endocytosis of simian virus 40 is followed by brefeldin A- sensitive transport to the endoplasmic reticulum, where the virus disassembles. *J. Virol.* **76**, 5156-66.

Oh, P., McIntosh, D. P. and Schnitzer, J. E. (1998). Dynamin at the neck of caveolae mediates their budding to form transport vesicles by GTP-driven fission from the plasma membrane of endothelium. *J. Cell Biol.* **141**, 101-14.

Okamoto, Y., Ninomiya, H., Miwa, S. and Masaki, T. (2000). Cholesterol oxidation switches the internalization pathway of endothelin receptor type A from caveolae to clathrin-coated pits in Chinese hamster ovary cells. *J. Biol. Chem.* **275**, 6439-46.

Orlandi, P. A., Curran, P. K. and Fishman, P. H. (1993). Brefeldin A blocks the response of cultured cells to cholera toxin. Implications for intracellular trafficking in toxin action. *J. Biol. Chem.* **268**, 12010-6.

Orlandi, P. A. and Fishman, P. H. (1998). Filipin-dependent inhibition of cholera toxin: evidence for toxin internalization and activation through caveolae-like domains. *J. Cell Biol.* **141**, 905-15.

Palade, G. E. (1953). Fine structure of blood capillaries. *J. Appl. Phys.* **24**, 1424.

Parton, R. G., Joggerst, B. and Simons, K. (1994). Regulated internalization of caveolae. *J. Cell Biol.* **127**, 1199-215.

Pelkmans, L., Kartenbeck, J. and Helenius, A. (2001). Caveolar endocytosis of simian virus 40 reveals a new two-step vesicular-transport pathway to the ER. *Nat. Cell Biol.* **3**, 473-83.

Pelkmans, L., Puntener, D. and Helenius, A. (2002). Local actin polymerization and dynamin recruitment in SV40-induced internalization of caveolae. *Science* **296**, 535-9.

Puri, V., Watanabe, R., Singh, R. D., Dominguez, M., Brown, J. C., Wheatley, C. L., Marks, D. L. and Pagano, R. E. (2001). Clathrin-dependent and -independent internalization of plasma membrane sphingolipids initiates two Golgi targeting pathways. *J. Cell Biol.* **154**, 535-47.

Richards, A. A., Stang, E., Pepperkok, R. and Parton, R. G. (2002). Inhibitors of COP-mediated Transport and Cholera Toxin Action Inhibit Simian Virus 40 Infection. *Mol. Biol. Cell* **13**, 1750-64.

Riddell, D. R., Christie, G., Hussain, I. and Dingwall, C. (2001). Compartmentalization of beta-secretase (Asp2) into low-buoyant density, noncaveolar lipid rafts. *Curr. Biol.* **11**, 1288-93.

Ross, C. A., Meldolesi, J., Milner, T. A., Satoh, T., Supattapone, S. and Snyder, S. H. (1989). Inositol 1,4,5-trisphosphate receptor localized to endoplasmic reticulum in cerebellar Purkinje neurons. *Nature (Lond.)* **339**, 468-70.

Sabharanjak, S., Sharma, P., Parton, R. G. and Mayor, S. (2002). GPI-anchored proteins are delivered to recycling endosomes via a distinct cdc42-regulated, clathrin-independent pinocytic pathway. *Dev. Cell* **2**, 411-23.

Sharp, A. H., Snyder, S. H. and Nigam, S. K. (1992). Inositol 1,4,5-trisphosphate receptors. Localization in epithelial tissue. *J. Biol. Chem.* **267**, 7444-9.

Shin, J. S., Gao, Z. and Abraham, S. N. (2000). Involvement of cellular caveolae in bacterial entry into mast cells. *Science* **289**, 785-8.

Shogomori, H. and Futerman, A. H. (2001a). Cholera toxin is found in detergent-insoluble rafts/domains at the cell surface of hippocampal neurons but is internalized via a raft- independent mechanism. *J. Biol. Chem.* **276**, 9182-8.

Shogomori, H. and Futerman, A. H. (2001b). Cholesterol depletion by methyl-beta-cyclodextrin blocks cholera toxin transport from endosomes to the Golgi apparatus in hippocampal neurons. *J. Neurochem.* **78**, 991-9.

Stan, R.-V., Roberts, W. G., Predescu, D., Ihida, K., Saucan, L., Ghitescu, L. and Palade, G. E. (1997). Immunoisolation and partial characterization of endothelial plasmalemmal vesicles (caveolae). *Mol. Biol. Cell* **8**, 595-605.

Sukumaran, S. K., Quon, M. J. and Prasadarao, N. V. (2002). Escherichia coli K1 internalization via caveolae requires caveolin-1 and protein kinase C-alpha interaction in human brain microvascular endothelial cells. *J. Biol. Chem.* **16**, 16.

Thomsen, P., Roepstorff, K., Stahlhut, M. and van Deurs, B. (2002). Caveolae are highly immobile plasma membrane microdomains, which are not involved in constitutive endocytic trafficking. *Mol. Biol. Cell* **13**, 238-50.

Tiruppathi, C., Song, W., Bergenfeldt, M., Sass, P. and Malik, A. B. (1997). Gp60 activation mediates albumin transcytosis in endothelial cells by tyrosine kinase-dependent pathway. *J. Biol. Chem.* **272**, 25968-75.

Torgersen, M. L., Skretting, G., van Deurs, B. and Sandvig, K. (2001). Internalization of cholera toxin by different endocytic mechanisms. *J. Cell Sci.* **114**, 3737-47.

Tran, D., Carpentier, J. L., Sawano, F., Gorden, P. and Orci, L. (1987). Ligands internalized through coated or noncoated invaginations follow a common intracellular pathway. *Proc. Natl. Acad. Sci. U.S.A.* **84**, 7957-61.

Uittenbogaard, A., Everson, W. V., Matveev, S. V. and Smart, E. J. (2002). Cholesteryl ester is transported from caveolae to internal membranes as part of a caveolin-annexin II lipid-protein complex. *J. Biol. Chem.* **277**, 4925-31.

van der Bliek, A. M., Redelmeier, T. E., Damke, H., Tisdale, E. J., Meyerowitz, E. M. and Schmid, S. L. (1993). Mutations in human dynamin block an intermediate stage in coated vesicle formation. *J. Cell Biol.* **122**, 553-63.

Wang, H.-J., Benlimame, N. and Nabi, I. R. (1997). The AMF-R tubule is a smooth ilimaquinone-sensitive subdomain of the endoplasmic reticulum. *J. Cell Sci.* **110**, 3043-3053.

Wang, H.-J., Guay, G., Pogan, L., Sauve, R. and Nabi, I. R. (2000). Calcium regulates the association between mitochondria and a smooth subdomain of the endoplasmic reticulum. *J. Cell Biol.* **150**, 1489-1498.

Wolf, A. A., Fujinaga, Y. and Lencer, W. I. (2002). Uncoupling of the cholera toxin-G(M1) ganglioside receptor complex from endocytosis, retrograde Golgi trafficking, and downstream signal transduction by depletion of membrane cholesterol. *J. Biol. Chem.* **277**, 16249-56.

Yamada, E. (1955). The fine structure of the gall bladder epithelium of the mouse. *J. Biophys. Biochem. Cytol.* **1**, 445-448.

Zhang, W., Razani, B., Altschuler, Y., Bouzahzah, B., Mostov, K. E., Pestell, R. G. and Lisanti, M. P. (2000). Caveolin-1 inhibits epidermal growth factor-stimulated lamellipod extension and cell migration in metastatic mammary adenocarcinoma cells (MTLn3). Transformation suppressor effects of adenovirus-mediated gene delivery of caveolin-1. *J. Biol. Chem.* **275**, 20717-25.

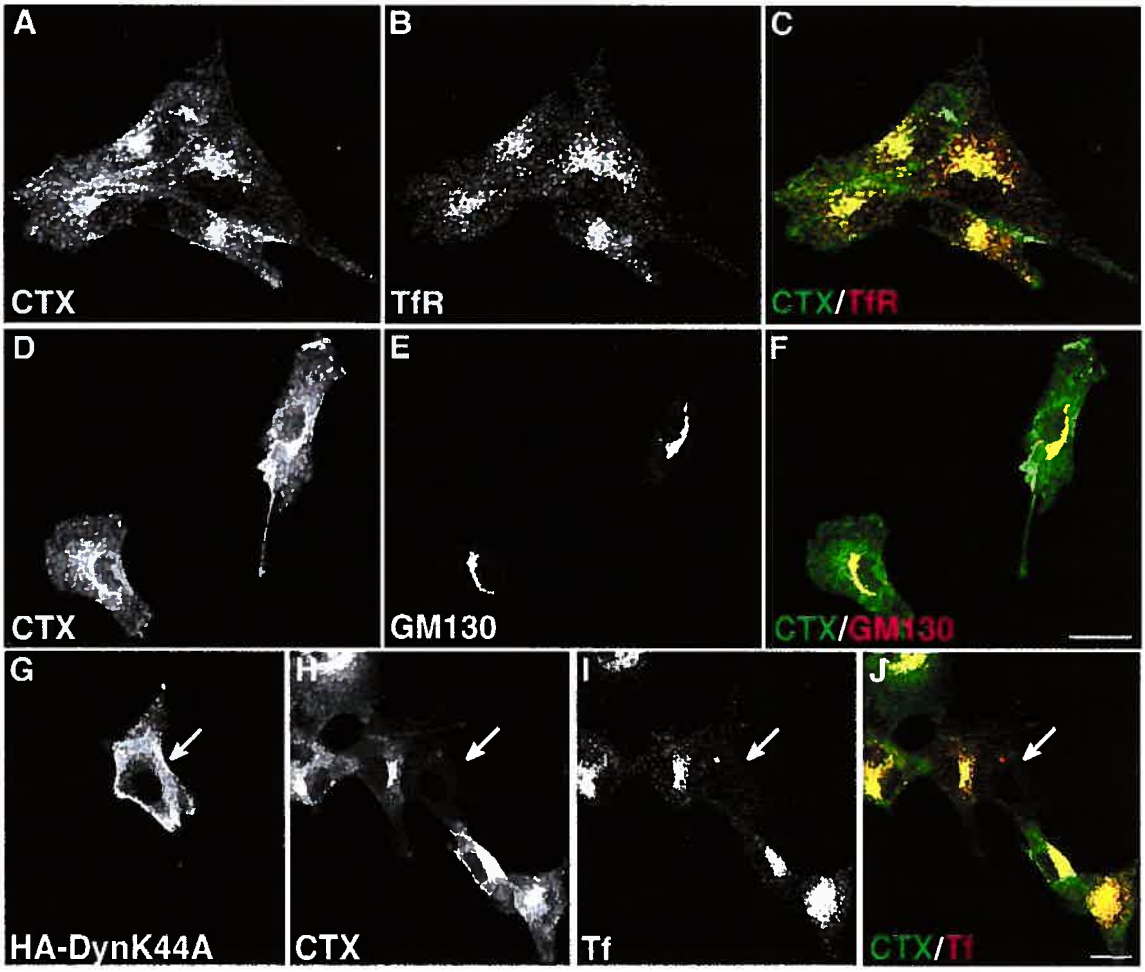


Fig. 1. DynK44A inhibits CTX internalization into endosomes and the Golgi apparatus. FITC-CTX internalized for 30 minutes at 37°C (A,D) is localized to both endosomes labeled for TfR (B) and the Golgi apparatus labeled for GM130 (E). The merged confocal images present FITC-CTX in green (C,F) and TfR (C) or GM130 (F) in red and colocalization in yellow. Alternatively, NIH-3T3 cells were infected with both the tTA and dynK44A adenoviruses (G-J), and after 36 hours, the cells were coincubated with FITC-CTX (H) and Rh-Tf (I) for 30 minutes at 37°C prior to fixation. HA-tagged dynK44A-infected cells (arrows) were identified by postfixation labeling with anti-HA antibodies followed by Alexa647 anti-mouse secondary antibodies (G). The merged confocal image presents FITC-CTX in green, Rh-Tf in red and colocalization in yellow (J). Dramatic reduction in CTX and Rh-Tf internalization was observed in the dynK44A expressing cell. Bar, 20 μm.

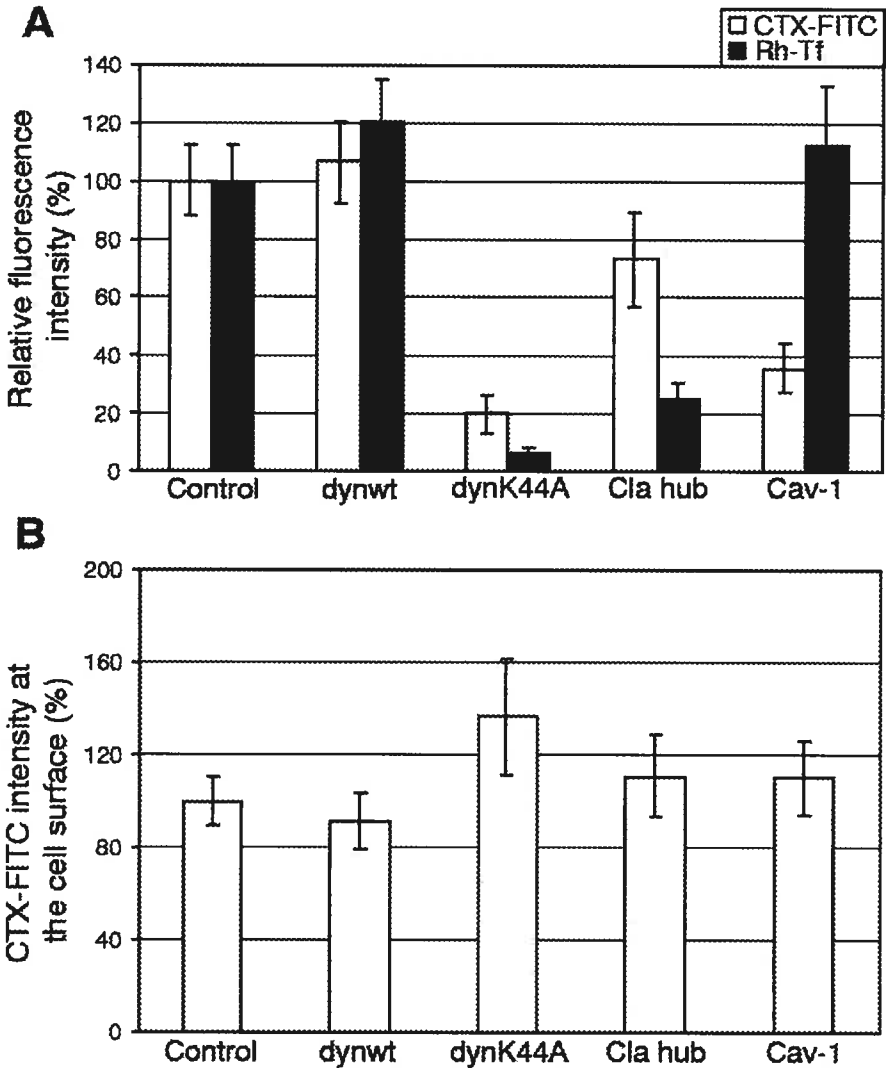


Fig. 2. Caveolae mediate CTX endocytosis. FITC-CTX (empty bars) and Rh-Tf (filled bars) were endocytosed for 30 minutes at 37°C in cells infected with wild-type dynamin, dynK44A, clathrin hub and caveolin-1 adenoviruses. Endocytosis into the perinuclear region was quantified in uninfected cells and in infected cells identified (as indicated) by postfixation labeling for the appropriate epitope marker. The degree of endocytosis is presented as the percentage of fluorescence intensity relative to uninfected control cells (A). Cell -surface FITC-CTX binding at 4°C was quantified in the adenovirus-infected cells and is presented as the percentage of fluorescence intensity relative to uninfected control cells (B). The data represent the average of three different experiments (±s.e.m.). The ability of dynK44A, but not the clathrin hub, to inhibit CTX endocytosis together with its reduction in caveolin-1 infected cells demonstrates the existence of a caveolae-mediated CTX endocytic pathway.

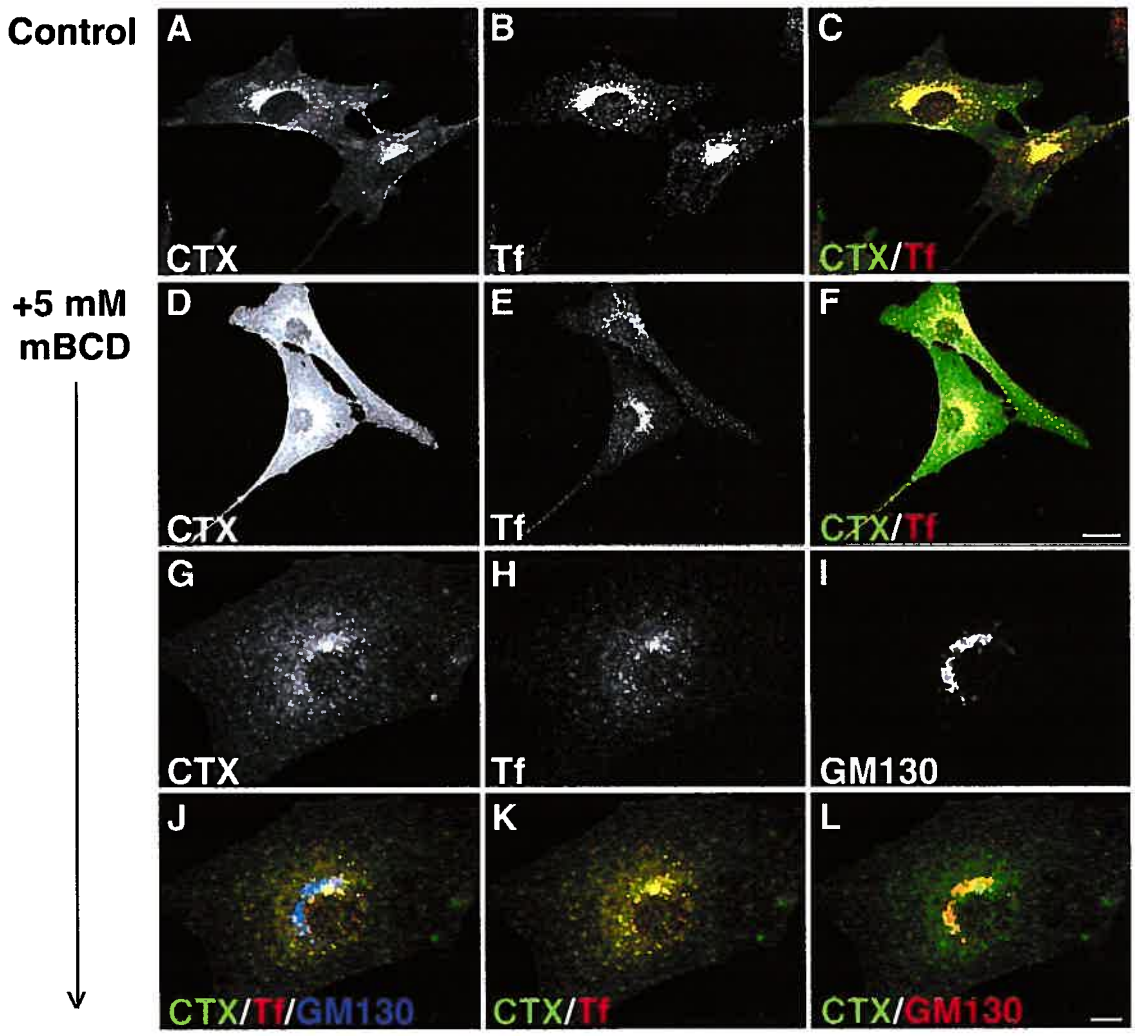


Fig. 3. MßCD blocks CTX delivery to the Golgi but not to endosomes. NIH-3T3 cells were either left untreated (A-C) or pretreated with 5 mM of mßCD (D-L) for 30 minutes then pulse labeled with FITC-CTX (A,D,G) and Rh-Tf (B,E,H) for 30 minutes at 37°C. Images presenting FITC-CTX internalization in the absence or presence of mßCD (A,D) were obtained using the same acquisition parameters. A smaller pinhole (0.6 Airy units) and increased zoom (G-L) were used to assess the overlap between internalized FITC-CTX (G), Rh-Tf-labeled endosomes (H) and the GM130-labeled Golgi apparatus (I). Merged confocal images present FITC-CTX in green and either Rh-Tf in red (C,F,K) or GM130 in red (L) and colocalization in yellow. A triple merge shows FITC-CTX in green, Rh-Tf in red and GM130 in blue (J). In mßCD-treated cells, cell-surface FITC-CTX labeling is significantly increased, and CTX is still internalized with transferrin to endosomes but not to the Golgi. Bars: A-F in F, 20 μm; G-L in L, 8 μm.

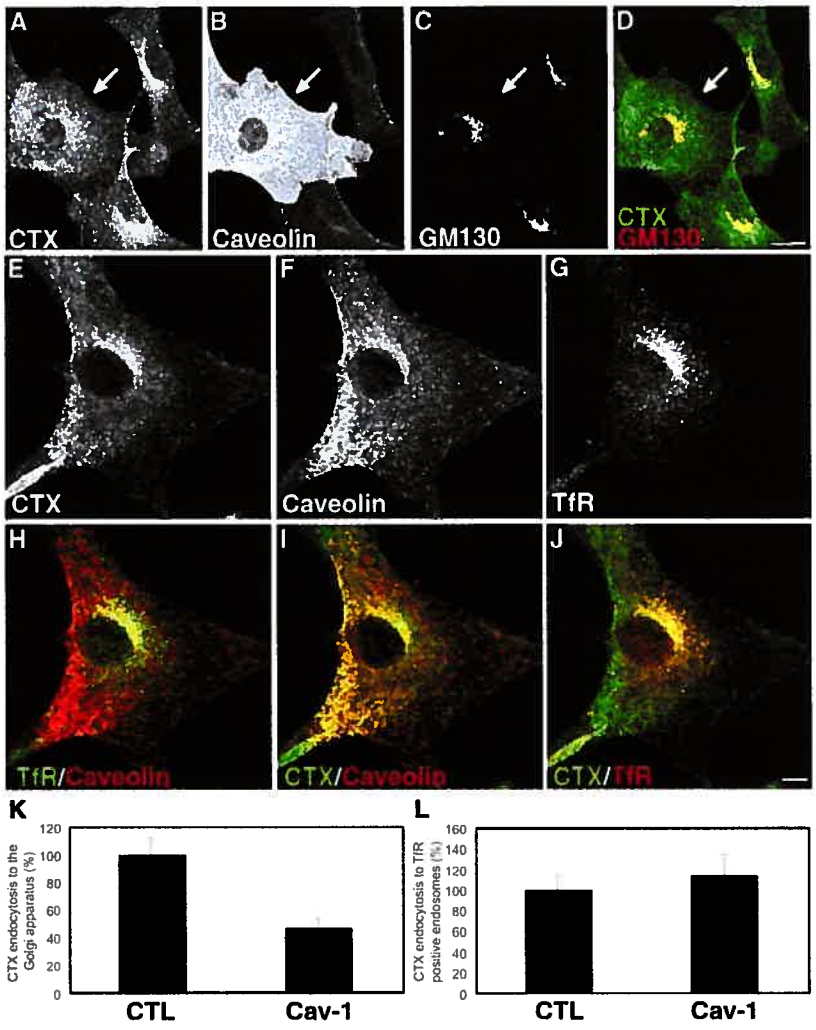


Fig. 4. Caveolin-1 overexpression negatively regulates CTX internalization to the Golgi apparatus but not to endosomes. NIH-3T3 cells were infected with the tTA and caveolin-1 adenoviruses and after 36 hours pulse labeled with FITC-CTX (A) for 30 minutes at 37°C prior to fixation. Cells were then triple labeled with anti-caveolin antibodies and Alexa 568 anti-rabbit antibodies (B) and anti-GM130 and Alexa 647 anti-mouse antibodies (C). The infected cell is indicated by an arrow, and the merged confocal image (FITC-CTX in green and GM130 in red) shows reduced FITC-CTX internalization to the Golgi in the caveolin-1-infected cell (D). Increased zoom of a caveolin-1-overexpressing cell (E-J) shows internalized FITC-CTX (E) and postfixation caveolin (F) and TfR (G) labeling. Merged confocal images present TfR in green and caveolin in red (H) and FITC-CTX in green and either caveolin (I) or TfR (J) in red. Quantification by mask overlay of FITC-CTX internalization to the GM130-positive Golgi (K) or to TfR-positive endosomes (L) shows that in cells overexpressing caveolin-1, FITC-CTX delivery to the Golgi apparatus is reduced. Bars: A-D in D, 20 μm; E-J in J, 8 μm.

