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Characterization of the Autocrine Motility Factor Receptor Tubule, A Subdomain of the Endoplasmic Reticulum

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SUMMARY

Autocrine motility factor (AMF) is a cytokine produced and secreted by tumor cells inducing both random and directed cell migration of producing cells. Interaction of AMF with its receptor, AMF-R, is able to stimulate cellular motility in both normal and cancer cells. AMF-R is expressed on the surface of motile cells, and has also been found within a Triton X-100 extractable intracellular compartment. By immunofluorescence microscopy, AMF-R labeling is localized to a distinct tubular membranous organelle that is oriented towards the periphery of the cell. AMF-R tubules can be distinguished from other organelles including the endosomes, lysosomes, Golgi apparatus and the rough endoplasmic reticulum. Electron microscopy shows AMF-R to be localized predominantly to smooth vesicular and tubular membranous organelles. Some smooth tubules labeled by anti-AMF-R antibody are continuous with rough endoplasmic reticulum, suggesting that AMF-R tubules are associated with the endoplasmic reticulum.

To better characterize AMF-R tubules and their relationship with other organelles in the cell, immunofluorescence microscopy and immunoelectron microscopy were used to study AMF-R tubules *in vivo* and *in vitro*. Ilimaquinone (IQ) is a sea sponge metabolite that was previously shown to induce the complete vesiculation of the Golgi apparatus. By double immunofluorescence labeling, the punctate AMF-R label resembles that of the Golgi membranes in cells treated with IQ, however, the two labels can be distinguished by confocal microscopy. Electron microscopy of IQ treated cells reveals that AMF-R is distributed to fenestrated networks of narrow interconnected tubules distinct from the uniform Golgi derived vesicles, and morphologically equivalent to smooth ER. Smooth AMF-R labeled tubules exhibit continuity with rough ER cisternae, and IQ selectively targets smooth and not rough ER. AMF-R tubules can be distinguished from the ER-Golgi

intermediate compartment labeled for ERGIC-53 by confocal microscopy and thus constitute a distinct IQ-sensitive subdomain of the smooth ER.

Significant overlap between the labeling of AMF-R tubules and mitochondria is observed by IF. By EM, anti-AMF-R labeled smooth tubules are often observed adjacent to mitochondria. Quantification of the post-embedding labeling of smooth tubules, rough ER and mitochondria reveals that the majority of AMF-R labeling is associated with smooth tubules and vesicles. The degree of overlap of AMF-R tubules and mitochondria was quantified from confocal images. The association between AMF-R tubules and mitochondria varies in different cell types and can be disrupted by incubation with PBS-CM.

In semi-intact MDCK cells produced by permeabilization with digitonin, the association of AMF-R tubules and mitochondria is maintained. Decreased overlap is observed in semi-intact MDCK cells incubated with either rat liver cytosol, or cytosol plus GTPγS compared to control cells. The effect of cytosol is not NEM sensitive and does not require ATP, suggesting that homotypic fusion is not involved in this process. Cytosol depleted of calcium-binding proteins is ineffective might implicating calcium-binding proteins in the dissociation of AMF-R tubules from mitochondria.

In conclusion, we have demonstrated that AMF-R tubules are a distinct IQ-sensitive subdomain of the smooth ER that is associated with mitochondria. Dissociation of AMF-R tubules from mitochondria is regulated by cytosolic proteins, and is not mediated by homotypic fusion. The fact that mitochondria are the major energy producer of the cell may be the basis for the association between these two organelles. Howover, the relationship between AMF-R and mitochondria remains unclear.

Key words: Autocrine motility factor receptor (AMF-R) tubule, Smooth endoplasmic reticulum, Ilimaquinone, Mitochondria

SOMMAIRE

L'AMF est une cytokine sécrétée par les cellules tumorales qui induit la migration cellulaire aléatoire et directionelle des cellules productrices. L'interaction de l'AMF avec son récepteur, L'AMF-R, est apte à stimuler la motilité des cellules normales et cancéreuses. L'AMF-R est exprimé à la fois à la surface des cellules motiles ainsi qu'au niveau d'un compartiment intracellulaire extractable au Triton X-100. La microscopie à fluorescence montre la localisation de l'AMF-R au niveau d'un organite tubulaire membranaire orienté vers la péripherie de la cellule distinct des autres organites intracellulaires incluant les endosomes, les lysosomes, l'appareil de Golgi, et le réticulum endoplasmique. La microscopie électronique permet de localiser l'AMF-R principalement au niveau des membranes vésiculaires et tubulaires lisses. Aussi, quelques tubules lisses exprimant l'AMF-R sont en continuité avec le réticulum endoplasmique rugueux, suggèrant que les tubules d'AMF-R sont associés avec le réticulum endoplasmique.

Afin de mieux caractériser les tubules d'AMF-R ainsi que leur relation avec les autres organites cellulaires, ces tubules furent étudiés *in vivo* et *in vitro* par microscopies à fluorescence et électronique. L'ilimaquinone (IQ) est un métabolite d'une éponge de mer qui induit la vésicularisation de l'appareil de Golgi. Suite à un traitement à l'IQ, le marquage ponctuel de l'AMF-R observé en immunofluorescence ressemble à celui de l'appareil de Golgi; cependant, les vésicules provenant de ces deux organites peuvent être distinguées par microscopie confocale. De plus, la microscopie électronique démontre que l'AMF-R se trouve au niveau des réseaux fenestrés des tubules minces dans les cellules traitées avec IQ. Ces réseaux ne correspondent pas aux vésicules uniformes dérivées de l'appareil de Golgi et resemblent morphologiquement au réticulum endoplasmique lisse. Les tubules lisses d'AMF-R montrent une continuité avec les tubules rugueux du

réticulum endoplasmique, mais l'IQ n'en affecte que la portion lisse. Les tubules d'AMF-R sont distingués du compartiment intermédiaire RE-Golgi (ERGIC) par microscopie confocale et constituent donc un sous-domaine distinct du réticulum endoplasmique lisse sensible à l'IQ.

Par microscopie à fluorescence, on remarque un fort chevauchement entre le marquage des tubules d'AMF-R et celui des mitochondries. Aussi, la microscopie électronique montre que les tubules lisses marqués par l'AMF-R sont souvent étroitement associés aux mitochondries. La quantification du marquage de l'anti-AMF-R en post-enrobage démontre que la majorité du marquage de l'AMF-R est associé aux tubules lisses et non au réticulum endoplasmique rugueux, ni aux mitochondries. L'association entre les tubules d'AMF-R et les mitochondries varie entre les types cellulaires et est modulée par l'incubation des cellules avec PBS-CM.

Dans les cellules MDCK semi-intactes perméabilisées avec la digitonine, l'association entre les tubules d'AMF-R et les mitochondries est maintenue. Cette association est réduite par une incubation avec le cytosol extrait de foie de rat et également par ce cytosol en présence de GTPγS. L'effet du cytosol n'est cependant pas inhibé par le NEM et ne nécessite pas l'ATP, suggèrant que la fusion homotypique n'est pas responsable de la dissociation des tubules d'AMF-R et des mitochondries. Suite à la déplétion de l'extrait cytosolique des protéines liant le calcium, le cytosol n'a plus d'effet sur l'association entre les tubules d'AMF-R et les mitochondries. Les protéines cytosoliques liant le calcium penvent donc être impliquées dans la dissociation de ces tubules et des mitochondries.

En conclusion, nous avons demontré que le tubule d'AMF-R est un sous-domaine lisse du réticulum endoplasmique sensible à l'IQ et associé aux mitochondries. La dissociation des tubules AMF-R des mitochondries est regulée par les protéines cytosoliques et non pas par la fusion homotypique. Les mitochondries sont le producteur majeur de l'énérgie de la cellule, une fonction qui peut être impliquée dans l'association

entre ces deux organites. Cependant, la nature de la relation entre les tubules d'AMF-R et les mitochondries reste à élucider.

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ABBREVIATION

AMF:	Autocrine Motility Factor
AMF-R:	Autocrine Motility Factor Receptor
ARS:	ATP Regenerating System
BFA:	Brefeldin A
BSA:	Bovine Serum Albumin
CM:	Calcium and Magnesium
COP:	Coat protein
CSB:	Cytoskeleton Stabilizing Buffer
CBPDC:	Calcium-Binding Protein Depleted Cytosol
DMEM:	Dulbecco's Modified Eagle Medium
DTT:	Dithiothreitol
ER:	Endoplasmic Reticulum
GA:	Golgi Apparatus
GTP:	Guanosine Triphosphate
LAMP:	Lysosomal Associated Membrane Protein
IQ:	Ilimaquinone
mAb:	Monoclonal antibody
MDCK:	Madin-Darby Canine Kidney
MTOC:	Microtubule Organizing Center
NEM:	N-ethylmaleimide
NSF:	N-ethylmaleimide Sensitive Factor
PBS:	Phosphate-Bufferd Saline
PT:	Pertussis Toxin
SNAPs:	Soluble NSF Associated Proteins

TGN: Trans- Golgi Network

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1. INTRODUCTION

1.1 Autocrine Motility Factor and Autocrine Motility Factor Receptor

1.1.1 Autocrine Motility Factor

Autocrine motility factor (AMF), is a 55 kDa cytokine produced and secreted by tumor cells which induces both random and directed cell migration of producing cells (Liotta et al., 1986). It has been purified from human A2058 melanoma (Liotta et al., 1986), murine B16-F1 melanoma (Silletti et al., 1991) and human HT-1080 fibrosarcoma cells (Watanabe et al., 1991b) and these three purified AMFs appear to represent identical or homologous molecules which can stimulate cell motility.