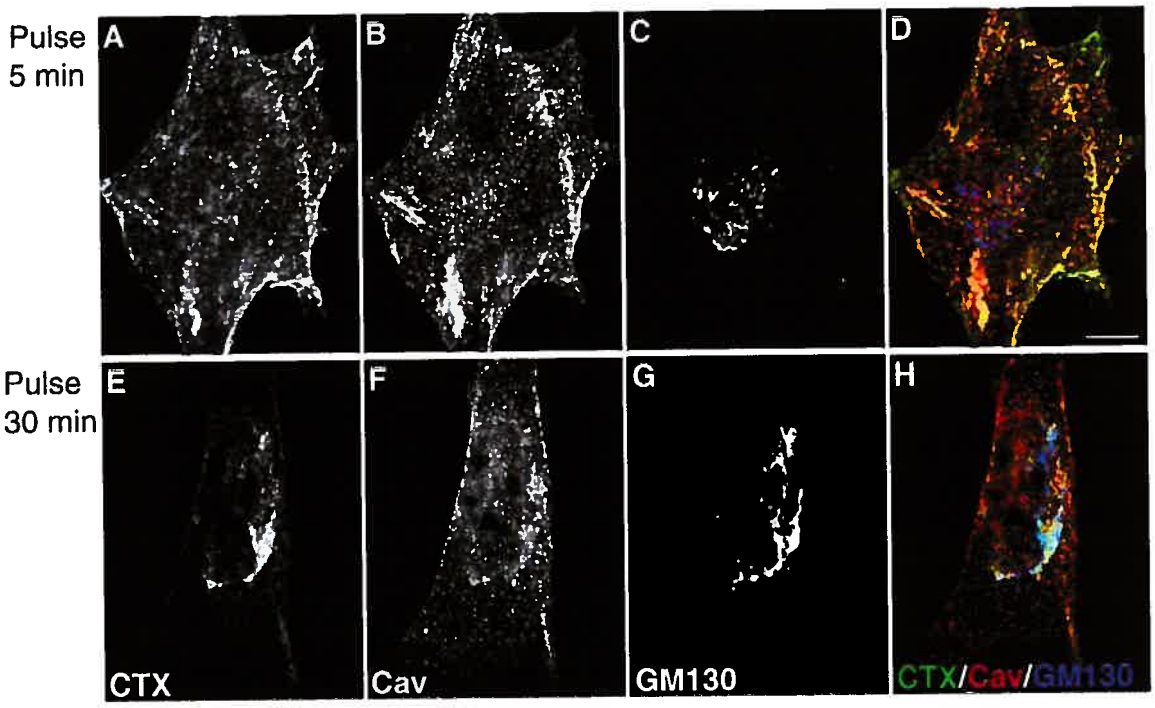


Fig. 5. CTX endocytosis in clathrin-hub-expressing cells. NIH-3T3 cells infected for 36 hours with the tTA and clathrin hub adenoviruses were incubated at 37°C with FITC-CTX for 5 (A-D) or 30 minutes (E-H). The distribution of FITC-CTX was visualized directly (A,E) and post-fixation labeling with anti-caveolin (B,F) and anti-GM130 (C,G) antibodies revealed with the appropriate Alexa-568- and Alexa-647-conjugated secondary antibodies, respectively. Merged confocal images (D,H) present FITC-CTX in green, caveolin in red and GM130 in blue. After five minutes of endocytosis, CTX is primarily associated with caveolae and with time accumulates in the perinuclear region, where, after 30 minutes, it colocalizes extensively with the Golgi apparatus. Bar, 8 μm.

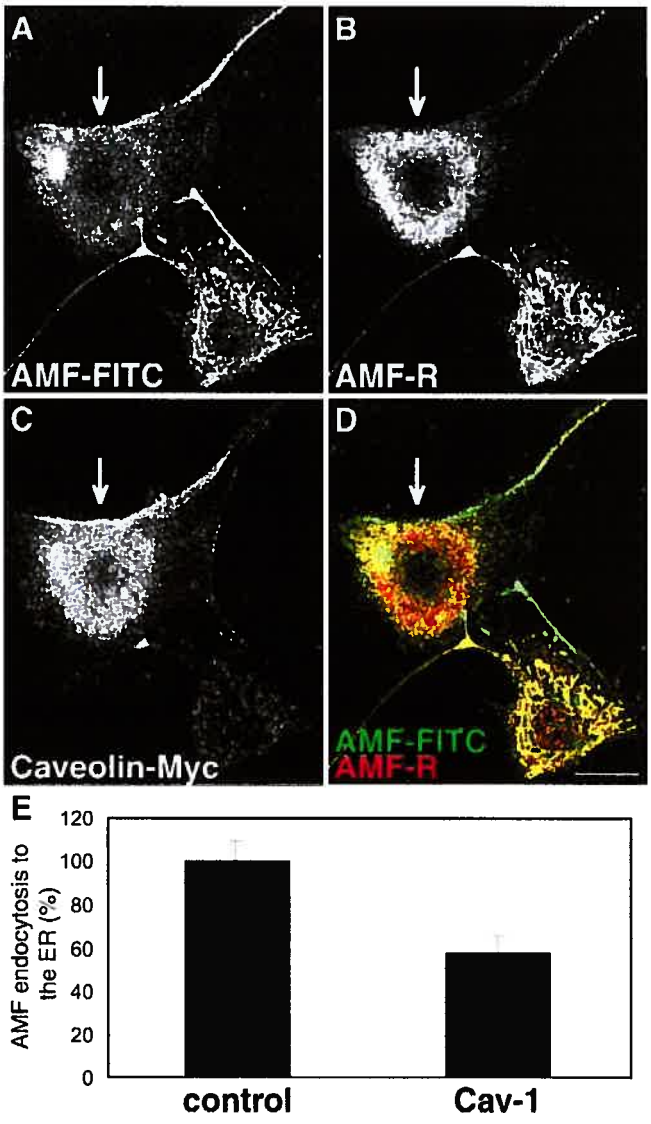


Fig. 6. Caveolin-1 overexpression reduces delivery of AMF-FITC to the smooth ER. NIH-3T3 cells were infected with the tTA and caveolin-1 adenoviruses and after 36 hours pulse labeled with AMF-FITC for 60 minutes at 37°C prior to fixation. AMF-FITC was revealed with rabbit anti-FITC followed by Alexa 488 anti-rabbit antibodies (A) and the smooth ER labeled with anti-AMF-R mAb followed by Alexa 647-anti-rat IgM antibodies (B). The caveolin-1-overexpressing cell (arrow) was detected with anti-c-Myc antibodies followed by Texas-Red anti-mouse antibodies (C). The merged confocal image presents AMF-FITC in green, the AMF-R-labeled smooth ER in red and colocalization in yellow (D). Quantification of AMF endocytosis to AMF-R-positive smooth ER tubules (E) shows that caveolin-1-expressing cells exhibit a significant decrease in the caveolae-mediated endocytosis of AMF. Bar, 20 μm.

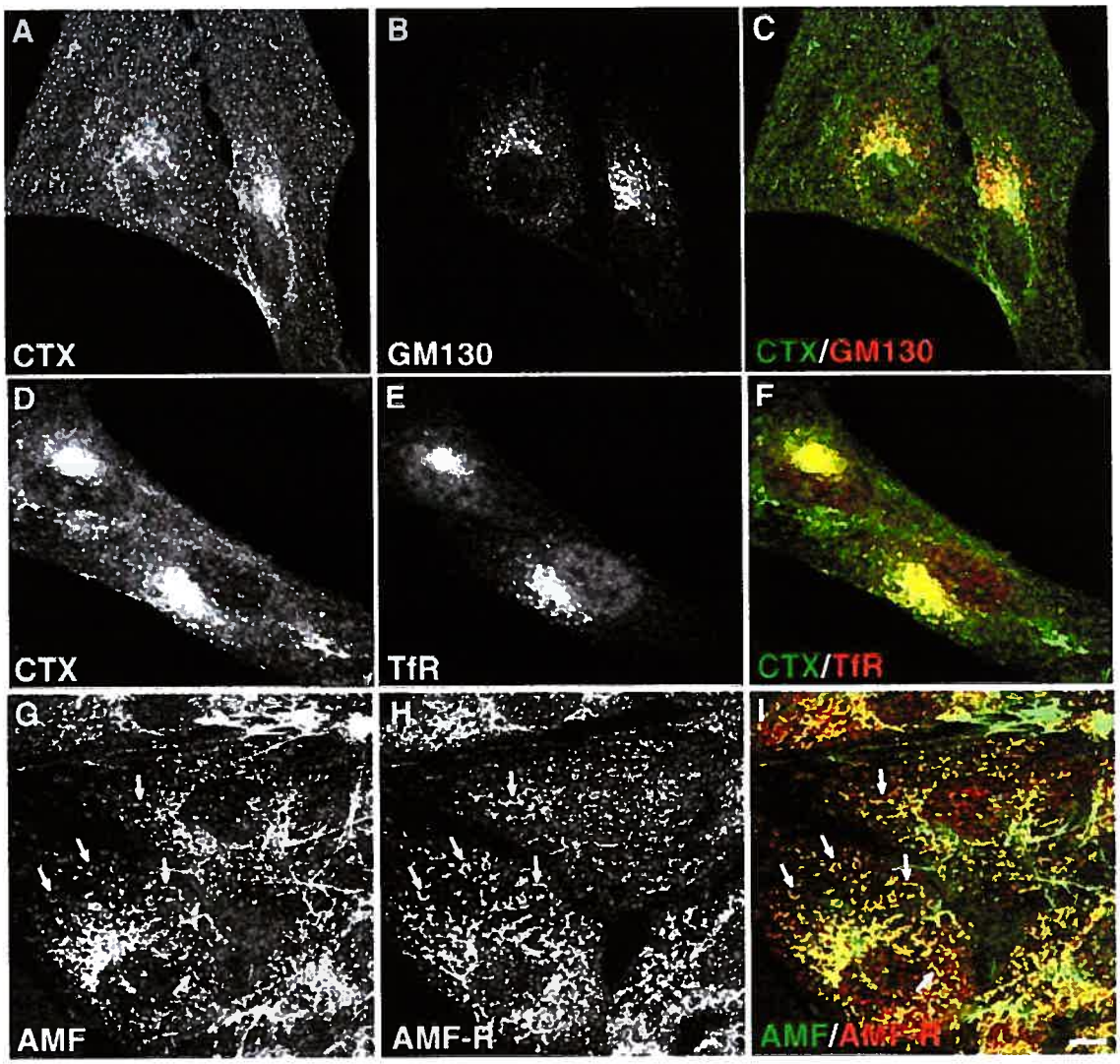


Fig. 7. BFA treatment inhibits the caveolae-mediated endocytosis of CTX to the Golgi apparatus but not of AMF to the smooth ER. NIH-3T3 cells were pretreated with 10 µg/ml BFA for 30 minutes at 37°C and then incubated in the presence of BFA with 5 µg/ml FITC-CTX for 30 minutes at 37°C (A-F) or with 50 µg/ml AMF-FITC for 60 minutes at 37°C (G-I). Cells incubated with FITC-CTX were labeled with either anti-GM130 (B) or anti-TfR (E) antibodies and merged confocal images present FITC-CTX in green and GM130 (C) or TfR (F) in red and colocalization in yellow. Internalized AMF-FITC was revealed with rabbit anti-FITC (G) and AMF-R tubules with anti-AMF-R mAb (H) followed by the appropriate secondary antibodies. The merged confocal image (I) presents the AMF in green and AMF-R in red and colocalization in yellow. In the presence of BFA, CTX is delivered to the endosomes but not to Golgi fragments, whereas AMF is still delivered to AMF-R-labeled smooth ER (arrows). Bar, 8 µm.

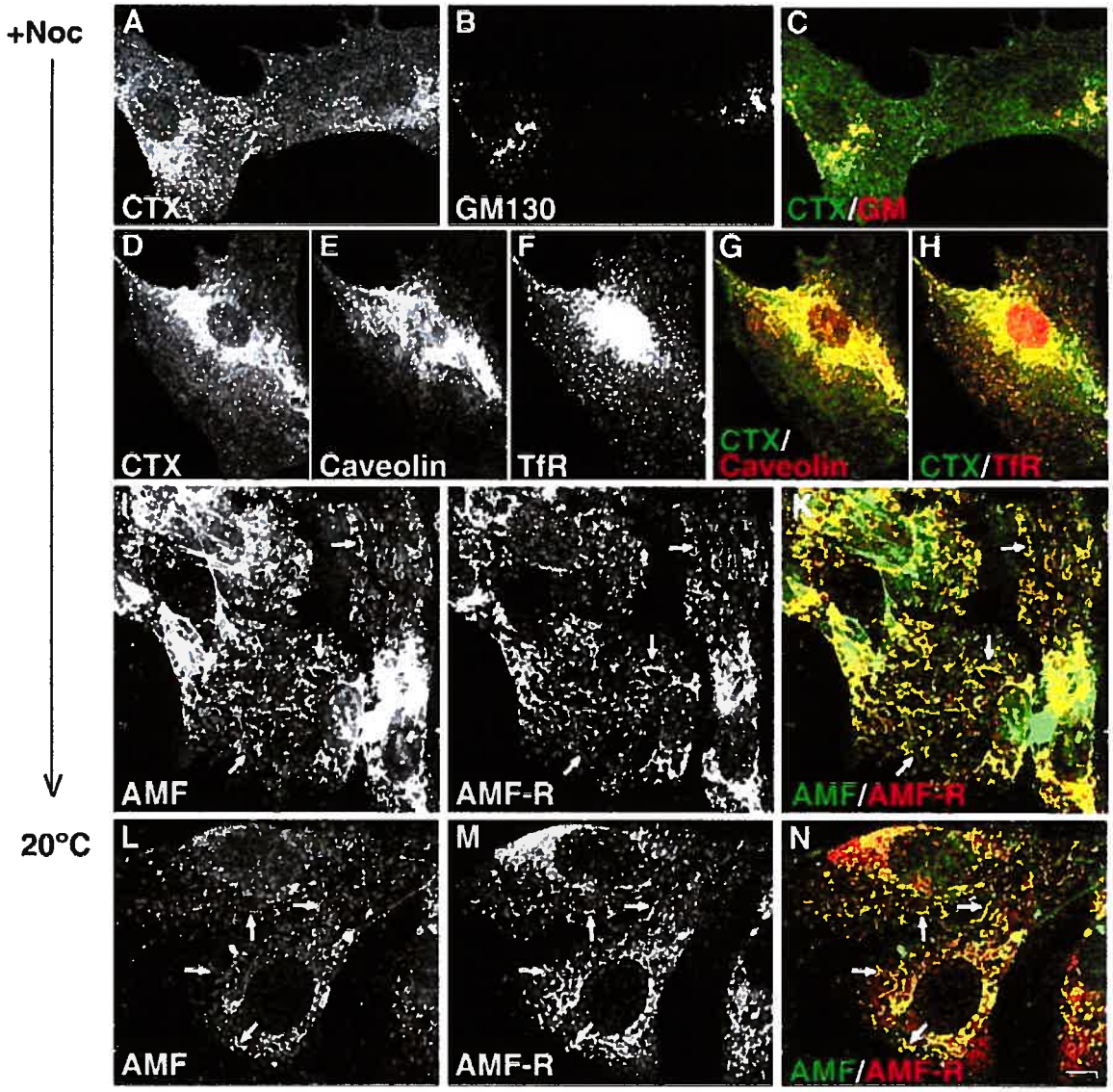


Fig. 8. Nocodazole treatment and a 20°C temperature block inhibit the caveolae-mediated endocytosis of CTX to the Golgi but not of AMF to the smooth ER. NIH-3T3 fibroblasts pretreated with 10 µM nocodazole at 37°C (A-K) were pulse labeled with either 5 µg/ml FITC-CTX for 30 minutes at 37°C (A-H) or with 50 µg/ml AMF-FITC for 60 minutes at 37°C (I-K) in the presence of nocodazole. Alternatively, cells were incubated with 50 µg/ml AMF-FITC for 60 minutes at 20°C (L-N). Overlap of endocytosed FITC-CTX (A,D) with anti-GM130 (B), anti-caveolin (E) or anti-TfR (F) labeling and of AMF-FITC (I, L) with anti-AMF-R (J, M) labeling was determined. The merged confocal images (C,G,H,K,N) present the indicated labels in red and green and colocalization in yellow. In the presence of nocodazole FITC-CTX is not delivered to the fragmented Golgi (A-C) but remains associated with caveolin and TfR-positive endosomes (D-H). Neither nocodazole treatment (I-K) nor a 20°C temperature block (L-N) prevent AMF delivery to the smooth ER (arrows). Bar, 8 µm.

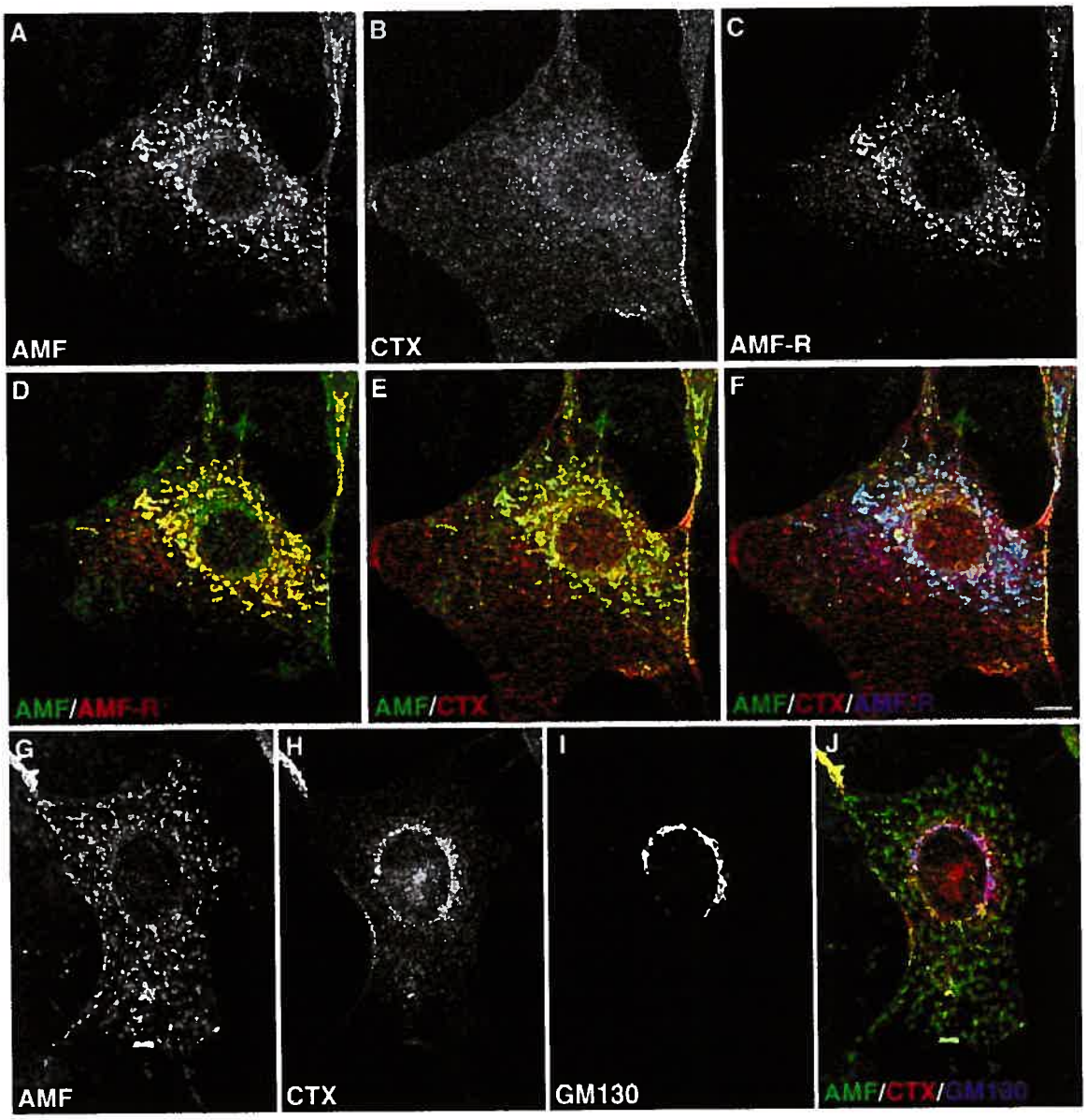


Fig. 9. AMF and CTX do not cointernalize. NIH-3T3 cells were coincubated with 50 µg/ml of AMF-FITC and 5 µg/ml of Alexa 594-CTX at 37°C for 5 minutes (A-F) or 30 minutes (G-J) prior to fixation with precooled methanol/acetone. The distributions of AMF-FITC, revealed with rabbit anti-FITC followed by Alexa 488 anti-rabbit antibodies (A and G), and of Alexa-594–CTX (B, H) were compared after five minutes with the smooth ER labeled with anti-AMF-R mAb and Alexa 647 anti-rat IgM antibodies (C) or at 30 minutes with the Golgi apparatus labeled with anti-GM130 mAb and Alexa 647 anti-mouse antibodies (I). Merged confocal images present AMF-FITC in green and either AMF-R (D) or Alexa 594-CTX (E) in red. Triple merges show AMF-FITC in green, Alexa 594-CTX in red and either AMF-R (F) or GM130 (J) in blue. Bar, 8 µm.