AMF stimulates the motility of A2058 human melanoma, HeLa human cervical carcinoma, MCF-7 human breast carcinoma, HT-1080 human fibrosarcoma, UV-2237-IP3 murine fibrosarcoma, J82 human bladder carcinoma, NIH-3T3 and Balb/c-3T3-A31 untransformed murine fibroblasts, the metastatic angiosarcoma variant of the Balb/c3T3-A31 fibroblast and various subpopulations of the K-1735 and B16 murine melanoma cell lines; however, AMF does not stimulate T lymphocyte or neutrophil motility (Liotta et al., 1986; Nabi et al., 1990; Nabi et al., 1992; Silletti et al., 1994; Silletti et al., 1993; Watanabe et al., 1991b).

Recent experiments demonstrated that AMF is identical to the previously cloned cytokine neuroleukin (NLK) and enzyme phosphohexose isomerase (PHI) (Watanabe et al., 1996). This polypeptide has been

implicated in cell motility and identified as a marker of cancer progression (Guirguis et al., 1987; Gurney, 1984). Murine AMF exhibits the enzymatic properties of PHI and rabbit heart PHI stimulated the motility of mouse fibrosarcoma cells in a similar fashion to AMF. Specific PHI inhibitors (carbohydrate phosphates) inhibited enzymatic activity and AMF-induced cell motility (Watanabe et al., 1996).

In vitro the secretion of AMF is confined to neoplastic transformed cells (Liotta et al., 1986), and in vivo AMF was detected in the urine of patients with bladder carcinoma, potentially allowing the development of prognosis and/or diagnostic markers for transitional cell carcinoma (TCC) of the bladder (Guirguis et al., 1988). In cultured cells, AMF production and secretion is restricted to transformed cells, but AMF stimulates the motility of both transformed and nontransformed cells which exhibit an equivalent motile response to AMF (Liotta et al., 1986). The transformed cells, which both produce AMF and express its receptor could stimulate their own motility in an autocrine fashion.

1. 1. 2. Autocrine Motility Factor Receptor

Autocrine motility factor receptor (AMF-R, gp78), a 78 kDa glycoprotein, was initally characterized in murine B16-F1 melanoma cells and human AMF-R has been cloned (Nabi et al., 1990; Silletti et al., 1991; Watanabe et al., 1991b) Identification of AMF-R as a motility factor receptor was initially obtained from the inhibition of binding of anti-gp78 mAb to gp78 in immunoblots by AMF-containing conditioned media; heat-inactivated AMF was incapable of blocking recognition of gp78 by the

antibody suggesting that AMF and anti-gp78 mAb bind to the same motility-activating epitope on gp78 (Nabi et al., 1990). Confirmation of the identity of gp78 as the receptor for AMF was obtained from the studies of gp78 immunopurified from B16-F1 melanoma cells and AMF purified from B16-F1 conditioned media by Sephacryl S-200 chromatography (Silletti et al., 1991).

AMF-R mediates cell motility via a receptor mediated signal pathway. The AMF motile response in melanoma occurs via a pertussis toxin (PT) sensitive G-protein-mediated pathway (Nabi et al., 1990; Silletti et al., 1991; Stracke et al., 1987). AMF binding to AMF-R initiates a signalling cascade which includes a bordella pertussis toxin-sensitive G-protein, inositol phosphate production, arachidonic acid metabolism, protein kinase C activity, and phosphorylation of AMF-R (Silletti and Raz, 1996). AMF binding to its receptor stimulates the increased incorporation of inositol into cellular lipids and inositol phosphates, in particular inositol triphosphates (Kohn et al., 1990). Inositol triphosphate expression levels are directly correlated with induced cell motility in response to different levels of AMF and are partially inhibited by pretreatment of the cells with pertussis toxin, but not by cholera toxin or other inhibitors of the cAMP metabolic pathway (Kohn et al., 1990).

AMF stimulates the extension of cell pseudopodia which exhibit prominant axial actin filament bundles. Pseudopodia isolated from cells stimulated by AMF contain 20 times more laminin and fibronectin receptors than those found in the plasma membrane of unstimulated cells (Guirguis et al., 1987). Thus AMF binding to its receptor initiates a signaling cascade, which induces pseudopodial extension and translocation of extracellular matrix receptor to the leading edge of the cell (Nabi et al., 1992).

Interaction of AMF-R with AMF stimulates cellular motility in both normal and cancer cells. In cultured cells, the secretion of AMF is restricted to transformed cells, while the expression of AMF-R is found both in transformed and nontransformed cells. NIH-3T3 fibroblasts, for example, do not secrete AMF (Liotta et al., 1986), but they do respond to tumor-derived AMF. AMF purified from HT-1080 human fibrosarcoma cells stimulates NIH-3T3 cell growth and motility. The expression of cell surface AMF-R is down-regulated in contact-inhibited NIH-3T3 cells and AMF stimulates the healing of experimentally wounded, density-arrested NIH-3T3 monolayer cultures (Silletti and Raz, 1993). Expression of AMF-R in normal cells is regulated by cell contact whereas in transformed cells AMF-R is constitutively expressed irrespective of cell density (Silletti et al., 1996). This motility inducing effect can be inhibited by PT, suggesting that AMF motility signal transduction in NIH-3T3 cells is the same as described previously for human (Silletti et al., 1991) and murine (Stracke et al., 1987) melanoma cells.

AMF-R expression in tumor cells is related to cellular invasive and metastatic ability. In epithelial cells, down-regulation of E-cadherin, a Ca^{++} dependent intercellular adhesion molecule, plays a role in tumor cell invasion and metastasis (Frixen et al., 1991; Schipper et al., 1991). Transformation of the polarized epithelial MDCK cell line with Moloney sarcoma virus generates the MSV-MDCK cell line which is highly invasive (Behrens et al., 1989). The increased cellular motility and decreased E-cadherin expression of MSV-MDCK cells is associated with significantly increased (8-fold) AMF-R levels compared to MDCK cells (Simard and Nabi, 1996). In human bladder carcinoma, positive expression of E-cadherin and negative expression of AMF-R were found to be associated with low

risk of clinical progression in superficial bladder carcinoma patients, while reduction in E-cadherin concomitantly with an increase in AMF-R expression was associated with poor prognosis (Otto et al., 1994). Inverse relation of AMF-R and E-cadherin expression may be characteristic of invasive tumor cells of epithelial origin.

AMF-R expression and distribution in tumor cells may have important implications for clinical cancer research. In human choriocarcinoma, AMF-R expression and location on the cell surface is related to motility of tumor cells; choriocarcinoma cells which exhibit AMF-R receptor clustering on the cell surface respond to AMF-R stimulation with increased cell motility, while term placenta cells which express decreased AMF-R levels and no receptor clustering are unresponsive (Yelian et al., 1996). AMF-R expression was also correlated with increased ability to colonize lungs in an experimental tumor system (Watanabe et al., 1991a). Up-regulation of AMF-R expression is associated with invasion and metastasis in experimental tumour systems (Watanabe et al., 1991b) and in human neoplasms including human gastric (Hirono et al., 1996), bladder (Otto et al., 1994) and colorectal (Nakamori et al., 1994) tumors. AMF-R detection in the urine is a possible marker for transitional cell carcinoma of the bladder (Korman et al., 1996). AMF-R expression therefore correlates with tumor progression and can serve as a marker for tumor malignancy.

1. 1. 3. AMF-R tubule

While AMF-R is expressed on the surface of motile cells, the bulk of the receptor is intracellular (Nabi et al., 1992). AMF-R has been found within a Triton X-100 extractable intracellular compartment (Benlimame et

al., 1995). By immunofluorescence microscopy, AMF-R labeling is localized to a distinct tubular membranous organelle that is oriented towards the periphery of the cell. The AMF-R tubules can be distinguished from endosomes, lysosomes, the Golgi apparatus and rough endoplasmic reticulum by double immunofluorescence labeling. Electron microscopy shows AMF-R is localized predominantly to smooth vesicular and tubular membranous organelles with a variable diameter of 50-250nm, which can form elaborate networks (Benlimame et al., 1995). However, ribosome studded tubules and some labeled smooth tubules are continuous with rough ER, suggesting that AMF-R tubules are associated with the ER (Benlimame et al., 1995). Cycloheximide treatment shows that the AMF-R tubule is not a biosynthetic subcompartment of ER (Benlimame et al., 1995).

The linear morphology, extension and orientation of AMF-R tubules is dependent on microtubule integrity. In cells preincubated in a cytoskeleton-stabilizing buffer (Schliwa et al., 1981), fixed in cold methanol and labeled for AMF-R by indirect immunofluoresence, AMF-R tubules are localized to a network of elongated tubular-vesicular structures in both the periphery and center of the cell aligned with microtubules. Following depolymerization or stabilization of microtubules by nocodazole or taxol in subconfluent MDCK cells, AMF-R tubules lose their linear extension and orientation towards the periphery of the cell (Benlimame et al., 1995).