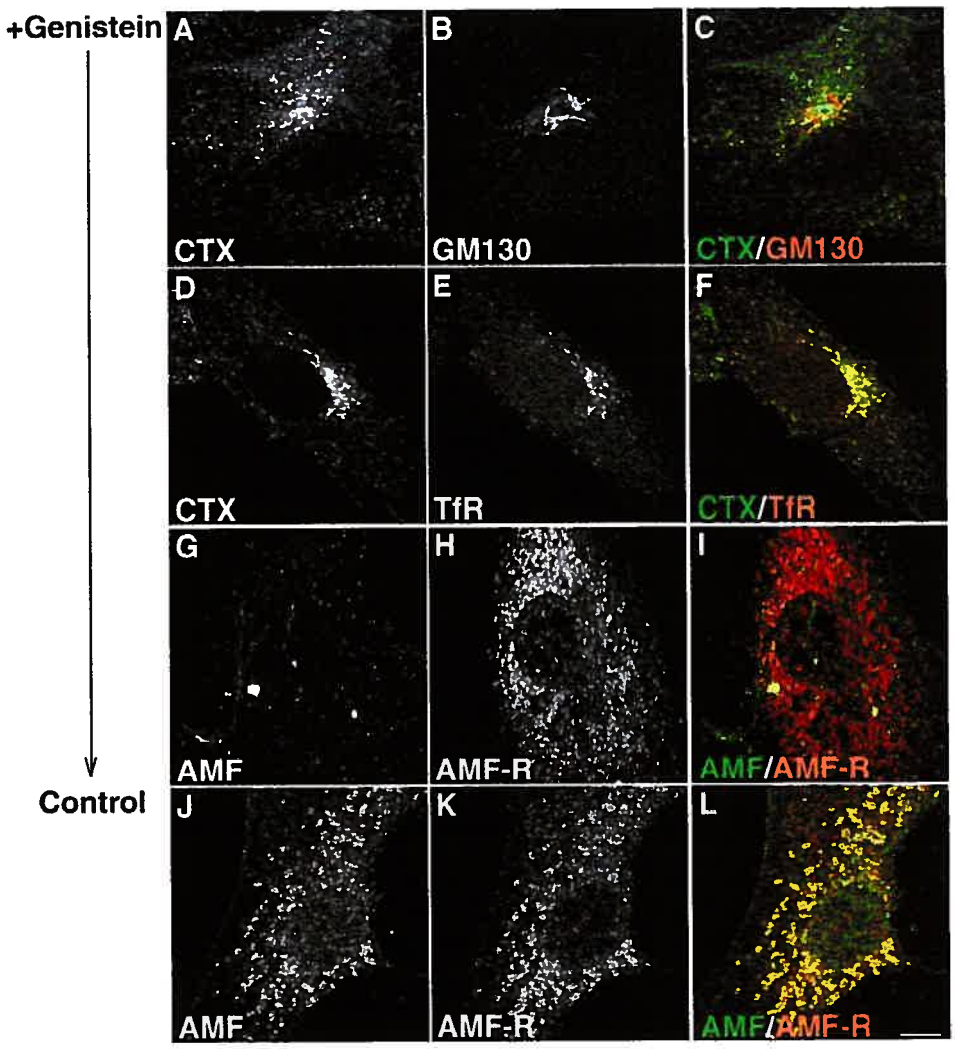


Fig. 10. Targeting of AMF to the ER and CTX to the Golgi apparatus is tyrosine kinase dependent. NIH-3T3 cells were pretreated with genistein (100 µg/ml) for 30 minutes during ligand incubation (A-I) or left untreated (J-L). Cells were pulse labeled with FITC-CTX for 30 minutes at 37°C (A,D) and labeled with monoclonal anti-GM130 (B) or anti-TfR antibodies (F) following by Alexa 568 anti-mouse secondary antibody. The merged confocal images present FITC-CTX in green and either GM130 (C) or TfR (F) in red and colocalization in yellow (C,F). Genistein selectively inhibits CTX delivery to the Golgi but not to TfR-positive endosomes. Alternatively, NIH-3T3 cells were pulse labeled with AMF-FITC for 5 minutes at 37°C. AMF-FITC was revealed with rabbit anti-FITC followed by Alexa 488 anti-rabbit antibodies (G,J) and the smooth ER labeled with anti-AMF-R followed by rhodamine-red-X anti-rat IgM antibodies (H, K). The merged confocal images present AMF-FITC in green and AMF-R in red and colocalization in yellow (I,L). The image of AMF endocytosis in the presence of genistein (G) was acquired at the same intensity level as the control in the absence of genistein (J), clearly demonstrating that genistein inhibits AMF delivery to the smooth ER. Bar, 8 µm.

III. DISCUSSION

5. L'AMF EST ENDOCYTÉ PAR DEUX VOIES DISTINCTES

L'AMF est une cytokine qui possède plusieurs fonctions et la régulation de son expression est très importante dans divers processus cellulaire. Nous avons montré que suite à la liaison de l'AMF à son récepteur, il est internalisé vers différents compartiments cellulaires. Dans les prochaines sections, nous allons discuter des différentes voies d'endocytose de l'AMF et les fonctions potentiels de chaque voie d'endocytose.

5.1 La localisation cellulaire de l'AMF-R

L'AMF-R est localisé au niveau d'un sous-compartiment du REL et il est un marqueur spécifique du REL (Benlimame et al., 1995; Wang et al., 1997; Wang et al., 2000). Une analyse par microscopie électronique du marquage de l'AMF-R en post-enrobage montre que l'AMF-R est majoritairement localisé au niveau du REL dans les cellules NIH-3T3, Hela et MDCK (Benlimame et al., 1995; Benlimame et al., 1998). Dans les cellules MDCK, une faible proportion de l'AMF-R est également détectée au niveau du RER ou dans le REL qui est en continuité avec le RER (Benlimame et al., 1995; Wang et al., 1997). Nous avions également observé par microscopie électronique que dans les cellules NIH-3T3 transformées avec l'oncogène H-ras (NIH-ras) ou v-abl (NIH-abl), une

augmentation de la distribution de l'AMF-R au niveau du RER en comparaison avec les cellules NIH-3T3 non-transformées (Le et al., 2002).

Des analyses quantitatives par microscopie électronique devront être faites afin de déterminer si la localisation de l'AMF-R au niveau du RER est significative dans les cellules NIH-3T3 transformées. Il était difficile de faire des études de colocalisation avec d'autres marqueurs du RE par microscopie à fluorescence car les cellules NIH-3T3 transformées ont de la difficulté à adhérer au pétri de culture et elles poussent les unes sur les autres. Il est probable que la distribution de l'AMF-R dans le REL ou dans le RER varie selon la lignée cellulaire, ou peut-être suite à une transformation cellulaire.

Une étude quantitative de la distribution de l'AMF-R a montré que dans les cellules NIH-3T3, 61% de l'AMF-R est localisé dans le RE, alors que 23% de l'AMF-R est localisé à la surface cellulaire de ces cellules. À la surface des cellules NIH-3T3, 13% de l'AMF-R est localisé au niveau des vésicules plasmalemmales lisses qui sont morphologiquement similaires aux cavéoles (Benlimame et al., 1998). La faible quantité (5%) d'AMF-R qui se trouve dans les cavéoles peut être due à la rapidité de l'endocytose de l'AMF-R ou de l'instabilité des cavéoles (Benlimame et al., 1998). Des images prises en microscopie confocale ou en microscopie électronique montrent une colocalisation partielle de l'AMF-R en surface avec la cavéoline et confirment la localisation de l'AMF-R dans les cavéoles (Benlimame et al., 1995; Le et al.,

2002). Des fractions insolubles au Triton-X-100 ont été isolées à partir des cellules NIH-3T3 et MDCK. Ces fractions sont enrichies par la cavéoline, mais l'AMF-R n'était pas détecté dans ces fractions. Même après plusieurs essais, l'AMF-R demeurait indétectable dans les fractions insolubles au Triton X-100 (résultats non-publiés).

L'AMF-R est donc localisé à la fois dans les cavéoles et à l'intérieur des cellules au niveau d'un sous-compartiment du REL, les tubules AMF-R, aussi bien que dans le RER. La double localisation de l'AMF-R suggère un recyclage entre ces deux compartiments. Le virus SV40 est également localisé au niveau des cavéoles et du REL et il a été montré que les cavéoles effectuent l'internalisation de SV40 vers le REL (Kartenbeck et al., 1989).

Les récepteurs muscariniques, de l'angiotensine II, de la bradykinin B2, de ß-adrénergique, de la cholecystokinin et de l'endothéline sont séquestrés dans les cavéoles. La séquestration de ces récepteurs dans ce compartiment leurs permettrait d'être désensibilisés suivant la liaison avec un agoniste (Chun et al., 1994; Roettger et al., 1995; Haasemann et al., 1998; Ishizaka et al., 1998; Dessy et al., 2000; Matveev and Smart, 2002). Les récepteurs EGF et PDGF sont localisés au niveau des radeaux lipidiques lorsqu'ils ne sont pas stimulés et phosphorylés. Cependant, au cours d'une longue exposition du ligand (60 minutes), les récepteurs sont recrutés et séquestrés dans les cavéoles afin d'empêcher d'autres liaisons du ligand avec son récepteur. De cette manière,

les cavéoles permettent l'inactivation de ces récepteurs. En fait, il a été suggéré que la liaison des récepteurs EGF et PDGF avec la cavéoline puisse être responsable de leur séquestration dans les cavéoles. Dans les cellules où il y a peu de cavéoline, ces récepteurs continuent d'être phosphorylés et d'être actifs (Matveev and Smart, 2002). L'implication de l'AMF-R au niveau des cavéoles reste à être déterminée.

5.2 La spécificité de l'AMF conjugué

Pour les études d'endocytose de l'AMF, nous avons utilisé la PGI, un homologue de l'AMF (Watanabe et al., 1996), qui est disponible commercialement. Afin de suivre l'internalisation de l'AMF, la PGI a été conjugué avec la biotine. L'AMF biotinylé (AMFb) peut être révélé par microscopie à fluorescence avec un marquage avec la streptavidine conjuguée à un fluorochrome ou par microscopie électronique avec la streptavidine conjuguée à une particule d'or.

Par immunobuvardage, l'AMFb est révélé avec la streptavidine-HRP et montre une seule bande. De plus, par microscopie confocale, un double marquage de l'AMFb et d'un anticorps anti-AMF-R à 4°C montre une colocalisation de l'AMFb avec l'anticorps anti-AMF-R. À certains endroits, nous avons observé que le marquage de l'AMFb se distingue du marquage de l'anti-AMF-R (Benlimame et al., 1998). Cette distinction peut refléter le fait que l'AMFb

entre en compétition avec l'anticorps anti-AMF-R pour la liaison avec le récepteur (Nabi et al., 1990). Ces études montrent que, tout comme l'anticorps anti-AMF-R, l'AMFb est bien reconnu par son récepteur en surface. Pour faciliter la détection de l'AMF endocyté, nous avons aussi conjugué la PGI directement avec des molécules de FITC, ce conjugué nous permet d'amplifier le signal avec un anticorps anti-FITC.

Une incubation des fibroblastes NIH-3T3 avec l'AMFb à 37°C pendant différentes périodes de temps montre une accumulation de l'AMFb dans des structures vésiculaires et tubulaires. Par microscopie confocale, les structures tubulaires colocalisent avec les tubules du REL qui sont marqués par l'anticorps anti-AMF-R (Benlimame et al., 1998; Le et al., 2000). Par microscopie électronique, ces tubules correspondent morphologiquement au REL (Le et al., 2002). L'internalisation de l'AMFb vers les tubules AMF-R montre une spécificité de l'AMFb pour l'AMF-R. De plus, l'incubation des cellules NIH-3T3 avec l'AMFb et de dix fois plus d'AMF non-conjugué a pour effet d'inhiber l'internalisation de l'AMFb (Le et al., 2000). Ces résultats indiquent que l'endocytose de l'AMF vers les structures vésiculaires et les tubules AMF-R est spécifiquement médiée par son récepteur AMF-R.

5.3 Les cavéoles/radeaux lipidiques médient l'endocytose de l'AMF vers le RE

Nous avons démontré dans les cellules NIH-3T3 que l'AMF est endocyté via les cavéoles/radeaux lipidiques vers le RE. Cette voie d'endocytose est bloquée en présence de la mβCD (Benlimame et al., 1998; Le et al., 2000; Le et al., 2002). Il faut noter qu'avec une forte concentration de mβCD (>10 mM), l'endocytose dépendante des vésicules de clathrine est aussi affectée (Rodal et al., 1999; Subtil et al., 1999). Dans les cellules NIH-3T3, nous avons déterminé qu'à une concentration de 5mM, la mβCD inhibe sélectivement l'endocytose dépendante des cavéoles/radeaux lipidiques et non pas l'endocytose médiée par les vésicules de clathrine (Le et al., 2000; Le et al., 2002).

La dynamine est impliquée dans la fission des vésicules de clathrine et des cavéoles et l'utilisation d'un mutant dominant négatif de la dynamine tel que la dynK44A bloque le détachement de ces vésicules de la membrane plasmique (Henley et al., 1999; Hinshaw, 2000). Nous avons également montré que l'endocytose de l'AMF vers le RE est bloquée par la dynK44A et elle est négativement régulée par la surexpression de la cavéoline-1 (Le et al., 2002). Ces études indiquent que l'internalisation de l'AMF vers le RE est effectuée par les cavéoles/radeaux lipidiques. Cette voie d'endocytose est donc définie comme dépendante de la dynamine, sensible au changement du niveau de cholestérol et négativement régulée par la cavéoline-1 (voir Figure 2).

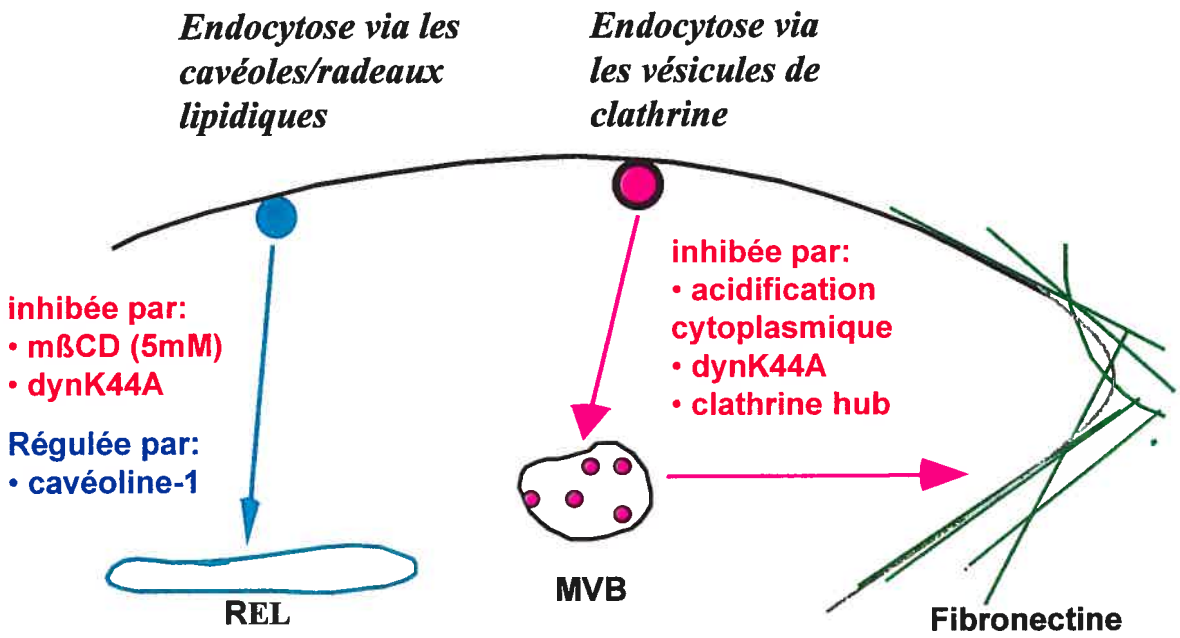


Figure 2. La double internalisation de l'AMF/AMF-R. Le complexe AMF/AMF-R est endocyté via: 1) les vésicules de clathrine vers les MVBs et il est ensuite recyclé vers les fibrilles de fibronectine. Cette voie d'endocytose est inhibée par une acidification cytoplasmique, par l'expression de la dynK44A ou de la clathrine hub; 2) les cavéoles/radeaux lipidiques vers le REL. Cette voie d'endocytose est bloquée en présence de la mßCD ou avec l'expression de la dynK44A et elle est négativement régulée par l'expression de la cavéoline-1.

5.4 L'endocytose de l'AMF vers les MVBs et son recyclage vers la fibronectine

Nous avons aussi montré que le complexe AMF/AMF-R est endocyté vers des structures vésiculaires qui ont été identifiées comme étant des corps multivésiculaires (MVBs) par microscopie électronique (Le et al., 2000). Les MVBs sont des endosomes qui contiennent des vésicules internes et qui sont l'intermédiaire entre les endosomes précoces et tardifs (Hopkins and Trowbridge, 1983; Dunn et al., 1986; Gruenberg et al., 1989). L'endocytose de l'AMF/AMF-R vers les MVBs est inhibée lors d'une acidification cytoplasmique et avec l'expression de la dynK44A. Cependant, en présence de 5mM de mβCD, l'endocytose de l'AMF vers les MVBs n'est pas bloquée. Donc ces études indiquent que les vésicules de clathrine sont responsable de l'internalisation de l'AMF vers les MVBs (Le et al., 2000; Le et al., 2002).

Nous avons également montré que suite à son endocytose vers les MVBs, l'AMF n'est pas transporté vers les lysosomes pour être dégradé. Il est plutôt recyclé vers les fibrilles à la surface cellulaire qui colocalisent avec la fibronectine, une protéine de la matrice extracellulaire. Un traitement des cellules avec mβCD n'a pas inhibé le recyclage de l'AMF vers les fibrilles de fibronectine, indiquant que l'endocytose de l'AMF vers le RE n'est pas impliquée dans le recyclage de l'AMF (Le et al., 2000). Une incubation des cellules NIH-3T3 avec l'AMF à 20°C pour bloquer le transport membranaire entre les

endosomes précoces et les endosomes tardifs (Dunn et al., 1980; Hopkins and Trowbridge, 1983; Griffiths et al., 1988) inhibe le recyclage de l'AMF (Le et al., 2000). Toutefois, une dépolymérisation des microtubules avec nocodazole pour bloquer le transport membranaire vers les endosomes précoces et les endosomes tardifs au niveau des MVBs (De Brabander et al., 1988; Gruenberg et al., 1989; Aniento et al., 1993) n'a pas empêché le recyclage de l'AMF (Le et al., 2000). Ces résultats montrent que les vésicules de clathrine médient l'endocytose de l'AMF vers les MVBs et à partir de ce compartiment l'AMF est recyclé vers les fibrilles de fibronectine (voir Figure 2).

5.5 Les potentiels fonctions de la double endocytose de l'AMF

Il a été suggéré que certains récepteurs couplés aux protéines G sont endocytés par différents mécanismes d'endocytose afin de cibler des organites intracellulaires distincts et aussi probablement pour différentes fonctions (Zhang et al., 1996). Par exemple, le récepteur de cholecystokinin est à la fois endocyté par les vésicules de clathrine et par les cavéoles. Les vésicules de clathrine médient l'internalisation du récepteur vers les endosomes et les lysosomes pour être dégradé. Alors que les cavéoles médient l'internalisation du récepteur vers un compartiment vésiculaire lisse adjacent à la membrane plasmique où le récepteur de cholecystokinin est rapidement recyclé à la surface membranaire (Roettger et al., 1995).

Une récente étude dans les lignées cellulaires fortement et faiblement métastasiques LMF-4 et HSC-3 a montré que les cellules LMF4 sont plus motiles que les cellules HSC-3. Une analyse de "scatchard" indique que les cellules LMF-4 et HSC-3 exprimaient deux types de récepteurs qui auraient différentes affinités pour l'AMF. Les cellules fortement métastasiques LMF-4 exprimeraient deux fois plus de récepteurs à faible affinité à sa surface comparées aux cellules faiblement métastasiques HSC-3 (Niinaka et al., 2002). La présence de deux types de récepteurs ou un changement de conformation de l'AMF-R qui affecterait son affinité pour l'AMF pourrait expliquer le fait que l'AMF soit internalisé par deux voies d'endocytose distinctes. Le rôle des différentes voies d'endocytose de l'AMF reste à déterminer. Voici quelques fonctions possibles de l'endocytose de l'AMF:

5.5.1 L'endocytose de l'AMF vers le RE

5.5.1.1 L'homéostasie calcique

Une pompe calcique à la membrane plasmique est essentielle dans la régulation du niveau de calcium libre dans le cytoplasme. Cette pompe calcique a été localisée dans les cavéoles. Il a également été rapporté qu'une protéine semblable au récepteur IP3 (IP3 receptor like protein) soit aussi concentrée dans les cavéoles. Les cavéoles pourraient donc être un appareil pour l'importation et l'exportation du calcium à partir du cytoplasme (Fujimoto et al.,

1992; Fujimoto, 1993). Il a aussi été montré que lors d'un traitement avec la thapsigargin pour enlever les réserves de calcium dans le RE, puis l'ajout de calcium dans le milieu extracellulaire, une concentration du calcium a été observée dans les cavéoles. Cette étude suggère que les molécules de signalisation qui contrôlent l'entrée du calcium sont concentrées dans les cavéoles (Isshiki et al., 2002). L'association des tubules AMF-R avec les mitochondries est dépendante du calcium cytoplasmique (Wang et al., 2000) et le RE est un lieu d'entreposage du calcium. Il est donc possible que l'internalisation de l'AMF vers le REL ait un rôle dans le transport des molécules impliquées dans la régulation du calcium.

5.5.1.2 Régulation de la motilité cellulaire

La migration des cellules est essentielle lors de l'invasion tumorale. Il a été proposé que l'exocytose des vésicules membranaires au côté guide de la cellule pourrait contribuer à l'apport de membrane pour l'extension des pseudopodes ou des lamellipodes au côté guide, ainsi que la création de force pour la locomotion des cellules (Bretscher, 1984; Singer and Kupfer, 1986; Hopkins et al., 1994). Il a été montré que suivant une incubation avec un anticorps anti-AMF-R à 20°C et d'une chasse de 60 minutes à 37°C, le complexe anti-AMF-R/AMF-R est internalisé puis il est ensuite redistribué vers le côté guide des fibroblastes A-31 (Nabi et al., 1992).

Les cellules MSV-MDCK-INV sont des cellules qui ont été isolées à partir des cellules MSV-MDCK (des cellules MDCK transformées avec le virus de sarcome de Moloney) qui ont traversées des filtres ayant des pores de 1 micron. Ces cellules sont fortement motiles et elles présentent de multiples pseudopodes (Le et al., 1998). Un marquage de l'AMF-R en immunofluorescence montre une accumulation de l'AMF-R à l'extrémité des pseudopodes (résultat non-publié). L'endocytose de l'AMF vers le REL et le recyclage de ce compartiment vers la membrane plasmique pourraient donc contribuer à un apport de membrane pour l'extension des pseudopodes ou des lamellipodes. Afin de vérifier cette hypothèse, il serait intéressant de faire des études in vivo de l'internalisation de l'AMF fluorescent ou des cellules qui expriment un AMF-R-GFP.

5.5.2 L'endocytose de l'AMF vers les MVBs

Les MVBs sont caractérisés par la présence de rab7 qui est le compartiment où les protéines sont triées pour être envoyées vers les lysosomes pour être dégradées (Mellman, 1996). Il a également été montré que TfR est limité à la membrane périphérique des MVBs et à partir de cet endroit, TfR peut être recyclé à la membrane plasmique. Par contre, la forme active de EGFR s'accumule dans les vésicules internes des MVBs (Futter and Hopkins, 1989; Hopkins et al., 1990; Futter et al., 1996). Lorsque tous les récepteurs qui sont destinés au recyclage de la membrane plasmique sont recyclés, les MVBs

fusionnent avec les lysosomes et le complexe EGF/EGF-R est rapidement dégradé dans les lysosomes (Futter et al., 1996; Mullock et al., 1998). Toutefois, une mutation de EGF-R qui cause une déficience de son activité tyrosine kinase, le complexe EGF/EGF-R n'est plus envoyé vers les vésicules internes pour éventuellement être dégradé par le lysosome. Mais, comme TfR, il reste à la périphérie des MVBs et il est recyclé à la membrane plasmique (Felder et al., 1990).

D'autres études ont montré que lorsque les réticulocytes deviennent des érythrocytes, ceux-ci vont secréter des vésicules membranaires, appelées exosomes, afin de libérer des protéines tels que TfR ou l'intégrine $\alpha 4\beta 1$ (Rieu et al., 2000). Dans les érythrocytes, les MVBs servent à la sécrétion des exosomes. Les exosomes sont des petites vésicules internes présentes dans les MVBs qui sont secrétées suite à la fusion des MVBs avec la membrane plasmique (Johnstone, 1992). Il a également été rapporté que par la sécrétion des exosomes, les cellules d'origine hématopoïétique libèrent des protéines qui sont impliquées dans la signalisation, l'adhésion cellulaire ou la présentation d'antigène (Denzer et al., 2000). Il est donc possible que le complexe AMF/AMF-R soit internalisé vers les MVBs où il y a un triage, puis il est recyclé vers la membrane plasmique afin de participer à la réorganisation de la matrice extracellulaire.