1. 2. The secretory apparatus and secretory pathway

The secretory apparatus is composed of the endoplasmic reticulum (ER) and Golgi apparatus (GA). Usually located in the perinuclear region of the cells, the GA presents a network of interconnected, highly convoluted

membranous cisternae and organized stacks of flattened saccules and associated secretory vesicles. Newly synthesized exocytic proteins traverse, sequentially, the ER and GA en route to the cell surface from which they are exocytosed (Copeland et al., 1988; Vertel et al., 1992). During this passage, the proteins are folded, assembled, and modified by glycosylation and trimming reactions, among numerous other modifications (Hirscherg and Snider, 1987). The mechanism by which proteins are transported from the ER to the GA and through the GA is thought to involve vesicular carriers. In addition to secretory proteins, membrane proteins and lysosomal enzymes also pass through the secretory apparatus to reach their final destinations. The important role of the GA in sorting is best characterized for the trans-Golgi network, although other elements in the secretory pathway may be involved (Mellman and Simons, 1992; Wong and Hong, 1993).

1. 2.1. Endoplasmic reticulum

The endoplasmic reticulum (ER) consists of a continuous membrane network linked to the nuclear envelope and is present throughout the cytoplasm of eukaryotic cells as a three dimensional network of continuous tubules and sheets. It is a dynamic structure and constitutes the major membrane pool of the cell. Classically, it is divided to three types which share direct membrane continuities and are regionally segregated: rough ER, smooth ER and transitional ER. Morphologically, rough ER is studded with ribosomes, smooth ER has no ribosomes bound and transitional ER consists of tubules which are half rough/half smooth and represents connections between rough and smooth ER (Palade, 1975). Functionally, rough ER is implicated in protein synthesis. Smooth ER is implicated in

lipid synthesis, glycogen storage and breakdown, the detoxification of harmful substance and some specialized functions (Vertel et al., 1992). Transitional ER connects both rough ER and smooth ER and is though to be the site from which exocytotic proteins are transported from the ER en route to the GA (Palade, 1975).

Specific markers for the RER, appear to be related, directly or indrectly, to ribosome binding and cotranslational translocation of polypeptide chains (Kreibich and Sabatini, 1992; Walter, 1992). For the SER, only a single marker, an epoxide hydrolase has been described (Galteau et al., 1985).

1. 2. 1. 1. Rough ER

The rough ER membrane is continuous with the nuclear envelope. Because of the presence of ribosomes on the ER membrane, the membrane seems "rough", and can be easily distinguished from smooth ER to which no ribosomes are bound. These membrane-bound ribosomes attached to the cytosolic side of the ER membrane are engaged in the synthesis of proteins that are concurrently translocated into the ER. Protein translation, the process by which the sequence of nucleotides in a messenger RNA molecule directs the incorporation of amino acids into a protein, and cotranslational activity are largely restricted to the rough ER subcompartment. For those proteins directed to the ER, ribosomes, mRNA, and the nascent polypeptide form a complex in the cytosol with a ribonucleoprotein signal recognition particle. This complex then interacts with signal recognition particle receptor and probably other proteins located in the membrane of the rough ER to form higher order complexes that function in the translation of the

nascent peptide (Vertel et al., 1992). After formation of the translocation complex, translocation continues and the nascent peptide is translocated across the rough ER membrane (Lingappa, 1991; Walter and Lingappa, 1986). In the ER lumen, the hydrophobic signal peptide is cleaved by signal peptidase and N-asparagine-linked oligosaccharides are added cotranslationally from a dolichol phosphate intermediate embedded in the ER membrane (Kornfeld and Kornfeld, 1985). Alterations in N-glycosylation, in many cases, have been implicated in the misfolding and faulty oligomerization of secretory glycoproteins (Machamer and Rose, 1988). Misfolded and incorrectly assembled proteins may be aggregated, retained, and sometimes degraded in the ER.

Free ribosomes in the cytosol which are unattached to the ER membrane synthesize all other proteins encoded by the nuclear genome. Membrane-bound and free ribosomes are structurally and functionally identical. They differ only in the proteins they synthesize at any given time and their association with the ER membrane (Alberts et al., 1994).

1. 2. 1. 2 Transitional ER

Transitional ER is the subcompartment of the ER that consist of part smooth/part rough tubules or sheets. It is the site where newly synthesized proteins exit from the ER en route to the Golgi (Palade, 1975). The transitional ER subcompartment is connected to both rough and smooth ER and morphologically represents sites of interaction between rough and smooth subdomains of the ER. The expansion of smooth ER in some cells suggests the existence of post-rough ER compartments, perhaps related to the transitional ER, that have some specialized functions. Several lines of

evidence suggest that one such compartment mediates oligosaccharide modification (Kabcenell and Atkinson, 1985). The budding compartment for mouse hepatitis virus A59 is located between the rough ER and cis-Golgi and bears morphological features characteristic of the transitional elements (Tooze et al., 1988). Mutant chondrocytes accumulate the truncated chondroitin sulfate proteoglycan precursor in smooth membrane-limited regions continuous with the rough ER and recycling of resident ER proteins via the KDEL-receptor also occurs in this compartment (Vertel et al., 1993). This compartment may be similar to what is now known as ER-Golgi intermediate compartment (see section 1. 2. 2.)

1. 2. 1. 3. Smooth ER

Smooth ER is a subcompartment of the ER. It is not involved in protein synthesis, but it does synthesize lipids in some cells. Although the smooth ER has no ultrastructural features that allow the clear discrimination of separate domains, it is likely that it is comprised of a number of separate subcompartments that serve a range of different functions.

Smooth ER in different cell lines plays different roles. It is abundant in cells active in lipid metabolism, steroidogenesis, glycogen storage and breakdown, and the detoxification of harmful substances (Christensen, 1975; Fawcett, 1981). For example, in testicular Leydig cells, the smooth ER comprises 60% of the total membrane area and functions in the synthesis of cholesterol and its conversion to testosterone (Mori and Christensen, 1980). Hepatocytes detoxify harmful substances through oxidation, hydroxylation or methylation reactions catalyzed by enzymes located in the smooth ER

(e.g. the cytochrome P450s) (Vertel et al., 1992). The amount of smooth ER in hepatocytes of hamster and rat liver increases rapidly when an animal is exposed to certain drugs such as phenobarbital, and this expansion correlates with drug tolerance. After removal of the drug, the smooth ER decreases by unknown mechanisms (Jones and Fawcett, 1966; Staubli et al., 1969).

The sarcoplasmic reticulum

The sarcoplasmic reticulum (SR), a unique form of smooth ER in the skeletal muscle cell, is comprised of longitudinal cisternae surrounding myofibrils and terminal cisternae specifically associated with the T tubules of the sarcolemma. The separate subcompartments of the SR are involved in different aspects of calcium regulation. Terminal cisternae function in signalling calcium release and storing high concentrations of calcium, while longitudinal cisternae function in the removal of calcium from cytosol via a calcium ATPase (Fleischer, 1985). In Purkinje neurons, the smooth ER appears to function in the uptake, storage, and release of intracellular calcium in a manner analagous to the muscle sarcoplasmic reticulum (Takei et al., 1992).

In various nonmuscle cell types, calciosomes (small cytoplasmic vacuoles) contain proteins similar to calsequestrin, the Ca^{++} storage protein of the sarcoplasmic reticulum, and are capable of both high affinity uptake and rapid triggered release of Ca^{++}. Individual calciosomes appear as small (105nm) membrane-bound vacuoles intermingled with, and often apposed to ER cisternae and mitochondria. Other calciosomes are scattered in the Golgi area, in between zymogen granules and beneath the plasma membrane. Calciosomes appear as unique cytological entities, ideally

equipped to play a role in the rapid-scale control of the cytosolic free Ca^{++} in nonmuscle cells (Hashimoto et al., 1988). It has been found that ER cisternal stacks do not represent independent Ca^{++} stores, but operate coordinately with adjacent ER cisternae. The greater Ca^{++} accumulation potential of the calciosome may allow it to function as a rapidly exchanging Ca^{++} store together with the ER (Villa et al., 1991).

The Crystalloid ER

The cystalloid ER, a specialized smooth ER of the compactin (inhibitor of reductase) resistant UT-1 cell (a line of chinese hamster ovary cells), is composed of multiple membrane tubules packed together in a hexagonal pattern. These membrane tubules contains large amounts of 3-hydroxy-3 methylglutaryl coenzyme A (HMG CoA) reductase, an integral membrane protein that enzymatically regulates endogenous cholesterol biosynthesis. Addition of cholesterol or related sterols, such as low density lipoprotein (LDL), to UT-1 cells promotes a rapid and stepwise disappearance of the crystalloid ER. HMG-CoA reductase is synthesized along the outer nuclear membrane and in response to an increased HMG CoA reductase synthesis, smooth ER cisterna emerge from the outer nuclear membrane and then transform into crystalloid ER tubules (Pathak et al., 1986).

1. 2. 1. 4. Degradation of proteins in the ER

Degradation of selected proteins within the ER serves at least two purposes: first, it plays a key regulatory role in cholesterol and lipoprotein metabolism, second, it is used for disposal of proteins that do not comply

with the demands of quality control (Bonifacino and Lippincott-Schwartz, 1991; Klausner and Sitia, 1990).