5.5.2.1 Le recyclage de l'AMF et son rôle dans la motilité cellulaire

L'AMF et son récepteur sont impliqués dans la motilité cellulaire et dans l'invasion des cellules tumorales (Silletti and Raz, 1996). Afin de déterminer si le recyclage de l'AMF vers les fibrilles de fibronectine a un effet sur la motilité cellulaire, les cellules NIH-3T3 ont été cultivées sur un substrat d'AMF, qui a pour effet d'attacher l'AMF-R au substrat et d'empêcher son internalisation. La culture des cellules sur un substrat d'AMF réduit la motilité des cellules NIH-3T3 et inhibe l'endocytose de l'AMF vers les MVBs ainsi que son recyclage vers les fibrilles de fibronectine. Des essais d'adhésion des cellules sur AMF montrent que cette réduction de la motilité n'est pas causée par une augmentation de l'adhésion des cellules au substrat (Le et al., 2000). Le recyclage de l'AMF/AMF-R est donc un élément limitant pour la motilité cellulaire.

Nous proposons que l'internalisation et le recyclage de l'AMF-R vers les sites de déposition de la fibronectine puissent être impliqués dans l'adhésion cellulaire et le remodelage de la matrice extracellulaire par les cellules tumorales motiles et invasives. Il a déjà été montré qu'à travers l'activation des intégrines β-1, l'AMF induit l'invasion des cellules de l'hépatome en stimulant l'adhésion et la motilité de ces cellules, ainsi que la sécrétion des métalloprotéases-2 (Torimura et al., 2001). Par conséquent le recyclage de l'AMF vers les fibrilles de fibronectine pourrait servir au transport des protéinases pour la dégradation de la matrice extracellulaire, un processus qui est important pour l'invasion des

cellules tumorales. Toutefois, une étude faite par Remacle et al., montrent que la forme active des métalloprotéinases de la matrice de type 1 (MT1-MMP) est endocytée par la voie de clathrine vers des compartiments endosomiales. Puis, les MT1-MMPs sont recyclés vers la membrane plasmique à partir des endosomes de recyclage qui sont marqués avec rab-4 (Remacle et al., 2003).

5.5.2.2 Le recyclage de l'AMF et son rôle dans le transport des intégrines

Les cellules tumorales doivent adhérer à une variété de protéines de la matrice extracellulaire afin d'envahir le tissu voisin (Akiyama et al., 1995). Les intégrines sont fondamentales pour médier l'adhésion des cellules aux protéines de la matrice (Hynes, 1992). La stimulation de l'AMF-R via sa liaison avec l'AMF ou avec l'anticorps anti-AMF-R augmente l'adhésion et l'étalement des cellules B16a (cellules des mélanomes murines) sur la fibronectine. De plus, l'activation de l'AMF-R stimule la translocation des intégrines αIIβ3 à la membrane plasmique (Timar et al., 1996). Le recyclage de l'AMF vers les sites de fibronectine pourrait donc servir à transporter les intégrines αIIβ3 au site d'adhésion avec la fibronectine.

Il a été récemment rapporté que l'adhésion à la fibronectine via l'intégrine α4β1 protège les cellules B de l'apoptose qui a été induite par la privation de sérum (Garcia-Gila et al., 2002).Une surexpression de l'AMF dans les cellules NIH-3T3 induit une résistance de ces cellules à l'apoptose également induite par

la privation de sérum (Tsutsumi et al., 2003b). Il reste à déterminer si les cellules NIH-3T3 qui surexpriment l'AMF recyclent plus d'AMF vers la fibronectine en comparaison avec les cellules NIH-3T3 contrôle.

6. LE RÔLE DE LA CAVÉOLINE DANS L'ENDOCYTOSE DES CAVÉOLES/ RADEAUX LIPIDIQUES

Les radeaux lipidiques sont définis comme des domaines enrichies en cholestérol et insolubles aux détergents. Bien que biochimiquement, les cavéoles pourraient être définis comme des radeaux lipidiques qui contiennent la cavéoline (Kurzchalia and Parton, 1999; Simons and Toomre, 2000) l'endocytose via les radeaux lipidiques et les cavéoles est sensible au cholestérol et est dépendante de l'activité GTPasique de la dynamine. Les cavéoles ne sont pas observées dans les cellules qui n'expriment pas la cavéoline et la réintroduction de la cavéoline-1 dans ces cellules induit de nouveau la formation des cavéoles (Fra et al., 1994; Fra et al., 1995; Engelman et al., 1997). Dans les sections qui suivent nous allons discuter de la nécessité de la cavéoline pour l'invagination et l'endocytose des cavéoles/radeaux lipidiques.

6.1 L'endocytose des cavéoles/radeaux lipidiques en absence de la cavéoline

Le niveau d'expression de la cavéoline et des cavéoles est réduit dans les cellules NIH-ras et absent dans les cellules NIH-abl comparé aux cellules NIH-3T3 non-transformées (Koleske et al., 1995; Le et al., 2002). Dans les cellules NIH-3T3 non-transformées, l'AMF est internalisé vers le REL (Le et al., 2000; Le et al., 2002). Dans les cellules NIH-3T3 transformées, malgré la réduction ou l'absence de la cavéoline et des cavéoles, l'AMF continue d'être endocyté vers les tubules du RE qui sont également marqués avec l'anticorps anti-AMF-R. Une quantification du taux d'AMF endocyté montre une augmentation de l'endocytose de l'AMF vers le RE dans les cellules transformées en comparaison avec les cellules NIH-3T3 contrôles (Le et al., 2002). De plus, autant dans les cellules NIH contrôles que dans les cellules NIH transformées, l'endocytose de l'AMF vers le RE est inhibée par la mβCD ou lors de l'expression de la dynK44A (Le et al., 2002).

Ces résultats suggèrent donc qu'en absence des cavéoles et de la cavéoline, les cavéoles/radeaux lipidiques effectuent l'endocytose de l'AMF vers le RE. Pour parfaire ces études de microscopie électronique et pour confirmer le rôle des cavéoles/radeaux lipidiques dans l'endocytose de l'AMF, nous pourrons effectuer des analyses biochimiques des fractions insolubles au Triton X-100 des cellules NIH-ras et NIH-abl qui expriment ou non la dynamine K44A. Ces

travaux vont nous permettre de déterminer qu'en présence de la dynamine K44A, l'AMF/AMF-R sera séquestré ou non dans les fractions insolubles au Triton X-100 qui correspondent aux cavéoles/radeaux lipidiques.

Il est possible que la cavéoline n'est pas nécessaire pour l'endocytose de l'AMF vers le RE et en absence de la cavéoline et des cavéoles/radeaux lipidiques endocytent l'AMF vers le RE de façon constitutive de sorte qu'une mesure du niveau d'expression de l'AMF-R en surface par FACS montre une diminution de la quantité d'AMF-R qui est exprimée dans les cellules NIH transformées comparée aux cellules non-transformées (Le et al., 2002). Ces résultats concordent avec le fait que les cellules fortement métastasiques, K1735-M1 et B16F10, ont moins d'AMF-R exprimée à la surface en comparaison avec les cellules faiblement métastasiques, K1735-C1-11 et B16-LR, respectivement (Watanabe et al., 1991b).

6.2 Les cavéoles/radeaux lipidiques s'invaginent indépendamment de la cavéoline

La surexpression de la dynK44A dans les cellules NIH-3T3 non-transformées montre une accumulation des cavéoles en surface. Dans les cellules NIH transformées, la dynK44A induit la formation des vésicules plasmalemmales lisses qui sont morphologiquement similaires aux cavéoles présentes dans les cellules NIH-3T3 contrôles et une réduction du niveau de

cholestérol en présence de la mβCD empêche la formation de ces vésicules (Le et al., 2002). Par conséquent, dans les cellules qui expriment peu ou pas de cavéoline, les cavéoles/radeaux lipidiques pourront s'invaginer, se détacher de la membrane pour former une vésicule endocytaire semblable aux cavéoles.

Les lymphocytes n'expriment pas la cavéoline et pourtant les protéines GPI se concentrent dans les vésicules morphologiquement équivalentes aux cavéoles avant d'être endocytées (Deckert et al., 1996). Il a été rapporté qu'interleukine-2 est associé avec les radeaux lipidiques et il est endocyté normalement dans les lymphocytes (Lamaze et al., 2001). Il est possible qu'en absence de la cavéoline, certaines cavéoles/radeaux lipidiques pourraient être des domaines très dynamiques qui s'invaginent et qui se détacheraient très rapidement de la membrane plasmique. Ces évidences suggèrent que les cavéoles/radeaux lipidiques sont capables d'internaliser et sont des structures instables à la membrane plasmique. La présence de la cavéoline n'est peut-être pas nécessaire à la formation des cavéoles, mais elle servirait à stabiliser les cavéoles/radeaux lipidiques. Ces résultats pourraient expliquer le fait qu'une déficience de la cavéoline-1 n'est pas létale chez les souris et pourtant elles n'ont pas de cavéole à la membrane plasmique (Drab et al., 2001; Razani et al., 2001a; Zhao et al., 2002). Il est à noter que quelques invaginations morphologiquement similaire aux cavéoles ont été observées dans les cellules des souris nulles de la cavéoline-1 (Drab et al., 2001).

Il a été rapporté que dans les cellules MDCK polarisées que la cavéoline 1 et 2 sont localisées du côté basolatéral, alors que seulement la cavéoline-1 est présente du côté apicale. De plus, les cavéoles ont été observées seulement au côté basolatéral, mais pas du côté apical (Scheiffele et al., 1998). L'expression d'une cavéoline mutante qui empêche la formation des complexes d'hétérodimère de la cavéoline-1 et 2 amène à une rétention de la cavéoline-2 à l'intérieur des cellules et à la disparition des cavéoles au côté basolatéral. Une surexpression de la cavéoline-2 à la membrane basolatérale montre une augmentation du nombre de cavéoles. Ces résultats démontrent que la cavéoline-2 puisse être nécessaire à la biogenèse des cavéoles (Lahtinen et al., 2003).

6.3 La cavéoline régule l'internalisation des cavéoles/radeaux lipidiques

L'expression de la dynK44A empêche la fission des cavéoles de la membrane plasmique (Henley et al., 1998; Oh et al., 1998). L'expression de la dynK44A dans les cellules NIH-3T3 transformées induit la formation des vésicules plasmalemmales lisses qui sont morphologiquement similaire aux cavéoles. Il a été montré que la réintroduction de la cavéoline dans les cellules NIH-ras et NIH-abl ou dans les lymphocytes induit de nouveau la formation des cavéoles (Fra et al., 1995; Engelman et al., 1997). Afin de déterminer si les vésicules plasmalemmales lisses qui sont dépourvues de la cavéoline et qui sont induites par l'expression de la dynK44A sont équivalentes aux cavéoles,

nous avons réintroduit la cavéoline-1 dans les cellules NIH-3T3 non-transformées et transformées (Le et al., 2002).

La surexpression de la cavéoline montre un plus grand nombre de cavéoles dans les cellules NIH-3T3 contrôles et elle induit la formation des cavéoles dans les cellules NIH-ras et NIH-abl. De plus, la surexpression de la cavéoline dans les cellules NIH-3T3 bloque l'internalisation de l'AMF vers le RE. La réintroduction de la cavéoline dans les cellules transformées réduit le niveau d'internalisation de l'AMF vers le RE au même niveau que les cellules NIH-3T3 contrôles (Le et al., 2002). Ces résultats suggèrent que la surexpression de la cavéoline stabilise la formation des cavéoles à la membrane plasmique et ralentit l'endocytose des cavéoles/radeaux lipidiques. Ce qui concorde avec le fait que l'expression de la cavéoline-1-GFP montre que la majorité des cavéoles sont immobiles et que seulement une petite population des cavéoles est mobile (Thomsen et al., 2002).

6.4 L'endocytose de CTX vers l'appareil de Golgi

CTX est localisée au niveau des cavéoles à la membrane plasmique (Parton, 1994) et il a été rapporté que les cavéoles médient l'endocytose de CTX (Orlandi and Fishman, 1998). Cependant, de récentes publications ont rapporté que CTX n'est pas seulement internalisée via les cavéoles mais également via les vésicules de clathrine (Shogomori and Futerman, 2001a) et

même par les vésicules indépendantes de la clathrine (Torgersen et al., 2001). De plus, il a été proposé que CTX puisse être séquestrée dans les cavéoles, mais elle est internalisée par d'autres voies d'endocytose.

Nous avons démontré dans les fibroblastes NIH-3T3 que l'endocytose de CTX vers les endosomes de recyclage est effectuée par les vésicules de clathrine. Toutefois, l'endocytose de CTX vers l'appareil de Golgi est médiée par les cavéoles, puisque cette voie d'endocytose peut être bloquée par la dynK44A et par la mßCD. De plus, nous avons montré que la cavéoline-1 régule cette voie d'endocytose (Le and Nabi, 2003). Ces résultats démontrent et confirment que l'internalisation de CTX vers l'appareil de Golgi est effectuée par les cavéoles/radeaux lipidiques et les vésicules de clathrine médient l'internalisation de CTX vers les endosomes de recyclage. De plus, ces résultats montrent que la cavéoline ne régule pas seulement l'endocytose de l'AMF mais également celle de CTX.

7. LE TRANSPORT INTRACELLULAIRE MÉDIÉ PAR LES CAVÉOLES/RADEAUX LIPIDIQUES

7.1 Le transport direct de l'AMF vers le RE

Les vésicules de clathrine internalisent leur cargo vers les endosomes et les lysosomes. L'endocytose médiée par les cavéoles/radeaux lipidiques

pourrait être une façon de cibler d'autres compartiments cellulaires. Des études *in vivo* ont montré que CTX et SV40 cointernalisent avec la cavéoline-1-GFP vers un compartiment intracellulaire qui est caractérisé par la présence de la cavéoline et qui se distingue des endosomes précoces par leur pH neutre et l'absence de la transferrine ou de son récepteur (Parton et al., 1994; Pelkmans et al., 2001; Nichols, 2002). Cet organite fut appelé cavéosome et il est localisé près du centrosome et son mouvement est dépendant des microtubules (Pelkmans et al., 2001; Mundy et al., 2002).

Il a été rapporté que l'internalisation de CTX et de SV40 vers l'appareil de Golgi et vers le RE est dépendante de beta-COP (Norkin et al., 2002; Richards et al., 2002), une composante impliquée dans la formation des coatamers de COP1 et qui est essentiel pour le transport rétrograde entre l'appareil de Golgi et le RE (Allan and Balch, 1999). De plus, une colocalisation partielle de beta-COP et de la cavéoline intracellulaire a été observée. Cette étude suggère que les cavéosomes contiendraient aussi beta-COP (Norkin et al., 2002). Une dépolymérisation des microtubules avec nocodazole bloque l'internalisation de SV40 au niveau des cavéosomes (Pelkmans et al., 2001). Nos études montrent que le nocodazole bloque l'internalisation de CTX vers l'appareil de Golgi, mais ne bloque pas l'endocytose de l'AMF vers le REL (Le et al., 2000; Le and Nabi, 2003). De plus, suite à son internalisation, l'AMF ne montre pas une colocalisation avec la cavéoline et le marquage intracellulaire de l'AMF-R ne montre aucune colocalisation avec la cavéoline. Ces résultats suggèrent que

l'endocytose de l'AMF vers le RE ne passe pas par l'intermédiaire des cavéosomes (Le and Nabi, 2003).

CTX est transporté vers l'appareil de Golgi et par un transport rétrograde vers le RE (Donta et al., 1993; Lencer et al., 1993; Nambiar et al., 1993; Orlandi et al., 1993). Par contre, il a été rapporté que le transport de SV40 vers le RE ne provient pas d'un transport rétrograde à partir de l'appareil de Golgi (Kartenbeck et al., 1989). Un traitement des cellules avec Brefeldin A ou une incubation à 20°C inhibe le transport de CTX vers l'appareil de Golgi et de SV40 vers le RE (Donta et al., 1993; Lencer et al., 1993; Nambiar et al., 1993; Orlandi et al., 1993; Morinaga et al., 2001; Norkin et al., 2002; Richards et al., 2002). Cependant, aucun de ces traitements n'affectent l'endocytose de l'AMF vers le RE (Le and Nabi, 2003).

Ces résultats indiquent que l'internalisation de l'AMF vers le REL n'est pas médiée par l'intermédiaire de l'appareil de Golgi et jusqu'à maintenant, aucun intermédiaire n'a été identifié dans l'internalisation de l'AMF vers le REL. L'AMF est donc probablement endocyté directement à partir des cavéoles/radeaux lipidiques vers le REL. Dans les cellules NIH-3T3 qui expriment la forme dominante négative de la clathrine (clathrine hub), une cointernalisation de CTX et de l'AMF pendant cinq minutes montre que la majorité de CTX est encore à la surface membranaire alors que l'AMF est déjà internalisé vers le REL qui est marqué par l'anticorps anti-AMF-R (Le and Nabi, 2003). Cette étude démontre

que l'AMF est directement internalisé vers le RE et l'AMF est internalisé via une route distincte de CTX.

Le RE est organisé en réseau qui s'étend à travers la cellule et sous la membrane plasmique, ainsi, la distance qui sépare les cavéoles et le RE est très petite (Bergeron et al., 1994). Dans les cellules musculaires lisses, il a été montré que le cholestérol est localisé dans les cavéoles et dans les tubules du RE qui sont distribués à la périphérie des cellules et en même temps, très proche des cavéoles. Toutefois, un contact directe entre le RE et les cavéoles n'a pas été observé dans ces cellules (Thyberg, 2000; Thyberg, 2002). Il a également été montré que le cholestérol nouvellement synthétisé dans le RE est transporté vers la membrane plasmique via sa liaison avec la cavéoline (Urbani and Simoni, 1990; Smart et al., 1996). Le cholestérol peut également être transporté de la membrane plasmique directement vers le RE (Lange, 1994).

Dans les cellules intestinales, il a été rapporté que les cavéoles ou les radeaux lipidiques concentrent l'excès de cholestérol à la membrane plasmique et le cholestérol est ensuite endocyté vers le RE (Field et al., 1998). En absence de GLUT2, un transporteur de glucose, la libération du glucose à partir des hépatocytes n'est pas affectée. Il a été suggéré qu'un mécanisme compensatoire, soit un trafic membranaire direct à partir du RE vers la surface cellulaire, est responsable pour la libération du glucose en absence de GLUT2 (Guillam et al., 1998). La localisation des tubules AMF-R à proximité des

cavéoles pourraient permettre un recyclage très rapide de l'AMF-R vers la surface des cellules en cas de nécessité tel qu'il a été décrit pour le récepteur de la cholecystokinin (Roettger et al., 1995).

7.2 L'existence de plusieurs classes de cavéoles

SV40 et CTX sont internalisés vers différentes populations de cavéosomes ce qui suggère que certains cavéosomes transportent leur contenu vers l'appareil de Golgi et d'autres cavéosomes dirigent leur cargo directement vers le RE (Nichols, 2002). La ségrégation des molécules cargo vers l'appareil de Golgi ou vers le RE se ferait donc avant leur internalisation vers différentes populations de cavéosomes. La ségrégation du cargo peut donc se faire directement à la membrane plasmique. Nos travaux sur la cointernalisation de l'AMF avec CTX montrent que ces deux ligands ne sont pas internalisés ensemble et que la cinétique de leur endocytose est différente (Le and Nabi, 2003). Un marquage de surface de l'AMF et de CTX montre que l'AMF ne colocalise pas avec CTX (résultat non-présenté). Ces résultats suggèrent que l'AMF et CTX peuvent se trouver et être internalisés par différentes populations de cavéoles. Cependant, il est possible qu'une même population de cavéoles médie l'endocytose de l'AMF et de CTX. Mais, c'est le temps de recrutement ou de séquestration de l'AMF qui diffère de CTX et pour cette raison, ces deux ligands ne colocalisent pas en surface.

Plusieurs évidences indiquent que les radeaux lipidiques/cavéoles sont hétérogènes. Par exemple, dans les lymphocytes T en migration, les gangliosides GM1 et GM3 sont redistribués de façon asymétrique. GM1 est enrichie du côté de l'uropode, alors que GM3 est localisé du côté guide (Gomez-Mouton et al., 2001). Les protéines GPI, l'alkaline phosphatase, et la prominin sont toutes présentes dans les fractions insolubles aux détergents. Toutefois, par immunofluorescence, ces protéines montrent une distribution distincte à la surface cellulaire (Roper et al., 2000). Ces études indiquent qu'il existe différentes populations de radeaux lipidiques/cavéoles dans la cellule et que les protéines peuvent être recrutées d'un endroit à un autre à la suite d'un stimuli. Il a été montré que SV40 prenait deux à quatre heures avant d'être endocyté vers le RE (Pelkmans et al., 2001), alors que l'AMF prend seulement cinq minutes pour être endocyté vers le RE (Le and Nabi, 2003). Ces résultats supportent l'idée que l'AMF puisse être internalisé par une autre population de cavéoles que celles qui endocytent SV40. Cependant, il n'est pas exclu que d'autres facteurs peuvent influencer la cinétique de ces deux ligands. Par exemple, l'AMF pourrait interagir avec d'autres protéines pour faciliter son endocytose ou, le temps de recrutement de SV40 par les cavéoles peut être plus long que pour l'AMF.

La cointernalisation de la cavéoline-1 GFP avec SV40 ou CTX démontre que les cavéoles sont des structures dynamiques capable d'être endocytées dans la cellule (Pelkmans et al., 2001; Nichols, 2002). Cependant, l'expression

de la cavéoline-1-GFP dans différents types cellulaires montre que la majorité des cavéoles à la membrane plasmique sont immobiles et seulement une minorité d'entres elles sont endocytées (Pelkmans et al., 2001; Mundy et al., 2002; Thomsen et al., 2002). La liaison de SV40 aux cavéoles induit une cascade de signalisation causant une réorganisation du cytosquelette d'actine et le recrutement de la dynamine-2 au niveau des cavéoles prêtes à internaliser le virus SV40. Donc, l'endocytose des cavéoles est un processus qui est médié à la suite de l'activation d'un signal (Pelkmans et al., 2002). Il reste à déterminer si la liaison de l'AMF à son récepteur induit son internalisation via les cavéoles ou si l'internalisation de l'AMF est un processus constitutif. Cependant, le fait que l'AMF soit internalisé vers le RE aussi rapidement pourrait suggérer que l'endocytose de l'AMF est constitutive.

IV. CONCLUSION

Globalement, nos études ont démontré que l'AMF est internalisé par deux voies distinctes : la première voie est médiée par les vésicules de clathrine vers les MVBs où l'AMF est ensuite recyclé vers les fibrilles de fibronectine et la deuxième voie est médiée par les cavéoles/radeaux lipidiques vers le RE. En utilisant l'AMF comme un marqueur pour l'internalisation effectuée par les cavéoles, nous avons défini l'endocytose dépendante des cavéoles comme étant sensible au niveau du cholestérol, dépendante de la dynamine et négativement régulée par la cavéoline-1. Nous avons également déterminé qu'en absence de la cavéoline, les radeaux lipidiques sont capables de former des invaginations cavéolaires instables et très dynamiques. Donc, la présence de la cavéoline-1 a pour but de stabiliser les invaginations cavéolaires et de réguler leur capacité endocytaire. Ces études ont aussi mis en évidence l'existence de multiples voies d'endocytose dépendante des cavéoles.