Proteins that fail to fold correctly or assemble into requisite oligomeric complexes are in most cases not released from the ER (Hurtley and Helenius, 1989; Lodish, 1988; Rose and Doms, 1988). They aggregate and are eventually degraded by a process referred to as pre-Golgi degradation or ER degradation (Klausner and Sitia, 1990; Lippincott-Schwartz, 1988). For example, in the case of secreted immunoglobulins, in the absence of light chains, heavy chains are retained in the ER where they bind to the heavy chain binding protein or chaperone, Bip, and are eventually degraded. Immunoglobulin M (IgM) requires further assembly into polymeric structures which are retained and degraded in the ER if they fail to oligomerize (Amitay et al., 1991). T cell antigen receptor (TCR), a mature antigen receptor found on the surface of T cells, is composed of at least seven transmembrane subunits. Partial complexes or individual subunits are not expressed on the cell surface and are degraded in the ER in T cells as well as in fibroblasts transfected with TCR subunits (Lippincott-Schwartz, 1988).

Degradation of newly synthesized proteins in the ER is distinct from lysosomal degradation. Detailed studies have established several important characteristics that distinguish ER degradation from other mechanisms of intracellular protein disposal. These include insensitivity to lysosomal inhibitors such as ammonium chloride and leupeptin, inhibition at low temperature, a high degree of substrate specificity, and extremely rapid degradation (Lippincott-Schwartz, 1988). The rapid nature of the degradation, combined with the detection of the degraded species only within the ER, suggested the ER itself as the site of proteolysis (Chen et al.,

1988; Lippincott-Schwartz, 1988). A permeabilized cell system identified the ER as a site of protein degradation. Use of chimeric integral membrane proteins such as Tac-TCRa and Tac-TCRb demonstrated that the machinery responsible for pre-Golgi degradation of newly synthesized proteins exists within the ER, and is different from lysosomal degradation as it can operate independently of exogenously added ATP and other cytosolic factors (Stafford and Bonifacino, 1991). A specific determinant present in the transmembrane domain was identified as a targeting signal for ER degradation (Bonifacino et al., 1990a; Bonifacino et al., 1990b). Chimeric proteins containing this degradative sequence were then used in conjunction with a permeabilized cell system and subcellular fractionation to confirm the ER as the site of degradation (Stafford and Bonifacino, 1991).

Proteasomes are large multicatalytic proteinase complexes that perform a large part of proteolytic enzyme activity of the cell. They are located both in the nucleus and in the cytoplasm; some of the cytoplasmic proteasomes are associated with cytosolic side of the ER membrane. It has been shown recently that proteasomes associated with ER membrane are involved in the ER-associated degradation of misfolded or improperly secreted proteins which are retained in the lumen of the ER (Biedered et al., 1996; Hiller et al., 1996; Werner et al., 1996; Yeung et al., 1996).

ER-associated degradation of misfolded proteins takes place on the cytoplasmic side of the ER where the proteasomes are located (Hiller et al., 1996; McCracken and Brodsky, 1996). Proteins for degradation must be translocated from the lumen of the ER into the cytosol for cytoplasmic proteolysis to occur. Recent data indicate that the Sec61 complex, involved in translocation of proteins into the ER, also provides the conduit through

which proteins are extruded back into the cytosol (Pilon et al., 1997; Wiertz et al., 1996).

ER-associated degradation of aberrant proteins such as apolipoprotein B is mediated by the ubiquitin-proteasome pathway degradation (Yeung et al., 1996). A mutated plasma membrane protein, the cystic fibrosis transmembrane conductance regulator (CFTR), is another example of a protein which is retained in the ER membrane and degraded by the ubiquitin-dependent proteasome pathway (Ward et al., 1995). Studies of yeast mutants defective in the breakdown of a mutated soluble vacuolar protein, carboxypeptidase yscY (CPY), has shown that PY entered the ER, was glycosylated and then transported back to the cytoplasmic domain of the ER where it was conjugated with ubiquitin and degraded (Hiller et al., 1996). Experiments have also demonstrated that proteasome mediated degradation of cytochrome P-450 (Roberts, 1997) and other proteins (Werner et al., 1996) may not be preceded by polyubiquitination of the target proteins. Recently it has been shown that proteasomes are also reponsible for degradation of some integral membrane proteins, such as the subunits of the Sec61p complex (Biedered et al., 1996) and HMG-CoA reductase (Hampton et al., 1996).

It is not clear where ER degradation actually takes place. It has been suggested that rapid proteolysis in the rough ER is followed by transport of residual proteins to a second compartment where degradation is completed at an even greater rate (Tsao et al., 1992). In morphological studies, a mutant proteoglycan precursor targeted for degradation accumulates within a smooth membrane-enclosed subcompartment of the ER (Vertel et al., 1992). One class of mutant low density lipoprotein receptor genes (class 2 mutations) produces a receptor synthesized and glycosylated in the ER that

is almost entirely confined to rough ER and irregular extensions of the rough ER or the transitional zone of the ER (Pathak et al., 1988). Class I major histocompatibility complex (MHC) molecules involve the interaction of two distinct polypetides (the heavy and light chains) with peptide antigen. In cell lines that synthesize both chains but express low levels of MHC class I molecules on their surface as a result of failure to assemble, the MHC class I molecules do not reach the Golgi apparatus and accumulate in an expanded ER-Golgi intermediate compartment (Hsu et al., 1991). Expression of Rubella virus in CHO cells in the absence of E2 glycoprotein, results in the accumulation of the E1 glycoprotein in a tubular network of smooth membranous tubular structures contiguous with the ER; this compartment is proximal to ERGIC and has distinctive properties from either the RER, Golgi and ERGIC (Hobman et al., 1992). Precursors of chondroitin sulfate proteoglycan (CSPG) have been localized to specialized regions of the ER; this ER compartment exhibits a variable tubulo-vesicular morphology but is invariably recognized as a smooth membrane bounded region continuous with typical ribosome studded elements of the rough ER (Vertel et al., 1989). In thymic epithelial cells derived from transgenic mice deficient for the transport associated with antigen presentation (TAP), the misfolded major histocompatibility complex class I molecules accumulate in a degradative compartment consisting of an expanded network of tubular and fenestrated membranes which contains ERGIC-53, a marker for the ERGIC (Raposo et al., 1995). Improperly folded or incompletely assembled proteins are degraded in a smooth ER or in a post-ER compartment, probably the ERGIC.

1. 2. 2. ER-Golgi intermediate compartment (ERGIC)

1. 2. 2.1. What is ERGIC

At 15°C, proteins synthesized in the ER en route to the Golgi are blocked in an ER-Golgi intermediate compartment (Saraste and Kuismanen, 1984; Schweizer et al., 1990). This compartment exhibits a dynamic structure which consists of pleomorphic membranous vesicular-tubular cluster structures, thought to constitute an intermediate station of transport between the ER and the Golgi complex (Lotti et al., 1992). This structure has been variously named: pre-Golgi vacuoles, intermediate compartment, intermediate elements, salvage compartment, cis-Golgi network and ER-Golgi intermediate comparment (ERGIC) (Hauri and Schweizer, 1992). The membrane boundaries in this region are unclear.

1. 2. 2. 2. The markers of the ERGIC

P53, a marker of ERGIC (Schweizer et al., 1990), is a non-glycosylated, homo-oligomeric transmembrane protein which is predominantly localized in tubulovesicular membranes near the cis-side of the Golgi apparatus (Schweizer et al., 1988; Schweizer et al., 1990). The p53 harboring membranes were shown to represent membranes involved in ER-Golgi transport by studying the transport of the G-protein of vesicular stomatitis virus (tsO45 mutant). When transport from the ER is blocked by incubation at 15°C (the temperature which arrests exocytic protein transport betwen ER and Golgi), newly synthesized G protein colocalized with p53, identifying p53 as a marker for the ER-Golgi intermediate elements which are now known as the ERGIC.

P58, a resident cis-Golgi protein (Saraste et al., 1987), has also been found within morphologically distinct smooth ER clusters outside the Golgi region. These pleiomorphic structures accumulated in cells at the site where protein transport between the ER and Golgi is arrested at 15°C (Lahtinen et al., 1992). The biochemical properties of p58 are very similar to p53 and antibodies against p58 and p53 react with the same protein species in computer-based, two-dimensional protein maps of human amnion cells. p58 is therefore the rat homologue of human p53 (Celis et al., 1990).

Immunofluorescence double labeling has shown that the small GTP-binding protein rab2 is colocalized precisely with p53 in tubulovesicular structures between the ER and Golgi complex, in close proximity to the Golgi stack and spread throughout the cytoplasm (Chavrier et al., 1990). The rab2 containing compartment consists of 30-40 separated units of clustered small vesicles and short tubules (Lotti et al., 1992) which is consistent with the morphology of the ERGIC.

KDEL receptors are also found in the ERGIC (Griffiths et al., 1994). The KDEL sequence is a common C-terminal sequence of resident soluble proteins in the lumen of the ER, necessary and sufficient for their retention in the ER (Tang et al., 1992). The retention signal works not by anchoring resident proteins in the lumen of the ER but by the selective retrieval of ER resident proteins after they have escaped from the ER. In the ERGIC and cis-Golgi network, a specific membrane-bound receptor protein binds to the ER retention signal and packages any proteins displaying the signal into special transport vesicles that return the proteins to the ER. When attached to an ER retention signal, the lysosomal protein cathepsin D is continuously retrieved from a post-ER compartment (Pelham, 1988). The KDEL receptor

has been found to concentrate in the ERGIC (Griffiths et al., 1994) as well as in the Golgi stack, rough ER and trans-Golgi network.

1. 2. 2. 3. The ERGIC may be a subdomain of the ER

Recently, experiments have demonstrated that the ERGIC is not a stable compartment, but rather a transport intermediate arising from the exit site of the ER and travelling along microtubules to the GA (Scales et al., 1997). There are four major ways in which the ERGIC could be structurally related to ER and Golgi (Hauri and Schweizer, 1992). First, it may be a physically separated membrane structure from ER and Golgi; thus two vesicular transport steps would be required from ER to Golgi. Second, ERGIC may not be a compartment but a pleomorphic transport intermediate. Third, the ERGIC may represent the first CGN tubulo-vesicular cisternae. In this case, there would be only one step of vesicular transport from the ER to Golgi and the 15°C block would have to be placed between the CGN and next cisternae. Last, the ERGIC may be a subcompartment of the ER, and is referred to as the trans-ER network (TEN), which would include the transitional elements. A 15°C would block one single round of vesicular transport between the TEN and the CGN.

Previously, experiments have shown that ERGIC is continuous with the rough ER and ERGIC can thus be considered to be a smooth subdomain of the ER (Hauri and Schweizer, 1992;Krijnse-Locker et al., 1994;Saraste and Svensson, 1991). Isolation of ERGIC has shown that the intermediate compartment defined by P53 has unique properties and does not exhibit typical features of rough ER and cis-Golgi (Krijnse-Locker et al., 1995; Schweizer et al., 1991).

1. 2. 3. Golgi Apparatus (GA)

The Golgi apparatus (GA) consists of stacks of cisternae located in the pericentriolar region of mammalian cells (Beams and Kessel, 1968), each cisternae comprising a flattened central portion that is closely apposed to adjacent cisternae in the stack and a peripheral rim that is often fenestrated and the site of both the budding and fusion of a COP-coat transport vesicles. Each Golgi stack has two distinct faces: a cis face (enter face) and a trans face (exit face). Both the cis and trans faces are closely connected to an extensive tubular reticulum termed the cis- and trans-Golgi networks. The cisternae form organized stacks and are organized as a sequential series of processing compartments. The polarity of the stacks has been shown biochemically, by histochemical stains specific for processing enzymes localized in specific regions (Dunphy et al., 1985; Kaplan et al., 1987), and immunocytochemically, by labeling for key glycosyltransferases and related proteins (Kaplan et al., 1987). The Golgi stacks are concentrated in the pericentriolar region and associated with microtubule organizing center (MTOC) (Rogalski and Singer, 1984; Thyberg and Moskalewski, 1985). The Golgi stacks are linked together laterally by tubules which connect equivalent cisternae in adjacent stacks generating a bifurcating, ribbon-like structure. These polarized stacks communicate with other intracellular compartments through vesicular and, perhaps, nonvesicular structures (Rambourg and Clermont, 1990).

The cis-Golgi network (CGN), is located to the side of the Golgi stack which faces the ER, and consists of a continuous array of tubules and networks. It functions in the receipt of newly synthesized proteins from the

ER and recycling of proteins and lipids that have escaped from the ER (Mellman and Simons, 1992). Soluble proteins bearing the KDEL ER retention marker that are present in ERGIC are also found in CGN (Griffiths et al., 1994). The medial Golgi corresponds to the cisternae and tubules in the middle of the Golgi stack, whose structure varies widely in different cells. Most trimming and glycosylation reactions (N-linked, O-linked, as well as glycolipid synthesis) occur in the medial Golgi (Mellman and Simons, 1992). Distinct from the CGN or trans-Golgi network (TGN), the medial cisternae represent a single functionally continuous compartment. The TGN is a tubular-vesicular network that acts primarily in sorting proteins to their final destination (Mellman and Simons, 1992).

Secretory glycoproteins, flowing from the ER and harboring partially trimmed N-linked oligosaccharides, undergo further modifications within the Golgi complex. In polarized cells, completely glycosylated products are sorted at the exit face of this organelle for secretion from the appropriate membrane domain (Mellman and Simons, 1992). These operations, requiring a sequence of ordered reactions, reflect the complex nature of the Golgi apparatus, the morphological integrity of which is maintained by its asssociation with microtubules (Ho et al., 1989; Rogalski and Singer, 1984).

1. 2. 3. 1. Integrity of the Golgi apparatus

(1). The Golgi apparatus can be fragmented by microtubule-depolymerizing drugs

The GA is a microtubule-associated organelle, and its morphology is dependent on integrity of microtubules. Two different types of association of Golgi elements with microtubules have been postulated: The first is

lateral along the length of microtubules permitting transport of Golgi elements along microtubules and the second is confinement to the region around the minus ends of microtubules or microtubule bundles (MTOC) that is seen in steady-state normal cells and in taxol-treated cells (Rogalski and Singer, 1984). The functional significance of the association between the GA and MTOC is to maintain cell polarity by establishing a membrane traffic along a polarized microtubule network (Rogalski and Singer, 1984;Thyberg and Moskalewski, 1985). When cells are treated with nocodozale (a drug that depolymerizes the microtubules), the compact structure and perinuclear location of the Golgi apparatus is disrupted and the Golgi apparatus is dispersed into a set of discrete elements which correspond to stacks of GA saccules scattered throughout the cells (Ho et al., 1989; Rogalski and Singer, 1984). These morphologically modified clusters are functionally normal; protein processing, such as glycosylation, is not modified in cells treated by microtubule depolymerizing drugs (Stults et al., 1989). Washout of the drug results in microtubules formation from the MTOC and also to the recompaction of GA elements to their normal configuration. During this recompaction process, GA elements were seen in close lateral apposition to microtubules (Ho et al., 1989). Molecular motors such as dynein are required for the relocation of Golgi stacks to the pericentriolar region (Corthésy-Theulaz et al., 1992).

(2). Mitosis

When animal cells enter mitosis, membrane traffic ceases and this event is accompanied by fragmentation of the Golgi apparatus, the nuclear

envelope and the ER. The Golgi apparatus is converted into thousands of small vesicles that become randomly distributed thoughout the mitotic cell cytoplasm and finally equally divided to the two daughter cells (Lucocq et al., 1989). These vesicles are very small and nonfunctional and are different from the Golgi clusters derived from nocodazole treated GA (Busson-Mabillot et al., 1982; Rogalski et al., 1984). The majority of mitotic Golgi fragments, accounting for two thirds of starting membrane, are uniform small vesicles (Lucocq et al., 1987; Orci et al., 1986). They are produced by a coat protomer-COPI dependent mechanism (Misteli and Warren, 1994; Misteli and Warren, 1995) which, during interphase, is responsible for both anterograde and retrograde transport through the Golgi stack. During telophase, reassembly occurs by essentially the reverse of this process. First, growth of a limited number of dispersed clusters by accretion and fusion of vesicles to form cisternal clusters next to a membranous "bud" on the ER, and then congregation and fusion of clusters to form the interphase Golgi stack in each daughter cell (Lucocq et al., 1989).

At the onset of mitosis, vesicle budding from the rim of each Golgi cisternae continues. These vesicles are COP-coated, and their fusion with their target membranes is inhibited (Misteli and Warren, 1994; Misteli and Warren, 1995). A role for COP-coated vesicles in the fragmentation process was initially demonstrated by adding GTPγS (non-hydrolyzable form of GTP) to the fragmentation assay. GTPγS prevents uncoating of COP-coated vesicles and lead to accumulating a large number of COP-coated vesicles. In a cell-free system, immunodepletion of the coatomer subunit of COP-coats from the mitotic cytosol abolished the formation of transport vesicles and inhibited fragmentation while readdition of purified coatomer reversed this effect (Misteli and Warren, 1994).

GTPγS acts on rab proteins (Melançon et al., 1987) and ARF (ADP-ribosylation factor) (Donaldson et al., 1991) and causes the accumulation of COP-coated vesicles (Orci et al., 1989). AlF acts on heterotrimeric G proteins (Gilman, 1987) and permits budding but not uncoating of COP-coated vesicles (Orci et al., 1989). GTPγS and AlF both prevent reassembly when added during fragmentation but not when added to the reassembly mixture (ATP and GTP in the presence of an ATP regenerating system and interphase cytosol). In fact, GTPγS stimulated reassembly such that all cisternae were stacked at the end of the incubation and comprised 40% of the total membrane (Rabouille et al., 1995). Golgi apparatus reassembly requires ATP, GTP and a 37°C incubation and is independent of microtubule and actin filaments.

Rab1 is specifically phosphorylated during mitosis and microinjection of a rab1 mutant protein causes fragmentation of the GA. A rab1 mutant affecting guanine nucleotide exchange promotes disassembly of the Golgi apparatus, thus GTP exchange and hydrolysis by GTPases of rab1 are required to form and maintain normal Golgi stacks. The similarity of Golgi disassembly seen with rab1 mutants to that occuring during mitosis, may point to a molecular basis involving rab1 for fragmentation of the Golgi apparatus during cell division (Wilson et al., 1995).

N-ethylmaleimide (NEM) treated cytosol also prevents reassembly of GA (Rabouille et al., 1995), meaning that N-ethylmaleimide sensitive factor (NSF), a protein known to promote fusion of transport vesicles with the target membrane in the exocytic and endocytic pathways, and soluble NSF attachment proteins (SNAPs), required for binding NSF to membranes (Rothman and Orci, 1992; Rothman and Warren, 1994), may be involved in GA membrane reassembly.