V. PERSPECTIVES

Maintenant que nous avons identifié les différentes voies d'endocytose que le complexe AMF/AMF-R utilise pour cibler le REL ou les MVBs. Des travaux supplémentaires pourront être faits pour déterminer les mécanismes de triage ou de la cinétique de l'endocytose de l'AMF/AMF-R via les cavéoles/radeaux lipidiques vers le REL, ou de son endocytose via la voie de clathrine et de son recyclage vers les fibrilles de fibronectine. Compte tenu de l'identification de l'AMF-R comme une ubiquitine ligase E3, il serait également intéressant de déterminer si l'ubiquitination du récepteur joue un rôle important dans la sélection de la voie d'endocytose utilisée par l'AMF/AMF-R.

Pour analyser la cinétique de l'internalisation de l'AMF/AMF-R, nous pourrions effectuer l'endocytose de l'AMF *in vivo* dans des cellules qui expriment la cavéoline-1-GFP. La cointernalisation de l'AMF avec la cavéoline-1-GFP permettrait : 1) de montrer que l'AMF est bien internalisé par les cavéoles; 2) de déterminer le temps que l'AMF/AMF-R prend pour se rendre au niveau du REL; 3) d'identifier un intermédiare (s'il y en a un) entre les cavéoles et le REL. Il serait aussi très intéressant d'étudier l'endocytose de l'AMF-R directement en co-transfectant par exemple, l'AMF-R-Texas-Red avec la cavéoline-1-GFP. Ainsi, il serait peut-être possible de déterminer si l'AMF-R est constitutivement endocyté vers le REL ou seulement lorsqu'il est stimulé par son ligand AMF.

Ensuite, pour étudier la cinétique du recyclage de l'AMF, nous pourrions incuber les cellules avec l'AMF-FITC pendant environ 30 minutes afin de permettre à l'AMF d'être endocyté vers les MVBs et *in vivo* via la microscopie confocale, nous pourrions mesurer le temps que l'AMF prend pour être recyclé vers les sites de fibronectine.

Afin de déterminer si l'ubiquitination est impliquée dans la sélection des voies d'endocytose de l'AMF/AMF-R, nous pourrions étudier l'endocytose de l'AMF dans les cellules qui expriment un AMF-R muté dans lequel le domaine Cue (domaine de liaison à l'ubiquitin) a été enlevé. De cette façon, nous pourrions déterminer si l'ubiquination est impliquée d'une façon quelconque dans l'endocytose de l'AMF/AMF-R et si elle influence la voie d'endocytose que l'AMF/AMF-R utilise pour être endocyté. Nous pourrions aussi effectuer l'endocytose de l'AMF en présence d'un inhibiteur du protéasome et regarder s'il y a un effet sur son endocytose.

Il a été démontré que la stimulation de la migration des cellules avec un "laminar shear stress" relocalise les cavéoles vers le côté arrière de la cellule et coïncide avec le début de la propagation du calcium. De plus, des études de microscopie électronique dans ces cellules, montrent que plusieurs cavéoles se retrouvent à proximité du REL (Isshiki et al., 2002b). Il serait donc très intéressant d'observer *in vivo,* l'endocytose de l'AMF dans des conditions où il y a une stimulation de la motilité.

Le développement d'un tel modèle pourrait nous permettre de déterminer si l'endocytose du complexe l'AMF/AMF-R s'effectue sélectivement au côté arrière de la cellule. De plus, au niveau de la région riche en cavéoles, nous pourrions observer si l'endocytose de l'AMF/AMF-R joue un rôle dans la libération du calcium. En même temps, nous pourrions déterminer si dans une cellule motile, l'endocytose (via les vésicules de clathrine) et le recyclage de l'AMF/AMF-R s'effectuent à partir du côté guide ou à l'arrière de la cellule.

Il a été montré que l'AMF stimule l'invasion des cellules et la sécrétion des métalloprotéases-2 (Torimura et al., 2001). Afin de déterminer s'il y a relation entre la motilité cellulaire, l'endocytose et le recyclage du complexe AMF/AMF-R, ainsi que la libération des métalloprotéinases; nous pourrions cointernaliser l'AMF avec un anticorps contre les métalloprotéases-2 ou effectuer l'endocytose de l'AMF et ensuite, faire un double marquage avec un anticorps contre les métalloprotéases-2 dans les cellules motiles.

VI. BIBLIOGRAPHIE

Aguilar RC, Boehm M, Gorshkova I, Crouch RJ, Tomita K, Saito T, Ohno H, Bonifacino JS. 2001. Signal-binding specificity of the mu4 subunit of the adaptor protein complex AP-4. *J. Biol. Chem.* 276:13145-13152.

Akiyama, S.K., K. Olden, and K.M. Yamada. 1995. Fibronectin and integrins in invasion and metastasis. *Cancer Met. Rev.* 14:173-89.

Allan, B., and W. Balch. 1999. Protein sorting by directed maturation of Golgi compartments. *Science.* 285:63-6.

Altankov, G., and F. Grinnell. 1993. Depletion of intracellular potassium disrupts coated pits and reversibly inhibits cell polarization during fibroblast spreading. *J. Cell Biol.* 120:1449-1459.

Altschuler, Y., S. Liu, L. Katz, K. Tang, S. Hardy, F. Brodsky, G. Apodaca, and K. Mostov. 1999. ADP-ribosylation factor 6 and endocytosis at the apical surface of Madin-Darby canine kidney cells. *J. Cell Biol.* 147:7-12.

Alves dos Santos, C.M., T. ten Broeke, and G.J. Strous. 2001. Growth Hormone Receptor Ubiquitination, Endocytosis, and Degradation Are Independent of Signal Transduction via Janus Kinase 2. *J. Biol. Chem.* 276:32635-32641.

Amraei, M., and I.R. Nabi. 2002. Species specificity of the cytokine function of phosphoglucose isomerase. *FEBS Lett.* 525:151-5.

Anderson, H.A., Y. Chen, and L.C. Norkin. 1996. Bound simian virus 40 translocates to caveolin-enriched membrane domains, and its entry is

inhibited by drugs that selectively disrupt caveolae. *Mol. Biol. Cell.* 7:1825-34.

Anderson, R.G. 1998. The caveolae membrane system. *Annu. Rev. Biochem.* 67:199-225.

Anderson, R.G.W., B.A. Kamen, K.G. Rothberg, and S.W. Lacey. 1992. Potocytosis: Sequestration and transport of small molecules by caveolae. *Science*. 253:410-411.

Aniento, F., N. Emans, G. Griffiths, and J. Gruenberg. 1993. Cytoplasmic dynein-dependent vesicular transport from early to late endosomes. *J. Cell Biol.* 123:1373-1387.

Baumann, M., K. Brand, J. Giedl, P. Hermanck, S. Ruf, J. Scheele, S. Hoferichter, and F.P. Gall. 1988. Significance of serum phosphohexose isomerase in gastrointestinal cancer at different stages. *Oncology*. 45:153-158.

Baumgart, E., H.D. Fahimi, A. Stich, and A. Volkl. 1996. L-lactate dehydrogenase A4- and A3B isoforms are bona fide peroxisomal enzymes in rat liver. Evidence for involvement in intraperoxisomal NADH reoxidation. *J. Biol. Chem.* 271:3846-55.

Bender, F.C., M.A. Reymond, C. Bron, and A.F. Quest. 2000. Caveolin-1 levels are down-regulated in human colon tumors, and ectopic expression of caveolin-1 in colon carcinoma cell lines reduces cell tumorigenicity. *Cancer Res.* 60:5870-8.

Benlimame, N., P.U. Le, and I.R. Nabi. 1998. Localization of autocrine motility factor receptor to caveolae and clathrin-independent internalization of its ligand to smooth endoplasmic reticulum. *Mol. Biol. Cell.* 9:1773-1786.

Benlimame, N., D. Simard, and I.R. Nabi. 1995. Autocrine motility factor receptor is a marker for a distinct membranous tubular organelle. *J. Cell Biol.* 129:459-71.

Benmerah, A., M. Bayrou, N. Cerf-Bensussan, and A. Dautry-Varsat. 1999. Inhibition of clathrin-coated pit assembly by an Eps15 mutant. *J. Cell Sci.* 112:1303-11.

Benmerah, A., C. Lamaze, B. Begue, S.L. Schmid, A. Dautry-Varsat, and N. Cerf-Bensussan. 1998. AP-2/Eps15 interaction is required for receptor-mediated endocytosis. *J. Cell Biol.* 140:1055-62.

Bergeron, M., G. Thiery, F. Lenoir, M.C. Giocondi, and C. Le Grimellec. 1994. Organization of the endoplasmic reticulum in renal cell lines MDCK and LLC-PK1. *Cell Tissue Res.* 277:297-307.

Biederer, T., C. Volkwein, and T. Sommer. 1997. Role of Cue1p in ubiquitination and degradation at the ER surface. *Science*. 278:1806-9.

Bodansky, O. 1954. Serum phosphohexose isomerase in cancer II. As an index of tumor growth in metastatic carcinoma of the breast. *Cancer.* 7:1200-1226.

Bretscher, M.S. 1984. Endocytosis: relation to capping and cell locomotion. *Science*. 226:681-686.

Brodsky, F.M., C.Y. Chen, C. Knuehl, M.C. Towler, and D.E. Wakeham. 2001. Biological basket weaving: formation and function of clathrin-coated vesicles. *Annu. Rev. Cell Dev. Biol.* 17:517-68.

Brown, C.M., M.G. Roth, Y.I. Henis, and N.O. Petersen. 1999. An internalization-competent influenza hemagglutinin mutant causes the redistribution of AP-2 to existing coated pits and is colocalized with AP-2 in clathrin free clusters. *Biochem.* 38:15166-73.

Brown, D. 2002. Structure and function of membrane rafts. *Int. J. Med. Microbiol.* 291:433-7.

Brown, D.A., and E. London. 1997. Structure of detergent-resistant membrane domains: does phase separation occur in biological membranes? *Biochem. Biophys. Res. Commun.* 240:1-7.

Brown, D.A., and J.K. Rose. 1992. Sorting of GPI-anchored proteins in glycolipid enriched membrane subdomains during transport to the apical cell surface. *Cell.* 68:533-544.

Capozza, F., T.M. Williams, W. Schubert, S. McClain, B. Bouzahzah, F. Sotgia, and M.P. Lisanti. 2003. Absence of caveolin-1 sensitizes mouse skin to carcinogen-induced epidermal hyperplasia and tumor formation. *Am J Pathol.* 162:2029-39.

Carbone, R., S. Fre, G. Iannolo, F. Belleudi, P. Mancini, P.G. Pelicci, M.R. Torrisi, and P.P. Di Fiore. 1997. eps15 and eps15R are essential components of the endocytic pathway. *Cancer Res.* 57:5498-504.

Carpentier, J.L., F. Sawano, D. Geiger, P. Gorden, A. Perrelet, and L. Orci. 1989. Potassium depletion and hypertonic medium reduce "non-coated" and clathrin-coated pit formation, as well as endocytosis through these two gates. *J Cell Physiol.* 138:519-26.

Chaput, M., V. Claes, D. Portetelle, I. Cludts, A. Cravador, A. Burny, H. Gras, and A. Tartar. 1988. The neurotrophic factor neuroleukin is 90% homologous with phosphohexose isomerase. *Nature (Lond.).* 332:454-457.

Chen, H., S. Fre, V.I. Slepnev, M.R. Capua, K. Takei, M.H. Butler, P.P. Di Fiore, and P. De Camilli. 1998. Epsin is an EH-domain-binding protein implicated in clathrin-mediated endocytosis. *Nature (Lond.).* 394:793-7.

Chen, W.S., C.S. Lazar, K.A. Lund, J.B. Welsh, C.P. Chang, G.M. Walton, C.J. Der, H.S. Wiley, G.N. Gill, and M.G. Rosenfeld. 1989. Functional independence of the epidermal growth factor receptor from a domain required for ligand-induced internalization and calcium regulation. *Cell.* 59:33-43.

Chen, Y., and L.C. Norkin. 1999. Extracellular simian virus 40 transmits a signal that promotes virus enclosure within caveolae. *Exp. Cell Res.* 246:83-90.

Chou, C.-C., Y.-J. Sun, M. Meng, and C.-D. Hsiao. 2000. The Crystal Structure of Phosphoglucose Isomerase/Autocrine Motility Factor/Neuroleukin Complexed with Its Carbohydrate Phosphate Inhibitors Suggests Its Substrate/Receptor Recognition. *J. Biol. Chem.* 275:23154-23160.

Chun, M., U.K. Liyanage, M.P. Lisanti, and H.F. Lodish. 1994. Signal transduction of a G protein-coupled receptor in caveolae: Colocalization of endothelin and its receptor with caveolin. *Proc. Natl. Acad. Sci. U.S.A.* 91:11728-11732.

Contamin, S., A. Galmiche, A. Doye, G. Flatau, A. Benmerah, and P. Boquet. 2000. The p21 Rho-activating toxin cytotoxic necrotizing factor 1 is endocytosed by a clathrin-independent mechanism and enters the cytosol by an acidic-dependent membrane translocation step. *Mol. Biol. Cell.* 11:1775-87.

Cook, L.B., C.-C. Zhu, and P.M. Hinkle. 2003. Thyrotropin-Releasing Hormone Receptor Processing: Role of Ubiquitination and Proteasomal Degradation. *Mol Endocrinol.* 17:1777-1791.

Cremona, O., and P. De Camilli. 2001. Phosphoinositides in membrane traffic at the synapse. *J. Cell Sci.* 114:1041-52.

Cremona, O., G. Di Paolo, M.R. Wenk, A. Luthi, W.T. Kim, K. Takei, L. Daniell, Y. Nemoto, S.B. Shears, R.A. Flavell, D.A. McCormick, and P. De Camilli. 1999. Essential role of phosphoinositide metabolism in synaptic vesicle recycling. *Cell.* 99:179-88.

Critchley, D.R., C.H. Streuli, S. Kellie, S. Ansell, and B. Patel. 1982. Characterization of the cholera toxin receptor on Balb/c 3T3 cells as a ganglioside similar to, or identical with, ganglioside GM1. No evidence for galactoproteins with receptor activity. *Biochem J.* 204:209-19.

Damke, H., T. Baba, A.M. van der Bliek, and S.L. Schmid. 1995. Clathrin-independent pinocytosis is induced in cells overexpressing a temperature-sensitive mutant of dynamin. *J. Cell Biol.* 131:69-80.

De Brabander, M., R. Nuydens, H. Geerts, and C.R. Hopkins. 1988. Dynamic behavior of the transferrin receptor followed in living epidermoid carcinoma (A431) cells with Nanovid microscopy. *Cell Motil. Cytoskel.* 9:30-47.

Deckert, M., M. Ticchioni, and A. Bernard. 1996. Endocytosis of GPI-anchored proteins in human lymphocytes: role of glycolipid-based domains, actin cytoskeleton, and protein kinases. *J. Cell Biol.* 133:791-799.

Dell' Angelica, E.C., Mullins, C. and Bonifacino, J.S. 1999. AP-4, a novel protein complex related to clathrin adaptors. *J. Biol. Chem.* 274: 7278-7285.

Denzer, K., M.J. Kleijmeer, H.F. Heijnen, W. Stoorvogel, and H.J. Geuze. 2000. Exosome: from internal vesicle of the multivesicular body to intercellular signaling device. *J. Cell Sci.* 113 Pt 19:3365-74.

Dessy, C., R.A. Kelly, J.L. Balligand, and O. Feron. 2000. Dynamin mediates caveolar sequestration of muscarinic cholinergic receptors and alteration in NO signaling [In Process Citation]. *EMBO J.* 19:4272-80.

Donta, S.T., S. Beristain, and T.K. Tomicic. 1993. Inhibition of heat-labile cholera and Escherichia coli enterotoxins by brefeldin A. *Infect Immun.* 61:3282-6.

Drab, M., P. Verkade, M. Elger, M. Kasper, M. Lohn, B. Lauterbach, J. Menne, C. Lindschau, F. Mende, F.C. Luft, A. Schedl, H. Haller, and T.V. Kurzchalia. 2001. Loss of caveolae, vascular dysfunction, and pulmonary defects in caveolin-1 gene-disrupted mice. *Science.* 293:2449-52.

Drevot, P., C. Langlet, X.J. Guo, A.M. Bernard, O. Colard, J.P. Chauvin, R. Lasserre, and H.T. He. 2002. TCR signal initiation machinery is pre-assembled and activated in a subset of membrane rafts. *EMBO J.* 21:1899-908.

Dunn, W.A., T.P. Connolly, and A.L. Hubbard. 1986. Receptor-mediated endocytosis of epidermal growth factor by rat hepatocytes: Receptor pathway. *J. Cell Biol.* 102:24-36.

Dunn, W.A., A.L. Hubbard, and N.N.J. Aronson. 1980. Low temperature selectively inhibits fusion between pinocytic vesicles and lysosomes during heterophagy of ^{125}I-asialofetuin by the perfused rat liver. *J. Biol. Chem.* 255:5971-5978.

Dupree, P., R.G. Parton, G. Raposo, T.V. Kurzchalia, and K. Simons. 1993. Caveolae and sorting in the trans-Golgi network of epithelial cells. *EMBO J.* 12:1597-605.

Engelman, J.A., C.C. Wykoff, S. Yasuhara, K.S. Song, T. Okamoto, and M.P. Lisanti. 1997. Recombinant expression of caveolin-1 in oncogenically transformed cells abrogates anchorage-independent growth. *J. Biol. Chem.* 272:16374-81.

Engelman, J.A., X. Zhang, F. Galbiati, D. Volonte, F. Sotgia, R.G. Pestell, C. Minetti, P.E. Scherer, T. Okamoto, and M.P. Lisanti. 1998a. Molecular genetics of the caveolin gene family: implications for human cancers, diabetes, Alzheimer disease, and muscular dystrophy. *Am J Hum Genet.* 63:1578-87.

Engelman, J.A., X.L. Zhang, and M.P. Lisanti. 1998b. Genes encoding human caveolin-1 and -2 are co-localized to the D7S522 locus (7q31.1), a known fragile site (FRA7G) that is frequently deleted in human cancers. *FEBS Lett.* 436:403-10.

Engelman, J.A., X.L. Zhang, and M.P. Lisanti. 1999. Sequence and detailed organization of the human caveolin-1 and -2 genes located near the D7S522 locus (7q31.1). Methylation of a CpG island in the 5' promoter region of the caveolin-1 gene in human breast cancer cell lines. *FEBS Lett.* 448:221-30.

Faik, P., J.I.H. Walker, A.A.M. Redmill, and M.J. Morgan. 1988. Mouse glucose-6-phosphate isomerase and neuroleukin have identical 3' sequences. *Nature (Lond.)*. 332:455-457.

Fang, S., M. Ferrone, C. Yang, J.P. Jensen, S. Tiwari, and A.M. Weissman. 2001. The tumor autocrine motility factor receptor, gp78, is a ubiquitin protein ligase implicated in degradation from the endoplasmic reticulum. *Proc. Natl. Acad. Sci. U.S.A.* 98:14422-7.

Farsad, K., N. Ringstad, K. Takei, S.R. Floyd, K. Rose, and P. De Camilli. 2001. Generation of high curvature membranes mediated by direct endophilin bilayer interactions. *J. Cell Biol.* 155:193-200.

Felder, S., K. Miller, G. Moehren, A. Ullrich, J. Schlessinger, and C.R. Hopkins. 1990. Kinase activity controls the sorting of the epidermal growth factor receptor within the multivesicular body. *Cell.* 61:623-634.

Field, F.J., E. Born, S. Murthy, and S.N. Mathur. 1998. Caveolin is present in intestinal cells: role in cholesterol trafficking? *J. Lipid Res.* 39:1938-1950.

Filella, X., R. Molina, J. Jo, E. Mas, and A.M. Ballesta. 1991. Serum phosphohexose isomerase activities in patients with colorectal cancer. *Tumor Biology*. 12:360-367.

Fingerhut, A., K. von Figura, and S. Honing. 2001. Binding of AP2 to sorting signals is modulated by AP2 phosphorylation. *J. Biol. Chem.* 276:5476-82.

Fishman, P.H. 1982. Role of membrane gangliosides in the binding and action of bacterial toxins. *J Membr Biol.* 69:85-97.

Ford, M.G., I.G. Mills, B.J. Peter, Y. Vallis, G.J. Praefcke, P.R. Evans, and H.T. McMahon. 2002. Curvature of clathrin-coated pits driven by epsin. *Nature (Lond.)*. 419:361-6.

Fra, A.M., E. Williamson, K. Simons, and R.G. Parton. 1994. Detergent-insoluble glycolipid microdomains in lymphocytes in the absence of caveolae. *J. Biol. Chem.* 269:30745-30748.

Fra, A.M., E. Williamson, K. Simons, and R.G. Parton. 1995. *De novo* formation of caveolae in lymphocytes by expression of VIP21-caveolin. *Proc. Natl. Acad. Sci. U.S.A.* 92:8655-8659.

Fujimoto, T. 1993. Calcium pump of the plasma membrane is localized in caveolae. *J. Cell Biol.* 120:1147-1157.

Fujimoto, T. 1996. GPI-anchored proteins, glycosphingolipids, and sphingomyelin are sequestered to caveolae only after crosslinking. *J. Histochem. Cytochem.* 44:929-941.

Fujimoto, T., H. Kogo, R. Nomura, and T. Une. 2000. Isoforms of caveolin-1 and caveolar structure. *J. Cell Sci.* 113 Pt 19:3509-17.

Fujimoto, T., S. Nakade, A. Miyawaki, K. Mikoshiba, and K. Ogawa. 1992. Localization of 1,4,5-triphosphate receptor-like protein in plasmalemmal caveolae. *J. Cell Biol.* 119:1507-1513.

Funasaka, T., A. Haga, A. Raz, and H. Nagase. 2001. Tumor autocrine motility factor is an angiogenic factor that stimulates endothelial cell motility. *Biochem. Biophys. Res. Commun.* 285:118-28.

Funasaka, T., A. Haga, A. Raz, and H. Nagase. 2002. Autocrine motility factor secreted by tumor cells upregulates vascular endothelial growth factor receptor (Flt-1) expression in endothelial cells. *Int. J. Cancer.* 101:217-23.

Futter, C.E., and C.R. Hopkins. 1989. Subfractionation of the endocytic pathway: isolation of compartments involved in the processing of internalised epidermal growth factor-receptor complexes. *J. Cell Sci.* 94 (Pt 4):685-94.

Futter, C.E., A. Pearse, L.J. Hewlett, and C.R. Hopkins. 1996. Multivesicular endosomes containing internalized EGF-EGF receptor complexes mature and then fuse directly with lysosomes. *J. Cell Biol.* 132:1011-23.

Galbiati, F., J.A. Engelman, D. Volonte, X.L. Zhang, C. Minetti, M. Li, H. Hou, Jr., B. Kneitz, W. Edelmann, and M.P. Lisanti. 2001. Caveolin-3 null mice show a loss of caveolae, changes in the microdomain distribution of the dystrophin-glycoprotein complex, and t- tubule abnormalities. *J. Biol. Chem.* 276:21425-33.

Galbiati, F., D. Volonte, J.A. Engelman, G. Watanabe, R. Burk, R.G. Pestell, and M.P. Lisanti. 1998. Targeted downregulation of caveolin-1 is sufficient to drive cell transformation and hyperactivate the p42/44 MAP kinase cascade. *EMBO J.* 17:6633-48.

Gallusser, A., and T. Kirchhausen. 1993. The beta 1 and beta 2 subunits of the AP complexes are the clathrin coat assembly components. *EMBO J.* 12:5237-44.

Garcia-Cardena, G., P. Martasek, B.S. Masters, P.M. Skidd, J. Couet, S. Li, M.P. Lisanti, and W.C. Sessa. 1997. Dissecting the interaction between nitric oxide synthase (NOS) and caveolin. Functional significance of the nos caveolin binding domain in vivo. *J. Biol. Chem.* 272:25437-40.