P115 is a cytosolic protein implicated in intra-Golgi transport (Waters et al., 1992), docking of transcytotic vesicles with the plasma membrane (Sztul et al., 1993; Sztul et al., 1991) and transport between the ER and the Golgi apparatus (Nakajima et al., 1991; Sapperstein et al., 1996). P115 has been shown to function in a docking step prior to membrane fusion (Barroso et al., 1995; Sapperstein et al., 1996). This vesicle docking protein shows saturable and high binding affinity to interphase Golgi membranes, but preincubation of p115 with mitotic cytosol reduced its affinity of binding to Golgi membranes 20-fold. Reduced p115 binding in mitotic cytosol may inhibit membrane fusion during mitosis preventing prior vesicle docking (Levine et al., 1996).

P97 was originally isolated as a ring shaped, hexameric ATPase (Peters et al., 1990). Sequencing showed that it is a member of a family of ATPases that includes NSF (Peters et al., 1990). P97 is bound to clathrin triskelion (Pleasure et al., 1993) and is needed for the budding of vesicles from the transitional ER (Zhang et al., 1994), suggesting a possible role for this protein in membrane traffic. In a cell-free system, Golgi cisternae can be reconstituted from mitotic Golgi fragments in the presence of either the NSF-like ATPase, p97, or NSF-SNAPs-p115. The morphology of cisternae regrown with p97 and NSF-SNAPs-p115 are different, and these factors may play distinct roles in the reconstitution of Golgi cisternae after mitosis (Rabouille et al., 1996).

There also exists a COP-independent pathway for reconstitution of the Golgi apparatus which accounts for the remaining third of membrane in mitosis. When the cells were incubated in the absence of the coatomer subunit of COP coats, transport vesicles were not formed, but the Golgi cisternae were converted into extensive tubular networks which then

slowly fragmented (Misteli and Warren, 1994;Misteli and Warren, 1995). Similar tubular networks were also observed in the presence of IQ that inhibited Golgi membranes to form COP-coated vesicles (Takizawa et al., 1993).

(3). Ilimaquinone (IQ)

IQ is a major metabolite of several dictyoceratid sponges. It was first obtained from *Hippospongia metachromia* (Luibrand et al., 1979). The chemical stucture of IQ is shown in Figure 1. IQ causes Golgi membranes to break down completely into 60-90nm diameter vesicles (vesiculated Golgi membrane-VGMs) dispersed throughout the cytoplasm in normal rat kidney (NRK) cells. IQ also depolymerizes cytoplasmic microtubules, but vesiculation of Golgi membranes and the reassembly of the vesicles into Golgi stacks are independent of microubules. Other intracellular organelles such as the ER, nucleus, mitochondria, and cytoskeletal elements such as actin filaments and intermediate filaments are not affected by IQ (Veit et al., 1993).

IQ inhibits protein transport between Golgi membranes and further transport along the secretory pathway by specifically inhibiting the formation of transport vesicles in Golgi membranes and not in the fusion of transport vesicles with the target Golgi membranes (Takizawa et al., 1993). IQ-mediated Golgi vesiculation occurs through activation of heterotrimeric G proteins and that it is the free ßg subunits, and not the activated a subunit, that triggers Golgi vesiculation (Jamora et al., 1997).

Like brefeldin A (BFA), a drug which blocks the cycle of association and dissociation of ß-Cop and ARF with Golgi membranes (Donaldson et

al., 1992;Helms and Rothman, 1992;Robinson and Kreis, 1992), IQ inhibits the association of the coat components ß-Cop and ADP-ribosylation factor (ARF) to Golgi membranes (Takizawa et al., 1993). However, unlike BFA, IQ does not block the transport of newly synthesized proteins from the ER to the cis-cisternae-derived VGMs and does not induce retrograde transport of Golgi enzymes to the ER. The effect of IQ is temperature and energy dependent (Takizawa et al., 1993); IQ has no affect on the structural organization of Golgi membranes at 4°C and 16°C (Takizawa et al., 1993; Veit et al., 1993). However, VGMs generated by IQ are capable of fusing and assembling into stacks of Golgi cisternae at 16°C (Veit et al., 1993). In contrast, after removal of BFA at 16°C Golgi resident enzymes fail to exit the ER.

In vitro, VGMs reassemble into stacks of Golgi cisternae by a reconstitution process that is temperature, ATP and cytosol dependent (Acharya et al., 1995b), but that does not require ARF and coatomers necessary for the formation of Golgi derived COPI vesicles (Jamora et al., 1997). Newly assembled stacks are functionally active in vesicular protein transport and contain processing enzymes that carry out Golgi-specific modification of glycoproteins (Acharya and Malhotra, 1995; Acharya et al., 1995b). Golgi reconstitution has two distinct processes: the first fusion event is NSF- dependent VGM fusion to form large vesicles of 200-300nm average; subsequently, the large vesicles extend into tubular elements and assembly into stacks of Golgi cisternae, which requires an NSF-like NEM-sensitive ATPase called p97 (Acharya et al., 1995a; Acharya et al., 1995b). This second step is a microtubule-dependent process by which the Golgi stacks are carried to their perinuclear location in the cells (Veit et al., 1993). Reconstitution of Golgi stacks from the Golgi fragments generated by IQ

treatment is a similar process to reassembly of Golgi stacks from mitotic fragments.

1.3. Project of research

Our laboratory is interested in the study of AMF-R tubules. My project concerns the characterization of the AMF-R tubule. I have studied modulation of AMF-R tubule morphology and their relationship with other organelles *in vivo* and *in vitro*.. These studies were performed primarily using immunofluorescence and immunoelectron microscopy.

2. ARTICLE

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The AMF-R tubule is a smooth ilimaquinone-sensitive subdomain of the endoplasmic reticulum

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SUMMARY

Autocrine motility factor receptor (AMF-R) is a marker for a distinct smooth membranous tubule. Ilimaquinone (IQ) is a sea sponge metabolite which induces the complete vesiculation of the Golgi apparatus and we show here that the addition of IQ to MDCK cells also results in the disruption of the AMF-R tubule. By immunofluorescence microscopy, the resultant punctate AMF-R label resembles the products of IQ-mediated vesiculation of the *trans*-Golgi network, however, the two labels can be distinguished by confocal microscopy. AMF-R tubule fragmentation occurs after nocodazole or taxol treatment of the cells demonstrating that the action of IQ on AMF-R tubules is not related to the ability of IQ to depolymerize microtubules. IQ activity is therefore not Golgi-specific. Electron microscopy of IQ-treated cells reveals that AMF-R is distributed to fenestrated networks of narrow interconnected tubules which are distinguishable from the uniform Golgi-derived vesicles and morphologically equivalent to smooth ER. Distinct fenestrations are visible in incompletely fragmented tubules which may represent intermediates in the fragmentation process. Smooth AMF-R labeled tubules exhibit continuity with rough ER cisternae and IQ selectively targets smooth and not rough ER. AMF-R tubules can be distinguished from the intermediate compartment labeled for ERGIC-53 by confocal microscopy and thus constitute a distinct IQ-sensitive subdomain of the smooth ER.

Key words: Autocrine motility factor receptor, Membrane tubule, Madin-Darby canine kidney, Ilimaquinone, Smooth endoplasmic reticulum

INTRODUCTION

Autocrine motility factor receptor (AMF-R) is a marker for a distinct tubular organelle (Benlimame et al., 1995). The AMF-R tubule is not equivalent to either endosomes or lysosomes and therefore not part of the endocytic pathway. Electron microscopic analysis of AMF-R distribution in MDCK, HeLa and NIH-3T3 cells shows that AMF-R is localized primarily to smooth tubular organelles of variable diameter (Benlimame et al., 1995; Benlimame and Nabi, 1997). In MDCK cells, significant labeling is also detected on ribosome studded tubules of the rough ER as well as transitional part rough/part smooth tubules demonstrating a relationship between smooth AMF-R tubules and the endoplasmic reticulum. However, by immunofluorescence, AMF-R tubules are clearly distinguished from rough ER tubules labeled for calnexin (Benlimame et al., 1995). Nevertheless, AMF-R tubules associate with the microtubule cytoskeleton in a similar fashion to that described for the endoplasmic reticulum (Terasaki et al., 1986; Lee et al., 1989; Benlimame et al., 1995; Nabi et al., 1997).

Disruption of microtubule integrity results in the loss of the organized cytoplasmic distribution of AMF-R tubules but does not effect their tubular morphology (Benlimame et al., 1995). Similarly, following microtubule depolymerization, the Golgi apparatus is dispersed into clusters which retain the stacked morphology and functionality of the Golgi apparatus (Rogalski et al., 1984; Thyberg and Moskalewski, 1985; Ho et al., 1989; Turner and Tartakoff, 1989; Cole et al., 1996). Subsequent vesiculation and loss of functionality of the Golgi apparatus occurs during mitosis (Lucocq et al., 1989). The dictyoceratid sea sponge metabolite ilimaquinone (IQ) also induces the breakdown of Golgi membranes to small vesicles and inhibits protein transport between Golgi stacks and protein secretion (Takizawa et al., 1993). Reformation of Golgi stacks after IQ treatment is a two step process involving NSF and p97 dependent steps, as described for Golgi reformation from mitotic Golgi fragments (Acharya et al., 1995a; Rabouille et al., 1995). The action of IQ is distinct from that of BFA in that IQ does not induce retrograde transport to the ER and does not block the transport of newly synthesized proteins from the ER to *cis*-Golgi vesicles (Takizawa et al., 1993; Veit et al., 1993). With respect to cellular organelles, to date the activity of IQ is specific to the Golgi apparatus and IQ affects neither fluid phase endocytosis nor mitochondrial or ER morphology (Takizawa et al., 1993; Veit et al., 1993). We show here that IQ also targets the AMF-R tubule demonstrating that IQ activity is not Golgi-specific. We further demonstrate that AMF-R tubules are not equivalent to the ER-Golgi intermediate compartment (ERGIC) and identify this organelle as a smooth IQ-sensitive subdomain of the ER.

MATERIALS AND METHODS

Chemicals and antibodies

IQ was the kind gift of Dr Vivek Malhotra (UCSD, San Diego, California). Paclitaxel (taxol) and nocodazole were purchased from

Sigma. Monoclonal antibody against AMF-R was used in the form of concentrated hybridoma supernatant (Nabi et al., 1990). Antibody against ERGIC-53 was kindly provided by Hans-Peter Hauri (Biozentrum, Basel, Switzerland). Antibodies to Tac (IL25) were purchased from AMAC (Westbrook, ME). Antibodies to α-tubulin were purchased from ICN (Mississauga, Ontario, Canada). Secondary antibodies conjugated to either FITC, Texas Red or 12 nm gold particles were purchased from Jackson Immunoresearch Laboratories (West Grove, PA). The fluorescent secondary antibodies were designated for use in multiple labeling studies and no interspecies cross-reactivity was detected. To detect the anti-AMF-R rat IgM, secondary antibodies specific for the μ chain of rat IgM were used.

Cells and cell culture

All cells were grown in an air-5% CO_2 incubator at constant humidity. MDCK cells transfected with a Tac-TGN38 chimeric protein (MDCK-TGG cells) (Humphrey et al., 1993; Rajasekaran et al., 1994) were obtained from Enrique Rodriguez-Boulan (Dyson Eye Institute, Cornell University Medical College, New York, New York). MDCK, MDCK-TGG and HeLa cells were grown in Dulbecco's minimum essential medium (DMEM) containing non-essential amino acids, vitamins, glutamine and a penicillin-streptomycin antibiotic mixture (Gibco, Burlington, Ontario, Canada) supplemented with 5% fetal calf serum (Immunocorp, Laval, Quebec, Canada). IQ was used at a concentration of 25 μM and added to cells in culture medium supplemented with 25 mM Hepes, pH 7.0.

Immunofluorescence

Cells were plated (25,000-50,000 cells/35 mm dish) on glass coverslips for 2 days before each experiment. Cells were fixed by the addition of precooled (−80°C) methanol/acetone (80%/20%, v/v) directly to the coverslips, and then placed at −20°C for 15 minutes. After fixation, the cells were rinsed extensively with PBS, pH 7.4, supplemented with 0.1 mM Ca^{2+} and 1 mM Mg^{2+} (PBS/CM), and then incubated for 15 minutes with PBS/CM containing 0.5% BSA (PBS/CM/BSA) at room temperature to reduce nonspecific binding. All washings and incubations with both primary and secondary (FITC and Texas Red conjugated) antibodies were done with PBS/CM/BSA. After labeling, the coverslips were mounted in Airvol (Air Products and Chemicals Inc, Allentown, PA). Labeled cells were viewed in a Zeiss Axioskop fluorescence microscope equipped with a ×63 Plan apochromat objective and selective filters. Images were photographed using Kodak T-Max

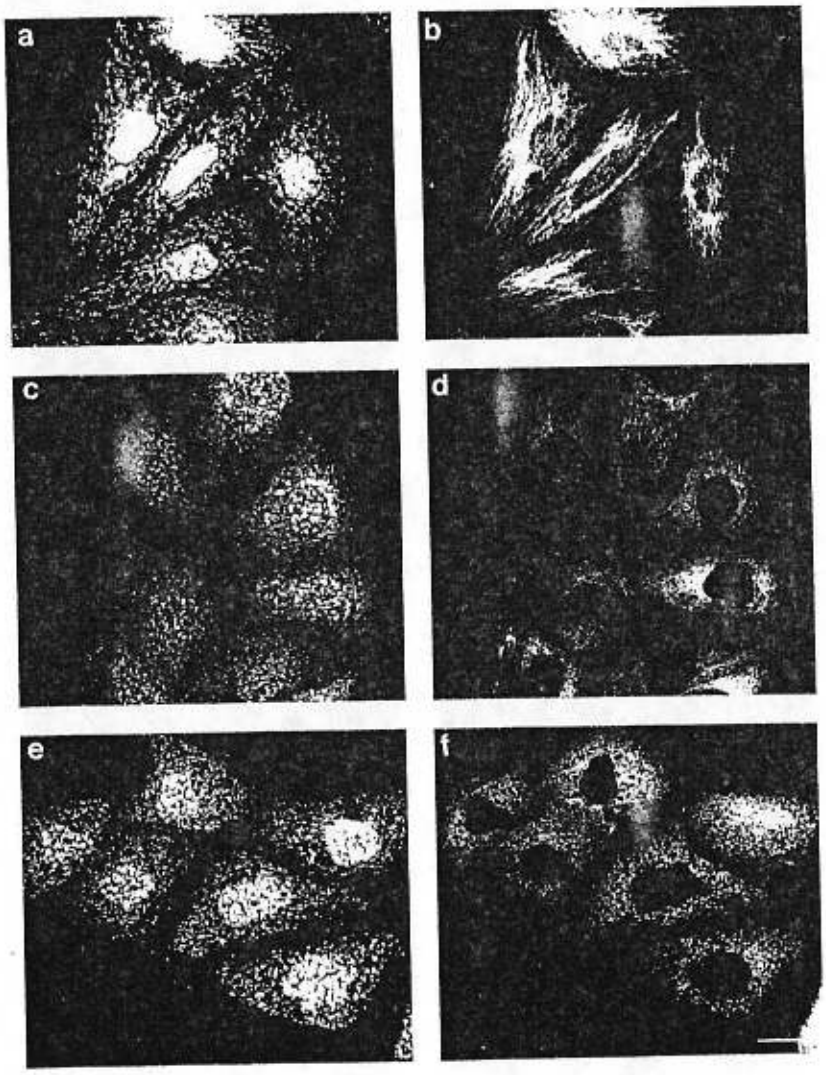

Fig. 1. Fragmentation of AMF-R tubules by ilimaquinone in MDCK cells. Untreated MDCK cells (a,b) or MDCK cells treated with 25 μM IQ for 30 (c,d) or 60 minutes (e,f) were double immunofluorescently labeled for AMF-R (a,c,e), and tubulin (b,d,f). In control cells (a,b), AMF-R tubules exhibit a linear morphology and are oriented towards the cell periphery of the cells in coordination with the microtubule cytoskeleton. In cells treated with 25 μM IQ (c,d), microtubules are depolymerized and AMF-R labeling is no longer localized to distinct tubular structures but exhibits a punctate distribution throughout the cytoplasm (c,d). Treatment with IQ for 60 minutes (e,f) results in the formation of larger AMF-R labeled structures (e). Bar, 20 μM.

400 film. Confocal analysis was performed with a Zeiss confocal microscope and printed with a Kodak XLS 8300 digital printer (Department of Cell Biology and Anatomy, McGill University, Montreal, Quebec).

Electron microscopy

Cells grown on Petri dishes were washed rapidly twice with Ringer's solution before fixing in the same solution containing 2% paraformaldehyde and 0.2% glutaraldehyde for 30 minutes at 37°C (Benlimame et al., 1995). 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, dehydrated and embedded in LR-White resin. Ultra-thin sections were blocked with 2% BSA in PBS/CM, and then incubated at room temperature with anti-AMF-R antibody followed by 12 nm gold-conjugated goat anti-rat secondary antibodies. The sections were stained with 5% uranyl acetate and visualized in a Philips 300 electron microscope.

RESULTS

Fragmentation of AMF-R tubules by IQ

IQ has previously been described to vesiculate the Golgi apparatus and depolymerize microtubules but not to affect other cellular organelles or cytoskeletal filaments (Takizawa et al., 1993; Veit et al., 1993). AMF-R tubules were previously characterized in MDCK cells (Benlimame et al., 1995), an IQ sensitive cell line (Takizawa et al., 1993), and we therefore tested the effect of IQ on AMF-R tubule morphology in MDCK cells (Fig. 1). In untreated cells, AMF-R tubules exhibit a linear morphology and extend to the cell periphery in alignment with the microtubule cytoskeleton (Fig. 1a,b). After treatment with IQ for 30 minutes the microtubules are extensively depolymerized and the AMF-R labeling exhibits a punctate distribution throughout the cell (Fig. 1c,d). Treatment with IQ for 60 minutes results in the formation of larger AMF-R labeled structures (Fig. 1e,f).