Garcia-Gila, M., E. Lopez-Martin, and A. Garcia-Pardo. 2002. Adhesion to fibronectin via alpha4 integrin (CD49d) protects B cells from apoptosis induced by serum deprivation but not via IgM or Fas/Apo-1 receptors. *Clin Exp. Immunol.* 127:455-62.

Ghitescu, L., and M. Bendayan. 1992. *J. Cell Biol.* 117:745-755.

Gilbert, A., J.P. Paccaud, M. Foti, G. Porcheron, J. Balz, and J.L. Carpentier. 1999. Direct demonstration of the endocytic function of caveolae by a cell-free assay. *J. Cell Sci.* 112:1101-10.

Glenney, J.R. 1989. Tyrosine phosphorylation of a 22-kDa protein is correlated with transformation by Rous Sarcoma virus. *J. Biol. Chem.* 264:20163-20166.

Golovina, V.A., and M.P. Blaustein. 1997. Spatially and functionally distinct Ca2+ stores in sarcoplasmic and endoplasmic reticulum. *Science.* 275:1643-1648.

Gomez-Mouton, C., J.L. Abad, E. Mira, R.A. Lacalle, E. Gallardo, S. Jimenez-Baranda, I. Illa, A. Bernad, S. Manes, and A.C. Martinez. 2001. Segregation of leading-edge and uropod components into specific lipid rafts during T cell polarization. *Proc. Natl. Acad. Sci. U.S.A.* 98:9642-7.

Goodman, O.B., Jr., J.G. Krupnick, V.V. Gurevich, J.L. Benovic, and J.H. Keen. 1997. Arrestin/clathrin interaction. Localization of the arrestin binding locus to the clathrin terminal domain. *J. Biol. Chem.* 272:15017-22.

Goodman, O.B., Jr., J.G. Krupnick, F. Santini, V.V. Gurevich, R.B. Penn, A.W. Gagnon, J.H. Keen, and J.L. Benovic. 1998. Role of arrestins in G-protein-coupled receptor endocytosis. *Adv Pharmacol.* 42:429-33.

Gorodinsky, A., and D.A. Harris. 1995. Glycolipid-anchored proteins in neuroblastoma cells form detergent- resistant complexes without caveolin. *J. Cell Biol.* 129:619-27.

Greener, T., X. Zhao, H. Nojima, E. Eisenberg, and L.E. Greene. 2000. Role of cyclin G-associated kinase in uncoating clathrin-coated vesicles from non-neuronal cells. *J. Biol. Chem.* 275:1365-70.

Griffiths, G., B. Hoflack, K. Simons, I. Mellman, and S. Kornfeld. 1988. The mannose-6-phosphate receptor and the biogenesis of lysosomes. *Cell.* 52:329-341.

Gruenberg, J., G. Griffiths, and K.E. Howell. 1989. Characterization of the early endosome and putative endocytic carrier vesicles in vivo and with an assay of vesicle fusion in vitro. *J. Cell Biol.* 108:1301-1316.

Guillam, M.-T., R. Burcelin, and B. Thorens. 1998. Normal hepatic glucose production in the absence of GLUT2 reveals an alternative pathway for glucose release from hepatocytes. *PNAS*. 95:12317-12321.

Guirguis, R., N. Javadpour, S. Sharareh, C. Biswas, W. el-Amin, I. Mansur, and J.S. Kim. 1990. A new method for evaluation of urinary autocrine motility factor and tumor cell collagenase stimulating factor as markers for urinary tract cancers. *J. Occup. Med.* 32:846-853.

Guirguis, R., E. Schiffmann, B. Liu, D. Birkbeck, J. Engel, and L. Liotta. 1988. Detection of autocrine motility factor in urine as a marker of bladder cancer. *J Natl Cancer Inst*. 80:1203-11.

Gurney, M.E., B.R. Apatoff, G.T. Spear, M.J. Baumel, J.P. Antel, M. Brown Bania, and A.T. Reder. 1986a. Neuroleukin: A lymphokine product of lectin-stimulated T cells. *Science*. 234:574-581.

Gurney, M.E., S.P. Heinrich, M.R. Lee, and H.-s. Yin. 1986b. Molecular cloning and expression of neuroleukin, a neurotropic factor for spinal and sensory neurons. *Science*. 234:566-574.

Haasemann, M., J. Cartaud, W. Muller-Esterl, and I. Dunia. 1998. Agonist-induced redistribution of bradykinin B2 receptor in caveolae. *J. Cell Sci.* 111:917-928.

Haglund, K., S. Sigismund, S. Polo, I. Szymkiewicz, P.P. Di Fiore, and I. Dikic. 2003. Multiple monoubiquitination of RTKs is sufficient for their endocytosis and degradation. *Nat. Cell Biol.* 5:461-6.

Hailstones, D., L.S. Sleer, R.G. Parton, and K.K. Stanley. 1998. Regulation of caveolin and caveolae by cholesterol in MDCK cells. *J. Lipid Res.* 39:369-379.

Hansen, S.H., K. Sandvig, and B. van Deurs. 1993. Molecules internalized by clathrin-independent endocytosis are delivered to endosomes containing transferrin receptors. *J. Cell Biol.* 123:89-97.

Harder, T., and K. Simons. 1997. Caveolae, DIGs, and the dynamics of sphingolipid-cholesterol microdomains. *Curr. Opin. Cell Biol.* 9:534-42.

Harris, T.W., E. Hartwieg, H.R. Horvitz, and E.M. Jorgensen. 2000. Mutations in synaptojanin disrupt synaptic vesicle recycling. *J. Cell Biol.* 150:589-600.

Henley, J.R., H. Cao, and M.A. McNiven. 1999. Participation of dynamin in the biogenesis of cytoplasmic vesicles. *FASEB J.* 13 Suppl 2:S243-7.

Henley, J.R., E.W. Krueger, B.J. Oswald, and M.A. McNiven. 1998. Dynamin-mediated internalization of caveolae. *J. Cell Biol.* 141:85-99.

Herreros, J., T. Ng, and G. Schiavo. 2001. Lipid Rafts Act as Specialized Domains for Tetanus Toxin Binding and Internalization into Neurons. *Mol. Biol. Cell.* 12:2947-2960.

Heuser, J. 1989. Effects of cytoplasmic acidification on clathrin lattice morphology. *J. Cell Biol.* 108:401-411.

Hinshaw, J.E. 2000. Dynamin and its role in membrane fission. *Annu. Rev. Cell Dev. Biol.* 16:483-519.

Hirono, Y., S. Fushida, Y. Yonemura, H. Yamamoto, H. Watanabe, and A. Raz. 1996. Expression of autocrine motility factor receptor correlates with disease progression in human gastric cancer. *Br. J. Cancer.* 74:2003-2007.

Hirst, J., Bright, N.A., Rous, B. and Robinson, M.S. 1999. Characterization of a fourth adaptor-related protein complex. *Mol. Biol. Cell.* 10:2787-2802.

Hopkins, C.R., A. Gibson, M. Shipman, and K. Miller. 1990. Movement of internalized ligand-receptor complexes along a continuous endosomal reticulum. *Nature (Lond.)*. 346:335-9.

Hopkins, C.R., A. Gibson, M. Shipman, D.K. Strickland, and I.S. Trowbridge. 1994. In migrating fibroblasts, recycling receptors are concentrated in narrow tubules in the pericentriolar area, and then routed to the plasma membrane of the leading lamella. *J. Cell Biol.* 125:1265-1274.

Hopkins, C.R., and I.S. Trowbridge. 1983. Internalization and processing of transferrin and the transferrin receptor in human carcinoma A431 cells. *J. Cell Biol.* 97:508-521.

Huttner, W.B., and A. Schmidt. 2000. Lipids, lipid modification and lipid-protein interaction in membrane budding and fission - insights from the roles of endophilin A1 and synaptophysin in synaptic vesicle endocytosis [In Process Citation]. *Curr Opin Neurobiol.* 10:543-51.

Hynes, R.O. 1992. Integrins: versatility, modulation, and signaling in cell adhesion. *Cell*. 69:11-25.

Ikezu, T., H. Ueda, B.D. Trapp, K. Nishiyama, J.F. Sha, D. Volonte, F. Galbiati, A.L. Byrd, G. Bassell, H. Serizawa, W.S. Lane, M.P. Lisanti, and T. Okamoto. 1998. Affinity-purification and characterization of caveolins from the brain: differential expression of caveolin-1, -2, and -3 in brain endothelial and astroglial cell types. *Brain Res*. 804:177-92.

Ilangumaran, S., S. Arni, G. van Echten-Deckert, B. Borisch, and D.C. Hoessli. 1999. Microdomain-dependent regulation of Lck and Fyn protein-tyrosine kinases in T lymphocyte plasma membranes. *Mol. Biol. Cell*. 10:891-905.

Ishizaka, N., K.K. Griendling, B. Lassegue, and R.W. Alexander. 1998. Angiotensin II Type 1 Receptor : Relationship With Caveolae and Caveolin After Initial Agonist Stimulation. *Hypertension*. 32:459-466.

Isshiki, M., Y.S. Ying, T. Fujita, and R.G. Anderson. 2002. A molecular sensor detects signal transduction from caveolae in living cells. *J. Biol. Chem.* 277:43389-98.

Isshiki, M., J. Ando, K. Yamanoto, T. Fujita, Y.S. Ying and R.G. Anderson, 2002b. Sites of Ca2+ wave initiation move with caveolae to the trailing edge of migrating cells. *J. Cell Sci.* 115:475-484.

Jacobson, K., and C. Dietrich. 1999. Looking at lipid rafts? *Trends Cell Biol.* 9:87-91.

Jacobson, K., E.D. Sheets, and R. Simson. 1995. Revisiting the fluid mosaic model of membranes. *Science*. 268:1441-2.

Jeffery, C. 1999. Moonlighting proteins. *Trends Biochem. Sci.* 24(1):8-11.

Jeffery, C.J., B.J. Bahnson, W. Chien, D. Ringe, and G.A. Petsko. 2000. Crystal structure of rabbit phosphoglucose isomerase, a glycolytic enzyme that moonlights as neuroleukin, autocrine motility factor, and differentiation mediator. *Biochem.* 39:955-64.

Jeffery, C.J., R. Hardre, and L. Salmon. 2001. Crystal structure of rabbit phosphoglucose isomerase complexed with 5- phospho-D-arabinonate identifies the role of Glu357 in catalysis. *Biochem.* 40:1560-6.

Johnstone, R.M. 1992. The Jeanne Manery-Fisher Memorial Lecture 1991. Maturation of reticulocytes: formation of exosomes as a mechanism for shedding membrane proteins. *Biochem Cell Biol.* 70:179-90.

Kalthoff, C., J. Alves, C. Urbanke, R. Knorr, and E.J. Ungewickell. 2002. Unusual structural organization of the endocytic proteins AP180 and epsin 1. *J. Biol. Chem.* 277:8209-16.

Kartenbeck, J., H. Stukenbrok, and A. Helenius. 1989. Endocytosis of simian virus 40 into the endoplasmic reticulum. *J. Cell Biol.* 109:2721-2729.

Kenworthy, A.K., and M. Edidin. 1998. Distribution of a Glycosylphosphatidylinositol-anchored Protein at the Apical Surface of MDCK Cells Examined at a Resolution of <100 A Using Imaging Fluorescence Resonance Energy Transfer. *J. Cell Biol.* 142:69-84.

Kenworthy, A.K., N. Petranova, and M. Edidin. 2000. High-Resolution FRET Microscopy of Cholera Toxin B-Subunit and GPI-anchored Proteins in Cell Plasma Membranes. *Mol. Biol. Cell.* 11:1645-1655.

Kim, J., Ahn, S., Guo, R. and Daaka, Y. 2003. Regulation of epidermal growth factor receptor internalization to protein-coupled receptors. *Biochemistry* 42:2887-2894.

Kim, W.T., S. Chang, L. Daniell, O. Cremona, G. Di Paolo, and P. De Camilli. 2002. Delayed reentry of recycling vesicles into the fusion-competent synaptic vesicle pool in synaptojanin 1 knockout mice. *PNAS.* 99:17143-17148.

Kirchhausen, T. 1999. Adaptors for clathrin-mediated traffic. *Annu. Rev. Cell Dev. Biol.* 15:705-32.

Kirchhausen, T. 2000. Clathrin. *Annu. Rev. Biochem.* 69:699-727.

Kohn, E.C., L.A. Liotta, and E. Schiffman. 1990. Autocrine motility factor stimulates a three-fold increase in inositol phosphate in human melanoma cells. *Biochem. Biophys. Res. Comm.* 166:757-764.

Koleske, A.J., D. Baltimore, and M.P. Lisanti. 1995. Reduction of caveolin and caveolae in oncogenically transformed cells. *Proc. Natl. Acad. Sci. U.S.A.* 92:1381-1385.

Kuchler, A. 2000. Unusual routes of protein secretion: the easy way out. *Trends Cell Biol.* 3:421-426.

Kugler, W., K. Breme, P. Laspe, H. Muirhead, C. Davies, H. Winkler, W. Schroter, and M. Lakomek. 1998. Molecular basis of neurological dysfunction coupled with haemolytic anaemia in human glucose-6-phosphate isomerase (GPI) deficiency. *Hum Genet.* 103:450-454.

Kurzchalia, T.V., P. Dupree, R.G. Parton, R. Kellner, H. Virta, M. Lehnert, and K. Simons. 1992. VIP21, a 21-kD membrane protein is an integral component of trans-Golgi- network-derived transport vesicles. *J. Cell Biol.* 118:1003-14.

Kurzchalia, T.V., and R.G. Parton. 1999. Membrane microdomains and caveolae. *Curr. Opin. Cell Biol.* 11:424-31.

Lahtinen, U., M. Honsho, R.G. Parton, K. Simons, and P. Verkade. 2003. Involvement of caveolin-2 in caveolar biogenesis in MDCK cells. *FEBS Lett.* 538:85-8.

Lamaze, C., A. Dujeancourt, T. Baba, C.G. Lo, A. Benmerah, and A. Dautry-Varsat. 2001. Interleukin 2 receptors and detergent-resistant membrane domains define a clathrin-independent endocytic pathway. *Mol. Cell.* 7:661-71.

Lange, Y. 1994. Cholesterol movement from plasma membrane to rough endoplasmic reticulum. Inhibition by progesterone. *J. Biol. Chem.* 269:3411-4.

Laporte, S.A., Oakley, R.H., Zhang, J., Holt, J.A., Ferguson, S.S.G., Caron, M.G. and Barak, L.S. 1999. The β2-adrenergic receptor/βarrestin complex recruits the clathrin adaptor AP-2 during endocytosis. *Proc.Natl. Acad. Sci. USA.* 96:3712-3717.

Le, P.U., N. Benlimame, A. Lagana, A. Raz, and I.R. Nabi. 2000. Clathrin-mediated endocytosis and recycling of autocrine motility factor receptor to

fibronectin fibrils is a limiting factor for NIH-3T3 cell motility. *J. Cell Sci.* 113:3227-3240.

Le, P.U., G. Guay, Y. Altschuler, and I.R. Nabi. 2002. Caveolin-1 is a negative regulator of caveolae-mediated endocytosis to the endoplasmic reticulum. *J. Biol. Chem.* 277:3371-9.

Le, P.U., and I.R. Nabi. 2003. Distinct caveolae-mediated endocytic pathways target the Golgi apparatus and the endoplasmic reticulum. *J. Cell Sci.* 116:1059-71.

Le, P.U., T.N. Nguyen, P. Drolet-Savoie, N. Leclerc, and I.R. Nabi. 1998. Increased ß-actin expression in an invasive Moloney sarcoma virus-transformed cell variant concentrates to the tips of multiple pseudopodia. *Cancer Res.* 58:1631-1635.

Lee, J.H., K.Z. Chang, V. Patel, and C.J. Jeffery. 2001. Crystal structure of rabbit phosphoglucose isomerase complexed with its substrate D-fructose 6-phosphate. *Biochem.* 40:7799-805.

Lee, S.W., C.L. Reimer, P. Oh, D.B. Campbell, and J.E. Schnitzer. 1998. Tumor cell growth inhibition by caveolin re-expression in human breast cancer cells. *Oncogene*. 16:1391-7.

Lencer, W.I., C. Constable, S. Moe, M.G. Jobling, H.M. Webb, S. Ruston, J.L. Madara, T.R. Hirst, and R.K. Holmes. 1995. Targeting of cholera toxin and Escherichia coli heat labile toxin in polarized epithelia: role of COOH-terminal KDEL. *J. Cell Biol.* 131:951-62.

Lencer, W.I., J.B. de Almeida, S. Moe, J.L. Stow, D.A. Ausiello, and J.L. Madara. 1993. Entry of cholera toxin into polarized human intestinal epithelial cells. Identification of an early brefeldin A sensitive event required for A1-peptide generation. *J Clin Invest.* 92:2941-51.

Li, L., G. Yang, S. Ebara, T. Satoh, Y. Nasu, T.L. Timme, C. Ren, J. Wang, S.A. Tahir, and T.C. Thompson. 2001. Caveolin-1 mediates testosterone-stimulated survival/clonal growth and promotes metastatic activities in prostate cancer cells. *Cancer Res.* 61:4386-92.

Li, S., J. Couet, and M.P. Lisanti. 1996. Src tyrosine kinases, Galpha subunits, and H-Ras share a common membrane-anchored scaffolding protein, caveolin. Caveolin binding negatively regulates the auto-activation of Src tyrosine kinases. *J. Biol. Chem.* 271:29182-90.

Li, S., T. Okamoto, M. Chun, M. Sargiacomo, J.E. Casanova, S.H. Hansen, I. Nishimoto, and M.P. Lisanti. 1995. Evidence for a regulated interaction between heterotrimeric G proteins and caveolin. *J. Biol. Chem.* 270:15693-15701.

Liang, J.-s., T. Kim, S. Fang, J. Yamaguchi, A.M. Weissman, E.A. Fisher, and H.N. Ginsberg. 2003. Overexpression of the Tumor Autocrine Motility Factor Receptor Gp78, a Ubiquitin Protein Ligase, Results in Increased Ubiquitinylation and Decreased Secretion of Apolipoprotein B100 in HepG2 Cells. *J. Biol. Chem.* 278:23984-23988.

Liotta, L.A., R. Mandler, G. Murano, D.A. Katz, R.K. Gordon, P.K. Chiang, and E. Schiffman. 1986. Tumor cell autocrine motility factor. *Proc. Natl. Acad. Sci. U.S.A.* 83:3302-3306.

Lisanti, M.P., M. Sargiacomo, and P.E. Scherer. 1999. Purification of caveolae-derived membrane microdomains containing lipid- anchored signaling molecules, such as GPI-anchored proteins, H-Ras, Src- family tyrosine kinases, eNOS, and G-protein alpha-, beta-, and gamma- subunits. *Methods Mol Biol.* 116:51-60.

Lisanti, M.P., Z. Tang, P.E. Scherer, and M. Sargiacomo. 1995. Caveolae purification and glycosylphosphatidylinositol-linked protein sorting in polarized epithelia. *Methods Enzymol.* 250:655-68.

Lisanti, M.P., Z.L. Tang, and M. Sargiacomo. 1993. Caveolin forms a hetero-oligomeric protein complex that interacts with an apical GPI-linked protein: implications for the biogenesis of caveolae. *J. Cell Biol.* 123:595-604.

Liu, P., and R.G. Anderson. 1995. Compartmentalized production of ceramide at the cell surface. *J. Biol. Chem.* 270:27179-85.

Liu, P., M. Rudick, and R.G. Anderson. 2002. Multiple functions of caveolin-1. *J. Biol. Chem.* 19:19.

Liu, P., Y. Ying, Y.G. Ko, and R.G. Anderson. 1996. Localization of platelet-derived growth factor-stimulated phosphorylation cascade to caveolae. *J. Biol. Chem.* 271:10299-303.

Liu, S.H., M.S. Marks, and F.M. Brodsky. 1998. A dominant-negative clathrin mutant differentially affects trafficking of molecules with distinct sorting

motifs in the class II major histocompatibility complex (MHC) pathway. *J. Cell Biol.* 140:1023-37.

Llorente, A., A. Rapak, S.L. Schmid, B. van Deurs, and K. Sandvig. 1998. Expression of mutant dynamin inhibits toxicity and transport of endocytosed ricin to the Golgi apparatus. *J. Cell Biol.* 140:553-63.

Llorente, A., B. van Deurs, O. Garred, P. Eker, and K. Sandvig. 2000. Apical endocytosis of ricin in MDCK cells is regulated by the cyclooxygenase pathway. *J. Cell Sci.* 113:1213-21.

Lobie, P. E., sadir, R., Graichen, R., Mertani, H. C., Morel, G. 1999. Caveolar internalization of growth hormone. *Exp. Cell Res.* 246:47-55.

Luo, Y., J.M. Long, C. Lu, S.L. Chan, E.L. Spangler, P. Mascarucci, A. Raz, D.L. Longo, M.P. Mattson, D.K. Ingram, and N.P. Weng. 2002. A link between maze learning and hippocampal expression of neuroleukin and its receptor gp78. *J Neurochem.* 80:354-61.

Mahaffey, D.T., M.S. Moore, F.M. Brodsky, and R.G. Anderson. 1989. Coat proteins isolated from clathrin coated vesicles can assemble into coated pits. *J. Cell Biol.* 108:1615-24.

Majoul, I.V., P.I. Bastiaens, and H.D. Soling. 1996. Transport of an external Lys-Asp-Glu-Leu (KDEL) protein from the plasma membrane to the endoplasmic reticulum: studies with cholera toxin in Vero cells. *J. Cell Biol.* 133:777-89.

Mao, Y., J. Chen, J.A. Maynard, B. Zhang, and F.A. Quiocho. 2001. A novel all helix fold of the AP180 amino-terminal domain for phosphoinositide

binding and clathrin assembly in synaptic vesicle endocytosis. *Cell.* 104:433-40.

Marjomaki, V., V. Pietiainen, H. Matilainen, P. Upla, J. Ivaska, L. Nissinen, H. Reunanen, P. Huttunen, T. Hyypia, and J. Heino. 2002. Internalization of Echovirus 1 in Caveolae. *J. Virol.* 76:1856-1865.

Maruyama, K., H. Watanabe, S. Hitoshi, T. Takayama, J. Gofuku, H. Yano, M. Inoue, S. Tamura, A. Raz, and M. Monden. 1995. Expression of autocrine motility factor receptor in human esophogeal squamous cell carcinoma. *Int. J. Cancer.* 64:316-321.

Matsumoto, I., M. Maccioni, D.M. Lee, M. Maurice, B. Simmons, M. Brenner, D. Mathis, and C. Benoist. 2002. How antibodies to a ubiquitous cytoplasmic enzyme may provoke joint- specific autoimmune disease. *Nat Immunol.* 3:360-5.

Matsumoto, I., A. Staub, C. Benoist, and D. Mathis. 1999. Arthritis provoked by linked T and B cell recognition of a glycolytic enzyme. *Science*. 286:1732-5.

Matveev, S.V., and E.J. Smart. 2002. Heterologous desensitization of EGF receptors and PDGF receptors by sequestration in caveolae. *Am J Physiol Cell Physiol.* 282:C935-946.

Mayor, S., K.G. Rothberg, and F.R. Maxfield. 1994. Sequestration of GPI-anchored proteins in caveolae triggered by cross-linking. *Science.* 264:1948-.

McMahon, H.T. 1999. Endocytosis: an assembly protein for clathrin cages. *Curr Biol.* 9:R332-5.

McNiven, M.A., H. Cao, K.R. Pitts, and Y. Yoon. 2000. The dynamin family of mechanoenzymes: pinching in new places. *Trends Biochem. Sci.* 25:115-20.

McPherson P.S., Garcia E.P., Slepnev V.I., David C., Zhang X., Grabs D., Sossin W.S., Bauerfeind R., Nemoto Y. and De Camilli P. 1996. *Nature* 279: 353-357.

Mellman, I. 1996. Endocytosis and molecular sorting. *Annu. Rev. Cell Dev. Biol.* 12:575-626.

Middlebrook, J.L., and R.B. Dorland. 1984. Bacterial toxins: cellular mechanisms of action. *Microbiol Rev.* 48:199-221.

Miller, K., M. Shipman, I.S. Trowbridge, and C.R. Hopkins. 1991. Transferrin receptors promote the formation of clathrin lattices. *Cell.* 65:621-32.

Miller, W.E., Maudsley, S., Ahn, s., Khan, k.D., luttrell, L.M. and Lefkowitz, R.J. 2000. β-Arrestin-1 interacts with the catalytic domain of the tyrosine kinase c-SRC. *J. Biol. Chem.* 275:11312-11319.

Mineo, C., and R.G. Anderson. 2001. Potocytosis. *Histochem Cell Biol.* 116:109-18.

Minshall, R.D., C. Tiruppathi, S.M. Vogel, W.D. Niles, A. Gilchrist, H.E. Hamm, and A.B. Malik. 2000. Endothelial cell-surface gp60 activates vesicle formation and trafficking via G(i)-coupled Src kinase signaling pathway. *J. Cell Biol.* 150:1057-70.

Mirre, C., L. Monlauzeur, M. Garcia, M.H. Delgrossi, and A. Le Bivic. 1996. Detergent-resistant membrane microdomains from Caco-2 cells do not contain caveolin. *Am J Physiol.* 271:C887-94.

Monier, S., R.G. Parton, F. Vogel, J. Behkle, A. Henske, and T.V. Kurzchalia. 1995. VIP21-caveolin, a membrane protein consituent of the caveolar coat, oligomerizes in vivo and in vitro. *Mol. Biol. Cell.* 6:911-927.

Montesano, R., J. Roth, A. Robert, and L. Orci. 1982. Non-coated membrane invaginations are involved in binding and internalization of cholera and tetanus toxins. *Nature (Lond.).* 296:651-653.

Mora, R., V.L. Bonilha, A. Marmorstein, P.E. Scherer, D. Brown, M.P. Lisanti, and E. Rodriguez-Boulan. 1999. Caveolin-2 localizes to the golgi complex but redistributes to plasma membrane, caveolae, and rafts when co-expressed with caveolin-1. *J. Biol. Chem.* 274:25708-17.

Morgan, J.R., K. Prasad, S. Jin, G.J. Augustine, and E.M. Lafer. 2001. Uncoating of clathrin-coated vesicles in presynaptic terminals: roles for Hsc70 and auxilin. *Neuron.* 32:289-300.

Morgan, J.R., K. Prasad, S. Jin, G.J. Augustine, and E.M. Lafer. 2003. Eps15 Homology Domain-NPF Motif Interactions Regulate Clathrin Coat Assembly during Synaptic Vesicle Recycling. *J. Biol. Chem.* 278:33583-33592.

Morinaga, N., Y. Kaihou, N. Vitale, J. Moss, and M. Noda. 2001. Involvement of ADP-ribosylation factor 1 in cholera toxin-induced morphological changes of Chinese hamster ovary cells. *J. Biol. Chem.* 276:22838-43.

Mosesson, Y., K. Shtiegman, M. Katz, Y. Zwang, G. Vereb, J. Szollosi, and Y. Yarden. 2003. Endocytosis of Receptor Tyrosine Kinases Is Driven by Monoubiquitylation, Not Polyubiquitylation. *J. Biol. Chem.* 278:21323-21326.

Moya, M., A. Dautry-Varsat, B. Goud, D. Louvard, and P. Boquet. 1985. Inhibition of coated pit formation in Hep2 cells blocks the cytotoxicity of diphtheria toxin but not that of ricin toxin. *J. Cell Biol.* 101:548-59.

Mukherjee, S., R.N. Ghosh, and F.R. Maxfield. 1997. Endocytosis. *Physiol. Rev.* 77:759-803.

Mukherjee, S., and F.R. Maxfield. 2000. Role of membrane organization and membrane domains in endocytic lipid trafficking. *Traffic.* 1:203-11.

Mullock, B.M., N.A. Bright, C.W. Fearon, S.R. Gray, and J.P. Luzio. 1998. Fusion of lysosomes with late endosomes produces a hybrid organelle of intermediate density and is NSF dependent. *J. Cell Biol.* 140:591-601.

Mundy, D.I., T. Machleidt, Y.S. Ying, R.G. Anderson, and G.S. Bloom. 2002. Dual control of caveolar membrane traffic by microtubules and the actin cytoskeleton. *J. Cell Sci.* 115:4327-39.

Munro, P., H. Kojima, J.L. Dupont, J.L. Bossu, B. Poulain, and P. Boquet. 2001. High sensitivity of mouse neuronal cells to tetanus toxin requires a GPI-anchored protein. *Biochem. Biophys. Res. Commun.* 289:623-9.

Murata, M., J. Peranen, R. Schreiner, F. Wieland, T.V. Kurzchalia, and K. Simons. 1995. VIP21/caveolin is a cholesterol-binding protein. *Proc. Natl. Acad. Sci. U.S.A.* 92:10339-43.

Nabi, I.R., G. Guay, and D. Simard. 1997. AMF-R tubules concentrate in a pericentriolar microtubule domain following transformation of MDCK epithelial cells. *J. Histochem. Cytochem.* 45:1351-1363.

Nabi, I.R. and Le, P.U. 2003. Caveolae/raft-dependent endocytosis. *J. Cell Biol.* 161:673-677.

Nabi, I.R., and A. Raz. 1987. Cell shape modulation alters glycosylation of a metastatic melanoma cell surface antigen. *Int. J. Cancer.* 40:396-401.

Nabi, I.R., H. Watanabe, and A. Raz. 1990. Identification of B16-F1 melanoma autocrine motility-like factor receptor. *Cancer Res.* 50:409-414.

Nabi, I.R., H. Watanabe, and A. Raz. 1992. Autocrine motility factor and its receptor: role in cell locomotion and metastasis. *Cancer Met. Rev.* 11:5-20.

Nakamori, S., H. Watanabe, M. Kameyama, S. Imaoka, H. Furukawa, O. Ishikawa, Y. Sasaki, T. Kabuto, and A. Raz. 1994. Expression of autocrine motility factor receptor in colorectal cancer as a predictor for disease recurrence. *Cancer.* 74:1855-1862.

Nambiar, M.P., T. Oda, C. Chen, Y. Kuwazuru, and H.C. Wu. 1993. Involvement of the Golgi region in the intracellular trafficking of cholera toxin. *J Cell Physiol.* 154:222-8.

Nasu, Y., T.L. Timme, G. Yang, C.H. Bangma, L. Li, C. Ren, S.H. Park, M. DeLeon, J. Wang, and T.C. Thompson. 1998. Suppression of caveolin expression induces androgen sensitivity in metastatic androgen-insensitive mouse prostate cancer cells. *Nat Med.* 4:1062-4.

Nichols, B.J. 2002. A distinct class of endosome mediates clathrin-independent endocytosis to the Golgi complex. *Nat. Cell Biol.* 15:15.

Nichols, B.J. and Lippincott-Schwartz. 2001. Endocytosis without clathrin coats. *Trends Cell Biol.* 11:406-12.

Nichols, B.J., A.K. Kenworthy, R.S. Polishchuk, R. Lodge, T.H. Roberts, K. Hirschberg, R.D. Phair, and J. Lippincott-Schwartz. 2001. Rapid cycling of lipid raft markers between the cell surface and Golgi complex. *J. Cell Biol.* 153:529-41.

Niinaka, Y., A. Haga, A. Negishi, H. Yoshimasu, A. Raz, and T. Amagasa. 2002. Regulation of cell motility via high and low affinity autocrine motility factor (AMF) receptor in human oral squamous carcinoma cells. *Oral Oncol.* 38:49-55.

Nishiyama, K., B.D. Trapp, T. Ikezu, R.M. Ransohoff, T. Tomita, T. Iwatsubo, I. Kanazawa, K.K. Hsiao, M.P. Lisanti, and T. Okamoto. 1999. Caveolin-3 upregulation activates beta-secretase-mediated cleavage of the amyloid precursor protein in Alzheimer's disease. *J. Neurosci.* 19:6538-48.

Norkin, L.C., H.A. Anderson, S.A. Wolfrom, and A. Oppenheim. 2002. Caveolar endocytosis of simian virus 40 is followed by brefeldin A- sensitive transport to the endoplasmic reticulum, where the virus disassembles. *J. Virol.* 76:5156-66.

Oh, P., D.P. McIntosh, and J.E. Schnitzer. 1998. Dynamin at the neck of caveolae mediates their budding to form transport vesicles by GTP-driven

fission from the plasma membrane of endothelium. *J. Cell Biol.* 141:101-14.

Ohno, H., J. Stewart, M.C. Fournier, H. Bosshart, I. Rhee, S. Miyatake, T. Saito, A. Gallusser, T. Kirchhausen, and J.S. Bonifacino. 1995. Interaction of tyrosine-based sorting signals with clathrin-associated proteins. *Science.* 269:1872-5.

Okamoto, T., A. Schlegel, P.E. Scherer, and M.P. Lisanti. 1998. Caveolins, a family of scaffolding proteins for organizing "preassembled signaling complexes" at the plasma membrane. *J. Biol. Chem.* 273:5419-22.

Orlandi, P.A. 1997. Protein-disulfide isomerase-mediated reduction of the A subunit of cholera toxin in a human intestinal cell line. *J. Biol. Chem.* 272:4591-9.

Orlandi, P.A., P.K. Curran, and P.H. Fishman. 1993. Brefeldin A blocks the response of cultured cells to cholera toxin. Implications for intracellular trafficking in toxin action. *J. Biol. Chem.* 268:12010-6.

Orlandi, P.A., and P.H. Fishman. 1998. Filipin-dependent inhibition of cholera toxin: evidence for toxin internalization and activation through caveolae-like domains. *J. Cell Biol.* 141:905-15.

Ostrom, R.S., C. Gregorian, R.M. Drenan, Y. Xiang, J.W. Regan, and P.A. Insel. 2001. Receptor number and caveolar co-localization determine receptor coupling efficiency to adenylyl cyclase. *J. Biol. Chem.* 276:42063-9.

Ostrom, R.S., J.D. Violin, S. Coleman, and P.A. Insel. 2000. Selective enhancement of beta-adrenergic receptor signaling by overexpression of

adenylyl cyclase type 6: colocalization of receptor and adenylyl cyclase in caveolae of cardiac myocytes. *Mol. Pharmacol.* 57:1075-9.

Otto, T., W. Birchmeier, U. Schmidt, A. Hinke, J. Schipper, H. Rübben, and A. Raz. 1994. Inverse relation of E-cadherin and autocrine motility factor receptor expression as a prognostic factor in patients with bladder carcinomas. *Cancer Res.* 54:3120-3123.

Palade, G.E. 1953. Fine structure of blood capillaries. *J. Appl. Phys.* 24:1424.

Pals-Rylaarsdam, R., V.V. Gurevich, K.B. Lee, J.A. Ptasienski, J.L. Benovic, and M.M. Hosey. 1997. Internalization of the m2 muscarinic acetylcholine receptor. Arrestin- independent and -dependent pathways. *J. Biol. Chem.* 272:23682-9.

Park, D.S., S.E. Woodman, W. Schubert, A.W. Cohen, P.G. Frank, M. Chandra, J. Shirani, B. Razani, B. Tang, L.A. Jelicks, S.M. Factor, L.M. Weiss, H.B. Tanowitz, and M.P. Lisanti. 2002. Caveolin-1/3 double-knockout mice are viable, but lack both muscle and non-muscle caveolae, and develop a severe cardiomyopathic phenotype. *Am J Pathol.* 160:2207-17.

Parolini, I., M. Sargiacomo, F. Galbiati, G. Rizzo, F. Grignani, J.A. Engelman, T. Okamoto, T. Ikezu, P.E. Scherer, R. Mora, E. Rodriguez-Boulan, C. Peschle, and M.P. Lisanti. 1999. Expression of caveolin-1 is required for the transport of caveolin-2 to the plasma membrane. Retention of caveolin-2 at the level of the golgi complex. *J. Biol. Chem.* 274:25718-25.

Parton, R.G. 1994. Ultrastructural localization of gangliosides; GM1 is concentrated in caveolae. *J Histochem Cytochem.* 42:155-66.

Parton, R.G., B. Joggerst, and K. Simons. 1994. Regulated internalization of caveolae. *J. Cell Biol.* 127:1199-215.

Parton, R.G., M. Way, N. Zorzi, and E. Stang. 1997. Caveolin-3 associates with developing T-tubules during muscle differentiation. *J. Cell Biol.* 136:137-54.

Patel, P.S., G.N. Rawal, R.M. Rawal, G.H. Patel, D.B. Balar, P.M. Shah, and D.C. Patel. 1995. Comparison between serum levels of carcinoembryonic antigen, sialic acid, and phosphohexose isomeras in lung cancer. *Neoplasia*. 42:271-274.

Pearse, B.M. 1988. Receptors compete for adaptors found in plasma membrane coated pits. *EMBO J.* 7:3331-6.

Pearse, B.M., and M.S. Robinson. 1990. Clathrin, adaptors, and sorting. *Annu Rev Cell Biol.* 6:151-71.

Pelkmans, L., and A. Helenius. 2002. Endocytosis via caveolae. *Traffic.* 3:311-20.

Pelkmans, L., J. Kartenbeck, and A. Helenius. 2001. Caveolar endocytosis of simian virus 40 reveals a new two-step vesicular-transport pathway to the ER. *Nat. Cell Biol.* 3:473-83.

Pelkmans, L., D. Puntener, and A. Helenius. 2002. Local actin polymerization and dynamin recruitment in SV40-induced internalization of caveolae. *Science*. 296:535-9.

Peters, K.R., W.W. Carley, and G.E. Palade. 1985. Endothelial plasmalemmal vesicles have a characteristic striped bipolar surface structure. *J. Cell Biol.* 101:2233-8.

Pishvaee, B., G. Costaguta, B.G. Yeung, S. Ryazantsev, T. Greener, L.E. Greene, E. Eisenberg, J.M. McCaffery, and G.S. Payne. 2000. A yeast DNA J protein required for uncoating of clathrin-coated vesicles in vivo. *Nat. Cell Biol.* 2:958-63.

Polo, S., S. Sigismund, M. Faretta, M. Guidi, M.R. Capua, G. Bossi, H. Chen, P. De Camilli, and P.P. Di Fiore. 2002. A single motif responsible for ubiquitin recognition and monoubiquitination in endocytic proteins. *Nature (Lond.)*. 416:451-5.

Ponting, C.P. 2000. Proteins of the endoplasmic-reticulum-associated degradation pathway: domain detection and function prediction. *Biochem J.* 351 Pt 2:527-35.

Pozzan, T., R. Rizzuto, P. Volpe, and J. Meldolesi. 1994. Molecular and cellular physiology of intracellular calcium stores. *Physiol. Rev.* 74:595-636.

Predescu, S.A., D.N. Predescu, and G.E. Palade. 1997. Plasmalemmal vesicles function as transcytotic carriers for small proteins in the continuous endothelium. *Am J Physiol.* 272:H937-49.

Puri, V., R. Watanabe, R.D. Singh, M. Dominguez, J.C. Brown, C.L. Wheatley, D.L. Marks, and R.E. Pagano. 2001. Clathrin-dependent and -independent internalization of plasma membrane sphingolipids initiates two Golgi targeting pathways. *J. Cell Biol.* 154:535-47.

Racine, C., M. Belanger, H. Hirabayashi, M. Boucher, J. Chakir, and J. Couet. 1999. Reduction of caveolin 1 gene expression in lung carcinoma cell lines. *Biochem. Biophys. Res. Commun.* 255:580-6.

Razani, B., J.A. Engelman, X.B. Wang, W. Schubert, X.L. Zhang, C.B. Marks, F. Macaluso, R.G. Russell, M. Li, R.G. Pestell, D. Di Vizio, H. Hou, Jr., B. Kneitz, G. Lagaud, G.J. Christ, W. Edelmann, and M.P. Lisanti. 2001a. Caveolin-1 null mice are viable but show evidence of hyperproliferative and vascular abnormalities. *J. Biol. Chem.* 276:38121-38.

Razani, B., A. Schlegel, J. Liu, and M.P. Lisanti. 2001b. Caveolin-1, a putative tumour suppressor gene. *Biochem Soc Trans.* 29:494-9.

Razani, B., X.B. Wang, J.A. Engelman, M. Battista, G. Lagaud, X.L. Zhang, B. Kneitz, H. Hou, Jr., G.J. Christ, W. Edelmann, and M.P. Lisanti. 2002a. Caveolin-2-deficient mice show evidence of severe pulmonary dysfunction without disruption of caveolae. *Mol. Cell Biol.* 22:2329-44.

Razani, B., S.E. Woodman, and M.P. Lisanti. 2002b. Caveolae: from cell biology to animal physiology. *Pharmacol Rev.* 54:431-67.

Remacle, A., Murphy, G. and Roghi G. 2003. Membrane type I-matrix metalloproteinase (MT1-MMP) is internalised by two different pathways and is recycled to the cell surface. *J. Cell Sci.* 116: 3905-3916.

Richards, A.A., E. Stang, R. Pepperkok, and R.G. Parton. 2002. Inhibitors of COP-mediated Transport and Cholera Toxin Action Inhibit Simian Virus 40 Infection. *Mol. Biol. Cell.* 13:1750-64.

Rieu, S., C. Geminard, H. Rabesandratana, J. Sainte-Marie, and M. Vidal. 2000. Exosomes released during reticulocyte maturation bind to fibronectin via integrin alpha4beta1. *Eur. J. Biochem.* 267:583-90.

Rijnboutt, S., G. Jansen, G. Posthuma, J.B. Hynes, J.H. Schornagel, and G.J. Strous. 1996. Endocytosis of GPI-linked membrane folate receptor-alpha. *J. Cell Biol.* 132:35-47.

Rodal, S.K., G. Skretting, Ø. Garred, F. Vilhardt, B. van Deurs, and K. Sandvig. 1999. Extraction of cholesterol with methyl-ß-cyclodextrin perturbs formation of clathrin-coated endocytic vesicles. *Mol. Biol. Cell.* 10:961-974.

Roettger, B.F., R.U. Rentsch, D. Pinon, E. Holicky, E. Hadac, J.M. Larkin, and L.J. Miller. 1995. Dual pathways of internalization of the cholecystokinin receptor. *J. Cell Biol.* 128:1029-1041.

Roper, K., D. Corbeil, and W.B. Huttner. 2000. Retention of prominin in microvilli reveals distinct cholesterol-based lipid micro-domains in the apical plasma membrane. *Nat. Cell Biol.* 2:582-92.

Roseberry, A., and M. Hosey. 2001. Internalization of the M2 muscarinic acetylcholine receptor proceeds through an atypical pathway in HEK293 cells that is independent of clathrin and caveolae. *J. Cell Sci.* 114:739-746.

Rosenberger, C.M., J.H. Brumell, and B.B. Finlay. 2000. Microbial pathogenesis: lipid rafts as pathogen portals. *Curr Biol.* 10:R823-5.

Rothberg, K.G., J.E. Heuser, W.C. Donzell, Y.-S. Ying, J.R. Glenney, and R.G.W. Anderson. 1992. Caveolin, a protein component of caveolae membrane coats. *Cell.* 68:673-682.

Rothberg, K.G., Y.S. Ying, B.A. Kamen, and R.G. Anderson. 1990a. Cholesterol controls the clustering of the glycophospholipid-anchored membrane receptor for 5-methyltetrahydrofolate. *J. Cell Biol.* 111:2931-8.

Rothberg, K.G., Y.S. Ying, J.F. Kolhouse, B.A. Kamen, and R.G. Anderson. 1990b. The glycophospholipid-linked folate receptor internalizes folate without entering the clathrin-coated pit endocytic pathway. *J. Cell Biol.* 110:637-49.

Rusk, N., P.U. Le, S. Mariggio, G. Guay, C. Lurisci, I.R. Nabi, D. Corda, and M. Symons. 2003. Synaptojanin 2 functions at an early step of clathrin-mediated endocytosis. *Curr Biol.* 13:659-63.

Rybin, V.O., X. Xu, M.P. Lisanti, and S.F. Steinberg. 2000. Differential targeting of beta -adrenergic receptor subtypes and adenylyl cyclase to cardiomyocyte caveolae. A mechanism to functionally regulate the cAMP signaling pathway. *J. Biol. Chem.* 275:41447-57.

Sabharanjak, S., P. Sharma, R.G. Parton, and S. Mayor. 2002. GPI-anchored proteins are delivered to recycling endosomes via a distinct cdc42-regulated, clathrin-independent pinocytic pathway. *Dev Cell.* 2:411-23.

Sandvig, K., S. Olsnes, O.W. Petersen, and B. van Deurs. 1987. Acidification of the cytosol inhibits endocytosis from coated pits. *J. Cell Biol.* 105:679-89.

Sandvig, K., and B. van Deurs. 1996. Endocytosis, intracellular transport, and cytotoxic action of Shiga toxin and ricin. *Physiol. Rev.* 76:949-66.

Santini, F., M.S. Marks, and J.H. Keen. 1998. Endocytic clathrin-coated pit formation is independent of receptor internalization signal levels. *Mol. Biol. Cell.* 9:1177-94.

Schaller, M., D.R. Burton, and H.J. Ditzel. 2001. Autoantibodies to GPI in rheumatoid arthritis: linkage between an animal model and human disease. *Nat Immunol.* 2:746-53.

Scheiffele, P., P. Verkade, A.M. Fra, H. Virta, K. Simons, and E. Ikonen. 1998. Caveolin-1 and -2 in the exocytic pathway of MDCK cells. *J. Cell Biol.* 140:795-806.

Scherer, P.E., and M.P. Lisanti. 1997. Association of phosphofructokinase-M with caveolin-3 in differentiated skeletal myotubes. Dynamic regulation by extracellular glucose and intracellular metabolites. *J. Biol. Chem.* 272:20698-705.

Scherer, P.E., M.P. Lisanti, G. Baldini, M. Sargiacomo, C.C. Mastick, and H.F. Lodish. 1994. Induction of caveolin during adipogenesis and association of GLUT4 with caveolin-rich vesicles. *J. Cell Biol.* 127:1233-43.

Schmid, S.L. 1997. Clathrin-coated vesicle formation and protein sorting: An integrated process. *Annu. Rev. Biochem.* 66:511-48.

Schmidt, A., M. Wolde, C. Thiele, W. Fest, H. Kratzin, A.V. Podtelejnikov, W. Witke, W.B. Huttner, and H.D. Soling. 1999. Endophilin I mediates synaptic vesicle formation by transfer of arachidonate to lysophosphatidic acid. *Nature (Lond.).* 401:133-41.

Schnitzer, J.E., J. Liu, and P. Oh. 1995a. Endothelial caveolae have the molecular transport machinery for vesicle budding, docking, and fusion including the VAMP, NSF, SNAP, annexins, and GTPases. *J. Biol. Chem.* 270:14399-14404.

Schnitzer, J.E., D.P. McIntosh, A.M. Dvorak, J. Liu, and P. Oh. 1995b. Separation of caveolae from associated microdomains of GPI-anchored proteins. *Science*. 269:1435-9.

Schnitzer, J.E., and P. Oh. 1994. Albondin-mediated capillary permeability to albumin. Differential role of receptors in endothelial transcytosis and endocytosis of native and modified albumins. *J. Biol. Chem.* 269:6072-82.

Schnitzer, J.E., P. Oh, and D.P. McIntosh. 1996. Role of GTP hydrolysis in fission of caveolae directly from plasma membranes [published erratum appears in Science 1996 Nov 15;274(5290):1069]. *Science*. 274:239-42.

Schnitzer, J.E., P. Oh, E. Pinney, and J. Allard. 1994. Filipin-sensitive caveolae-mediated transport in endothelium: reduced transcytosis, scavenger endocytosis, and capillary permeability of select macromolecules. *J. Cell Biol.* 127:1217-32.

Schubert, W., P.G. Frank, C.W. Chow, and M.P. Lisanti. 2001. Caveolae-deficient endothelial cells show defects in the uptake and transport of albumin In vivo. *J. Biol. Chem.* 31:31.

Schwab, W., F. Galbiati, D. Volonte, U. Hempel, K.W. Wenzel, R.H. Funk, M.P. Lisanti, and M. Kasper. 1999. Characterisation of caveolins from cartilage:

expression of caveolin-1, -2 and -3 in chondrocytes and in alginate cell culture of the rat tibia. *Histochem Cell Biol.* 112:41-9.

Schwartz, M.K. 1973. Enzymes in cancer. *Clin. Chem.* 19:10-22.

Schwencke, C., S. Okumura, M. Yamamoto, Y.J. Geng, and Y. Ishikawa. 1999. Colocalization of beta-adrenergic receptors and caveolin within the plasma membrane. *J Cell Biochem.* 75:64-72.

Sheets, E.D., G.M. Lee, R. Simson, and K. Jacobson. 1997. Transient confinement of a glycosylphosphatidylinositol-anchored protein in the plasma membrane. *Biochem.* 36:12449-58.

Shimizu, K., M. Tani, H. Watanabe, Y. Nagamachi, Y. Niinaka, T. Shiroishi, S. Ohwada, A. Raz, and J. Yokota. 1999. The autocrine motility factor receptor gene encodes a novel type of seven transmembrane protein. *FEBS Lett.* 456:295-300.

Shin, J.S., and S.N. Abraham. 2001. Caveolae as portals of entry for microbes. *Microbes Infect.* 3:755-61.

Shogomori, H., and A.H. Futerman. 2001a. Cholera toxin is found in detergent-insoluble rafts/domains at the cell surface of hippocampal neurons but is internalized via a raft- independent mechanism. *J. Biol. Chem.* 276:9182-8.

Shogomori, H., and A.H. Futerman. 2001b. Cholesterol depletion by methyl-beta-cyclodextrin blocks cholera toxin transport from endosomes to the Golgi apparatus in hippocampal neurons. *J Neurochem.* 78:991-9.

Simmen T, Honing S, Icking A, Tikkanen R, Hunziker W. 2002. AP-4 binds basolateral signals and participates in basolateral sorting in epithelial MDCK cells. *Nat. Cell Biol.* 4:154-159.

Silletti, S., and A. Raz. 1996. Regulation of autocrine motility factor receptor expression in tumor cell locomotion and metastasis. *Curr. Topics Microbiol. Immunol.* 213/II:137-169.

Silletti, S., J. Timar, K.V. Honn, and A. Raz. 1994. Autocrine motility factor induces differential 12-lipoxygenase expression and activity in high and low-metastatic K1735 melanoma cell variants. *Cancer Res.* 54:5752-5756.

Silletti, S., H. Watanabe, V. Hogan, I.R. Nabi, and A. Raz. 1991. Purification of B16-F1 melanoma autocrine motility factor and its receptor. *Cancer Res.* 51:3301-3311.

Simionescu, N., M. Siminoescu, and G.E. Palade. 1975. Permeability of muscle capillaries to small heme-peptides. Evidence for the existence of patent transendothelial channels. *J. Cell Biol.* 64:586-607.

Simons, K., and E. Ikonen. 1997. Functional rafts in cell membranes. *Nature (Lond.)*. 387:569-72.

Simons, K., and D. Toomre. 2000. Lipid rafts and signal transduction. *Nat. Rev. Mol. Cell Biol.* 1:31-9.

Simpson, J.C., D.C. Smith, L.M. Roberts, and J.M. Lord. 1998. Expression of mutant dynamin protects cells against diphtheria toxin but not against ricin. *Exp. Cell Res.* 239:293-300.

Simson, R., B. Yang, S.E. Moore, P. Doherty, F.S. Walsh, and K.A. Jacobson. 1998. Structural mosaicism on the submicron scale in the plasma membrane. *Biophys J.* 74:297-308.

Singer, S.J., and A. Kupfer. 1986. The directed migration of eukaryotic cells. *Ann. Rev. Cell Biol.* 2:337-365.

Skretting, G., M.L. Torgersen, B. van Deurs, and K. Sandvig. 1999. Endocytic mechanisms responsible for uptake of GPI-linked diphtheria toxin receptor. *J. Cell Sci.* 112:3899-909.

Smart, E.J., G.A. Graf, M.A. McNiven, W.C. Sessa, J.A. Engelman, P.E. Scherer, T. Okamoto, and M.P. Lisanti. 1999. Caveolins, liquid-ordered domains, and signal transduction. *Mol. Cell Biol.* 19:7289-304.

Smart, E.J., Y. Ying, W.C. Donzell, and R.G. Anderson. 1996. A role for caveolin in transport of cholesterol from endoplasmic reticulum to plasma membrane. *J. Biol. Chem.* 271:29427-35.

Smart, E.J., Y.S. Ying, P.A. Conrad, and R.G. Anderson. 1994. Caveolin moves from caveolae to the Golgi apparatus in response to cholesterol oxidation. *J. Cell Biol.* 127:1185-97.

Smythe, E., L.L. Carter, and S.L. Schmid. 1992. Cytosol- and clathrin-dependent stimulation of endocytosis in vitro by purified adaptors. *J. Cell Biol.* 119:1163-71.

Sofer, A., and A.H. Futerman. 1995. Cationic amphiphilic drugs inhibit the internalization of cholera toxin to the Golgi apparatus and the subsequent elevation of cyclic AMP. *J. Biol. Chem.* 270:12117-22.

Song, K.S., S. Li, T. Okamoto, L.A. Quilliam, M. Sargiacomo, and M.P. Lisanti. 1996a. Co-purification and direct interaction of Ras with caveolin, an integral membrane protein of caveolae microdomains. Detergent-free purification of caveolae microdomains. *J. Biol. Chem.* 271:9690-7.

Song, K.S., P.E. Scherer, Z. Tang, T. Okamoto, S. Li, M. Chafel, C. Chu, D.S. Kohtz, and M.P. Lisanti. 1996b. Expression of caveolin-3 in skeletal, cardiac, and smooth muscle cells. Caveolin-3 is a component of the sarcolemma and co-fractionates with dystrophin and dystrophin-associated glycoproteins. *J. Biol. Chem.* 271:15160-5.

Sorkin, A. 2000. The endocytosis machinery. *J. Cell Sci.* 113(Pt:4375-4376.

Spangler, B.D. 1992. Structure and function of cholera toxin and the related Escherichia coli heat-labile enterotoxin. *Microbiol Rev.* 56:622-47.

Stang, E., J. Kartenbeck, and R.G. Parton. 1997. Major histocompatibility complex class I molecules mediate association of SV40 with caveolae. *Mol. Biol. Cell.* 8:47-57.

Stracke, M.L., R. Guirguis, L.A. Liotta, and E. Schiffman. 1987. Pertussis toxin inhibits stimulated motility independently of the adenylate cyclase pathway in human melanoma cells. *Biochem. Biophys. Res. Comm.* 146:339-345.

Subtil, A., I. Gaidarov, K. Kobylarz, M.A. Lampson, J.H. Keen, and T.E. McGraw. 1999. Acute cholesterol depletion inhibits clathrin-coated pit budding. *Proc. Natl. Acad. Sci. U.S.A.* 96:6775-80.

Sun, Y.J., C.C. Chou, W.S. Chen, R.T. Wu, M. Meng, and C.D. Hsiao. 1999. The crystal structure of a multifunctional protein: phosphoglucose

isomerase/autocrine motility factor/neuroleukin. *Proc. Natl. Acad. Sci. U.S.A.* 96:5412-5417.

Tahir, S.A., G. Yang, S. Ebara, T.L. Timme, T. Satoh, L. Li, A. Goltsov, M. Ittmann, J.D. Morrisett, and T.C. Thompson. 2001. Secreted caveolin-1 stimulates cell survival/clonal growth and contributes to metastasis in androgen-insensitive prostate cancer. *Cancer Res.* 61:3882-5.

Takanami, I., K. Takeuchi, M. Naruke, S. Kodaira, F. Tanaka, H. Watanabe, and A. Raz. 1998. Autocrine motility factor in pulmonary adenocarcinomas: results of an immunohistochemical study. *Tumour Biol.* 19:384-9.

Takanami, I., K. Takeuchi, H. Watanabe, T. Yanagawa, and K. Takagishi. 2002. Autocrine motility factor receptor gene expression and cell motility in lung cancer cell lines. *Oncol Rep.* 9:125-8.

Takanami, I., K. Takeuchi, H. Watanabe, T. Yanagawa, K. Takagishi, and A. Raz. 2001. Significance of autocrine motility factor receptor gene expression as a prognostic factor in non-small-cell lung cancer. *Int. J. Cancer.* 95:384-7.

Takei, K., and V. Haucke. 2001. Clathrin-mediated endocytosis: membrane factors pull the trigger. *Trends Cell Biol.* 11:385-91.

Takei, K., V.I. Slepnev, V. Haucke, and P. De Camilli. 1999. Functional partnership between amphiphysin and dynamin in clathrin- mediated endocytosis. *Nat. Cell Biol.* 1:33-9.

Takizawa, P.A., J.K. Yucei, B. Veit, D.J. Faulkner, T. Deerinck, G. Soto, M. Ellisman, and V. Malhotra. 1993. Complete vesiculation of Golgi

membranes and inhibition of protein transport by a novel sea sponge metabolite, ilimaquinone. *Cell.* 73:1079-1090.

Tang, Z., P.E. Scherer, T. Okamoto, K. Song, C. Chu, D.S. Kohtz, I. Nishimoto, H.F. Lodish, and M.P. Lisanti. 1996. Molecular cloning of caveolin-3, a novel member of the caveolin gene family expressed predominantly in muscle. *J. Biol. Chem.* 271:2255-61.

Taniguchi, K., Y. Yonemura, N. Nojima, Y. Hirono, S. Fushida, T. Fujimura, K. Miwa, Y. Endo, H. Yamamoto, and H. Watanabe. 1998. The relation between the growth patterns of gastric carcinoma and the expression of hepatocyte growth factor receptor (c-met), autocrine motility factor receptor, and urokinase-type plasminogen activator receptor. *Cancer.* 82:2112-22.

Thomsen, P., K. Roepstorff, M. Stahlhut, and B. van Deurs. 2002. Caveolae are highly immobile plasma membrane microdomains, which are not involved in constitutive endocytic trafficking. *Mol. Biol. Cell.* 13:238-50.

Thyberg, J. 2000. Differences in Caveolae Dynamics in Vascular Smooth Muscle Cells of Different Phenotypes. *Lab Invest.* 80:915-929.

Thyberg, J. 2002. Caveolae and Cholesterol Distribution in Vascular Smooth Muscle Cells of Different Phenotypes. *J. Histochem. Cytochem.* 50:185-196.

Timar, J., M. Trikha, K. Szekeres, R. Baza, J. Tovari, S. Silletti, A. Raz, and K.V. Honn. 1996. Autocrine motility factor signals integrin-mediated metastatic melanoma cell adhesion and invasion. *Cancer Res.* 56:1902-1908.

Timme, T.L., A. Goltsov, S. Tahir, L. Li, J. Wang, C. Ren, R.N. Johnston, and T.C. Thompson. 2000. Caveolin-1 is regulated by c-myc and suppresses c-myc-induced apoptosis. *Oncogene*. 19:3256-65.

Tor, J., C. Pascual, R.M. Segura, J. Vilaseca, and S. Schwartz. 1982. Value of glutathione reductase and glucosephosphate isomerase measurements in infectious diseases. *Rev Clin Esp*. 164:15-8.

Tor, J., C. Pascual, R.M. Segura, J. Vilaseca, S. Schwartz, and J. Guardia. 1981. Glucose phosphate isomerase and glutathione reductase in benign and malignant extrahepatic cholestasis. *Clin Chem*. 27:634-5.

Torgersen, M.L., G. Skretting, B. van Deurs, and K. Sandvig. 2001. Internalization of cholera toxin by different endocytic mechanisms. *J. Cell Sci*. 114:3737-47.

Torimura, T., T. Ueno, M. Kin, R. Harada, T. Nakamura, T. Kawaguchi, M. Harada, R. Kumashiro, H. Watanabe, R. Avraham, and M. Sata. 2001. Autocrine motility factor enhances hepatoma cell invasion across the basement membrane through activation of beta1 integrins. *Hepatology*. 34:62-71.

Tran, D., J.L. Carpentier, F. Sawano, P. Gorden, and L. Orci. 1987. Ligands internalized through coated or noncoated invaginations follow a common intracellular pathway. *Proc. Natl. Acad. Sci. U.S.A*. 84:7957-61.

Tsutsumi, S., S.K. Gupta, V. Hogan, J.G. Collard, and A. Raz. 2002. Activation of small GTPase Rho is required for autocrine motility factor signaling. *Cancer Res*. 62:4484-90.

Tsutsumi, S., S.K. Gupta, V. Hogan, N. Tanaka, K.T. Nakamura, I.R. Nabi, and A. Raz. 2003a. The enzymatic activity of phosphoglucose isomerase is not required for its cytokine function. *FEBS Lett.* 534:49-53.

Tsutsumi, S., V. Hogan, I.R. Nabi, and A. Raz. 2003b. Overexpression of the autocrine motility factor/phosphoglucose isomerase induces transformation and survival of NIH-3T3 fibroblasts. *Cancer Res.* 63:242-9.

Umeda, A., A. Meyerholz, and E. Ungewickell. 2000. Identification of the universal cofactor (auxilin 2) in clathrin coat dissociation. *Eur. J. Cell Biol.* 79:336-42.

Urbani, L., and R.D. Simoni. 1990. Cholesterol and vesicular stomatitis virus G protein take separate routes from the endoplasmic reticulum to the plasma membrane. *J. Biol. Chem.* 265:1919-1923.

van Deurs, B., K. Roepstorff, A. Hommelgaard, and K. Sandvig. 2003. Caveolae: anchored, multifunctional platforms in the lipid ocean. *Trends Cell Biol.* 13:92-100.

van Deurs, B., T.I. Tonnessen, O.W. Petersen, K. Sandvig, and S. Olsnes. 1986. Routing of internalized ricin and ricin conjugates to the Golgi complex. *J. Cell Biol.* 102:37-47.

van Kerkhof, P., M. Smeets, and G.J. Strous. 2002. The Ubiquitin-Proteasome Pathway Regulates the Availability of the GH Receptor. *Endocrinology.* 143:1243-1252.

Varma, R., and S. Mayor. 1998. GPI-anchored proteins are organized in submicron domains at the cell surface. *Nature (Lond.).* 394:798-801.

Vickery, R.G., and M. von Zastrow. 1999. Distinct Dynamin-dependent and -independent Mechanisms Target Structurally Homologous Dopamine Receptors to Different Endocytic Membranes. *J. Cell Biol.* 144:31-43.

Vilhardt, F., M. Nielsen, K. Sandvig, and B. van Deurs. 1999. Urokinase-type plasminogen activator receptor is internalized by different mechanisms in polarized and nonpolarized Madin-Darby canine kidney epithelial cells. *Mol. Cell Biol.* 10:179-195.

Wang, H.-J., N. Benlimame, and I.R. Nabi. 1997. The AMF-R tubule is a smooth ilimaquinone-sensitive subdomain of the endoplasmic reticulum. *J. Cell Sci.* 110:3043-3053.

Wang, H.-J., G. Guay, L. Pogan, R. Sauve, and I.R. Nabi. 2000. Calcium regulates the association between mitochondria and a smooth subdomain of the endoplasmic reticulum. *J. Cell Biol.* 150:1489-1498.

Wang, L.H., K.G. Rothberg, and R.G. Anderson. 1993. Mis-assembly of clathrin lattices on endosomes reveals a regulatory switch for coated pit formation. *J. Cell Biol.* 123:1107-17.

Warren, R.A., F.A. Green, and C.A. Enns. 1997. Saturation of the endocytic pathway for the transferrin receptor does not affect the endocytosis of the epidermal growth factor receptor. *J. Biol. Chem.* 272:2116-21.

Watanabe, H., P. Carmi, V. Hogan, T. Raz, S. Silletti, I.R. Nabi, and A. Raz. 1991a. Purification of human tumor cell autocrine motility factor and molecular cloning of its receptor. *J. Biol. Chem.* 266:13442-13448.

Watanabe, H., I.R. Nabi, and A. Raz. 1991b. The relationship between motility factor receptor internalization and the lung colonizing capacity of murine melanoma cells. *Cancer Res.* 51:2699-2705.

Watanabe, H., K. Takehana, M. Date, T. Shinozaki, and A. Raz. 1996. Tumor cell autocrine motility factor is the neuroleukin/phosphohexose isomerase polypeptide. *Cancer Res.* 56:2960-3.

Werling, D., J. Hope, P. Chaplin, R. Collins, G. Taylor, and C. Howard. 1999. Involvement of caveolae in the uptake of respiratory syncytial virus antigen by dendritic cells. *J Leukoc Biol.* 66:50-58.

Wilde, A., E.C. Beattie, L. Lem, D.A. Riethof, S.H. Liu, W.C. Mobley, P. Soriano, and F.M. Brodsky. 1999. EGF receptor signaling stimulates SRC kinase phosphorylation of clathrin, influencing clathrin redistribution and EGF uptake. *Cell.* 96:677-87.

Williams, T.M., M.W. Cheung, D.S. Park, B. Razani, A.W. Cohen, W.J. Muller, D. Di Vizio, N.G. Chopra, R.G. Pestell, and M.P. Lisanti. 2003. Loss of caveolin-1 gene expression accelerates the development of dysplastic mammary lesions in tumor-prone transgenic mice. *Mol. Biol. Cell.* 14:1027-42.

Wolf, A.A., Y. Fujinaga, and W.I. Lencer. 2002. Uncoupling of the cholera toxin-G(M1) ganglioside receptor complex from endocytosis, retrograde Golgi trafficking, and downstream signal transduction by depletion of membrane cholesterol. *J. Biol. Chem.* 277:16249-56.

Xu, W., K. Seiter, E. Feldman, T. Ahmed, and J.W. Chiao. 1996. The differentiation and maturation mediator for human myeloid leukemia cells shares homology with neuroleukin or phosphoglucose isomerase. *Blood.* 87:4502-4506.

Yakirevich, E. and Y. Naot. 2000. Cloning of a glucose phosphate isomerase/neuroleukin-like sperm antigen involved in sperm agglutination. *Biol. Reprod.* 62:1016-23.

Yamada, E. 1955. The fine structure of the gall bladder epithelium of the mouse. *J. Biophys. Biochem. Cytol.* 1:445-448.

Yamamoto, M., Y. Toya, R.A. Jensen, and Y. Ishikawa. 1999. Caveolin is an inhibitor of platelet-derived growth factor receptor signaling. *Exp. Cell Res.* 247:380-8.

Yang, G., L.D. Truong, T.L. Timme, C. Ren, T.M. Wheeler, S.H. Park, Y. Nasu, C.H. Bangma, M.W. Kattan, P.T. Scardino, and T.C. Thompson. 1998. Elevated expression of caveolin is associated with prostate and breast cancer. *Clin Cancer Res.* 4:1873-80.

Yang, G., L.D. Truong, T.M. Wheeler, and T.C. Thompson. 1999. Caveolin-1 expression in clinically confined human prostate cancer: a novel prognostic marker. *Cancer Res.* 59:5719-23.

Zhang, B., Y.H. Koh, R.B. Beckstead, V. Budnik, B. Ganetzky, and H.J. Bellen. 1998. Synaptic vesicle size and number are regulated by a clathrin adaptor protein required for endocytosis. *Neuron.* 21:1465-75.

Zhao, X., T. Greener, H. Al-Hasani, S.W. Cushman, E. Eisenberg, and L.E. Greene. 2001. Expression of auxilin or AP180 inhibits endocytosis by mislocalizing clathrin: evidence for formation of nascent pits containing AP1 or AP2 but not clathrin. *J. Cell Sci.* 114:353-65.

Zhao, Y.Y., Y. Liu, R.V. Stan, L. Fan, Y. Gu, N. Dalton, P.H. Chu, K. Peterson, J. Ross, Jr., and K.R. Chien. 2002. Defects in caveolin-1 cause dilated cardiomyopathy and pulmonary hypertension in knockout mice. *Proc. Natl. Acad. Sci. U.S.A.* 99:11375-80.

Zhi, J., D.W. Sommerfeldt, C.T. Rubin, and M. Hadjiargyrou. 2001. Differential expression of neuroleukin in osseous tissues and its involvement in mineralization during osteoblast differentiation. *J Bone Miner Res.* 16:1994-2004.

Zundel, W., L.M. Swiersz, and A. Giaccia. 2000. Caveolin 1-mediated regulation of receptor tyrosine kinase-associated phosphatidylinositol 3-kinase activity by ceramide. *Mol. Cell Biol.* 20:1507-14.

Zwaagstra, J.C., M. El-Alfy, and M.D. O'Connor-McCourt. 2001. Transforming growth factor (TGF)-beta 1 internalization: modulation by ligand interaction with TGF-beta receptors types I and II and a mechanism that is distinct from clathrin-mediated endocytosis. *J. Biol. Chem.* 276:27237-45.

VII. LISTE DES PUBLICATIONS

1. **Phuong U. Le,** Trung N. Nguyen, Patrick Drolet-Savoie, Nicole Leclerc and Ivan R. Nabi **1998.** Increased ß-actin expression in an invasive MSV-MDCK cell variant concentrates to the tips ofmultiple pseudopodia. Cancer Res.58, 1381-1385.

2. Naciba Benlimame, **Phuong U. Le** and Ivan R. Nabi **1998.** Localization of Autocrine Motility Factor Receptor to caveolae and clathrin-independent internalization of its ligand to smooth endoplasmic reticulum. Mol. Biol. Cell 9, 1773-1786.

3. Lagana, A., J. Vadnais, **P.U. Le**, T. Nguyen, R. Laprade, I.R. Nabi, and J. Noël. **2000**. Regulation of the formation of tumor cell pseudopodia by the Na-H exchanger NHE1. *J. Cell Sci.* 113:3649-3662.

4. **Le, P.U.**, N. Benlimame, A. Lagana, A. Raz, and I.R. Nabi. **2000**. Clathrin-mediated endocytosis and recycling of autocrine motility factor receptor to fibronectin fibrils is a limiting factor for NIH-3T3 cell motility. *J. Cell Sci.* 113:3227-3240.

5. **Le, P.U.**, G. Guay, Y. Altschuler, and I.R. Nabi. **2002**. Caveolin-1 is a negative regulator of caveolae-mediated endocytosis to the endoplasmic reticulum. *J. Biol. Chem.* 277:3371-9, ainsi que la page de couverture.

6. **Le, P.U.** and I.R. Nabi. **2003**. Distinct caveolae-mediated endocytic pathways target the Golgi apparatus and the endoplasmic reticulum.. *J. Cell Sci.*116 :1059-1071.

7. Nabi, I.R. and **Le, P.U. 2003**. Caveolae/raft-dependent endocytosis. *J. Cell Biol.* 161:673-677.

8. Pang, H., **Le, P.U.** and Nabi, I.R. **2004**. Ganglioside GM1 levels are a determinant of the extent of caveolae/raft-dependent endocytosis of cholera toxin to the Golgi apparatus. *J. Cell Sci.* Mars 2.