In order to study in parallel the effect of IQ on AMF-R tubules and the TGN, we used MDCK cells transfected with a chimeric protein construct consisting of the ectodomain of IL25 (Tac) and the transmembrane and cytoplasmic domains of TGN38 which localizes to the TGN (MDCK-TGG cells) (Humphrey et al., 1993; Rajasekaran et al., 1994). AMF-R tubules do not colocalize with the β-COP labeled Golgi apparatus in transformed MSV-MDCK cells and anti-AMF-R antibodies do not label the Golgi apparatus by post-embedding immunoelectron microscopy (Benlimame et al., 1995; Nabi et al., 1997). The distribution of AMF-R tubules and the TGN in untreated cells is quite distinct and the two labels do not colocalize (Fig. 2a,c,e). Following IQ treatment for 30 minutes at 37°C, both labels exhibit a similar

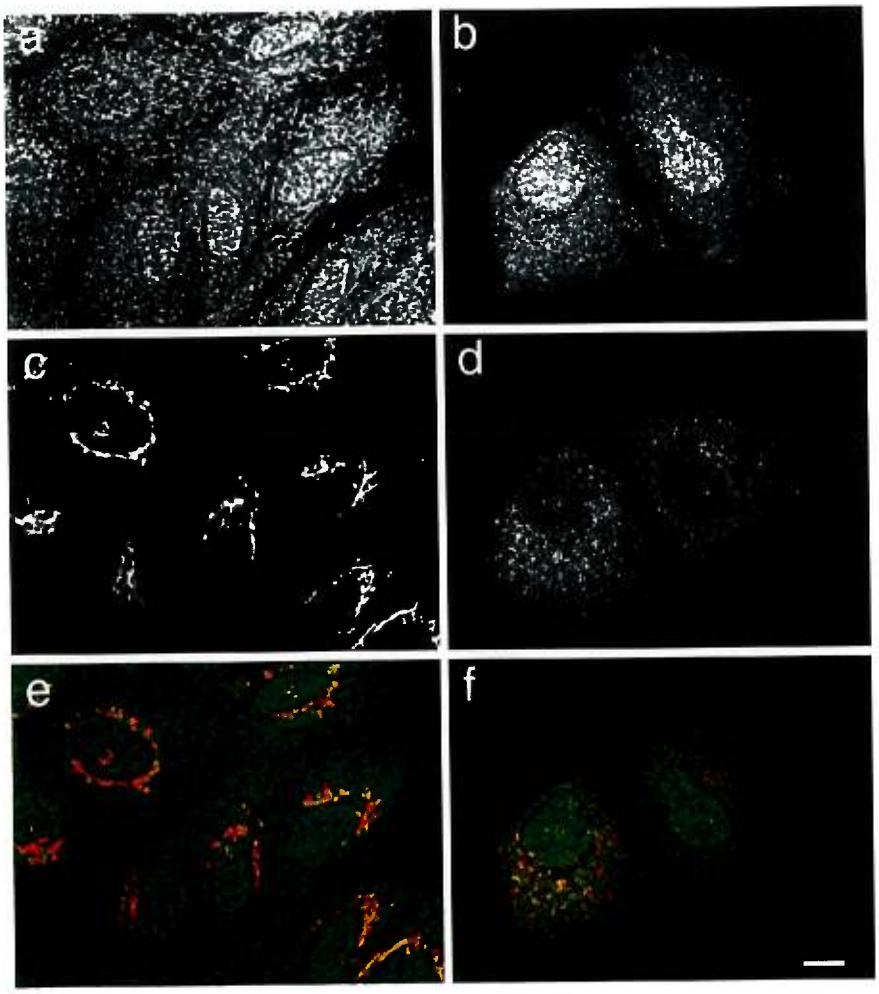

Fig. 2. IQ targets TGN membranes and AMF-R tubules. MDCK-TGG cells were untreated (a,c,e) or treated with 25 μM IQ for 30 minutes (b,d,f) and the cells were double immunofluorescently labeled for AMF-R (a,b) and with anti-Tac antibody to reveal TGN38 (c,d). Dual color merged confocal images reveal that the AMF-R (green) and TGN (red) labels can be distinguished in both control (e) and IQ treated (f) cells. Bar, 20 μM.

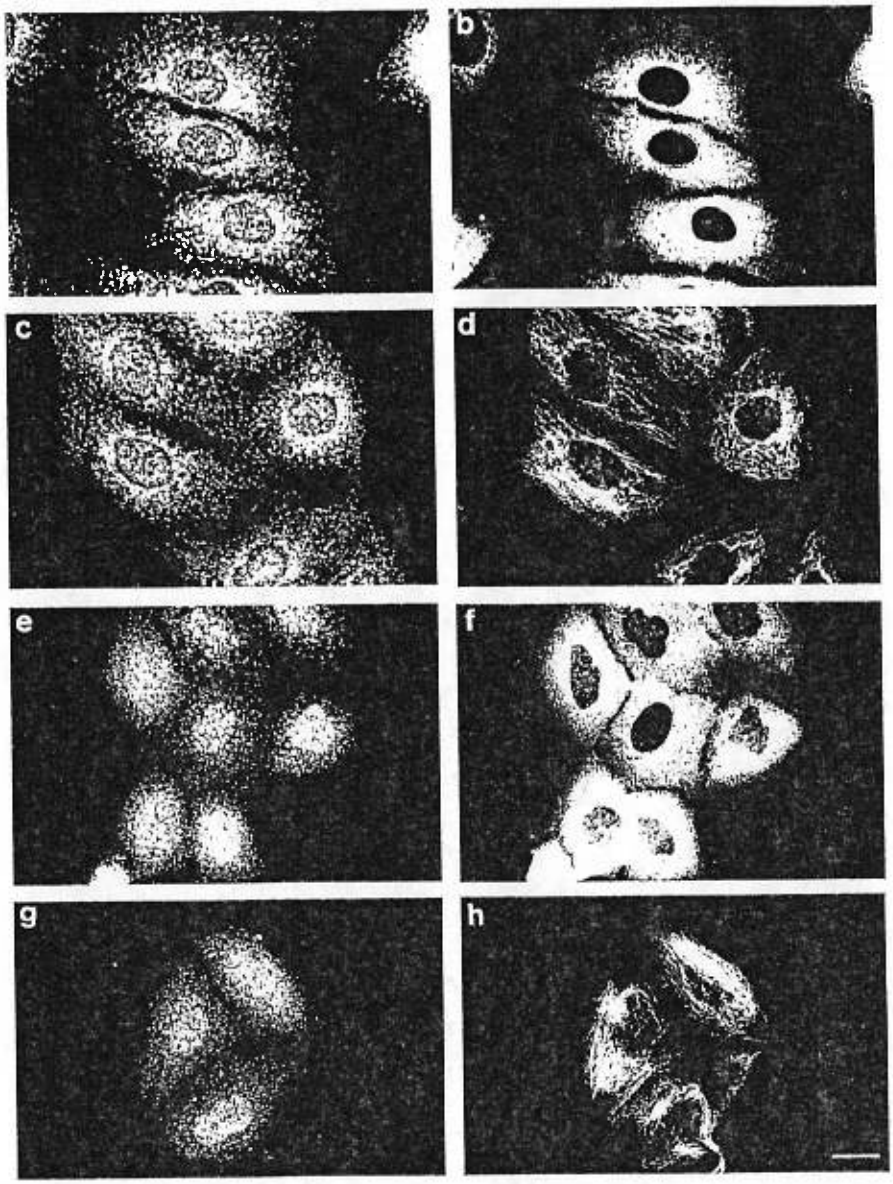

Fig. 3. IQ fragmentation of AMF-R tubules is microtubule-independent. MDCK cells were treated with 20 μM nocodazole for 30 minutes (a,b,e,f) or with 10 μM taxol (c,d,g,h) for 30 minutes and then incubated for a further 30 minutes in the presence of these drugs with (e,f,g,h) or without (a,b,c,d) the addition of 25 μM IQ. Cells were double immunofluorescently labeled for AMF-R (a,c,e,g) and for tubulin (b,d,f,g). Bar, 20 μM.

punctate distribution throughout the cell (Fig. 2b,d,f). Dual color merged images of the IQ treated cells (Fig. 2f, AMF-R in green and Tac-TGN38 in red) show that the AMF-R and TGN labels can be distinguished. This is particulary evident in the cell to the right which exhibits a more spread morphology. As described for the IQ-mediated breakdown of Golgi membranes, IQ does not affect AMF-R tubule morphology when cells are treated at 4°C or in the presence of azide (data not shown). IQ action on both Golgi and AMF-R tubule membranes is therefore similar.

The effect of IQ on AMF-R tubules is microtubule-independent

AMF-R tubules are microtubule-associated membranous organelles, however, disruption of microtubule integrity does not influence the tubular morphology of this organelle but rather affects their peripheral orientation and tubular extension (Benlimame et al., 1995). IQ also depolymerizes microtubules (Veit et al., 1993) and we questioned whether the effect of IQ on AMF-R tubules is related to the effect of IQ on microtubule integrity. MDCK cells were pretreated with either 20 μM nocodazole for 30 minutes to disrupt microtubules or with 10 μM taxol for 30 minutes to stabilize microtubules and then treated with 25 μM IQ for 30 minutes in the continued presence of either nocodazole or taxol. After treatment with either nocodazole or taxol, elongated tubules were still present (Fig. 3a,c). In both the absence of microtubules in nocodazole treated cells or in the presence of stabilized microtubules in taxol treated cells, IQ still disrupted the tubular morphology of AMF-R tubules (Fig. 3e,g). As described for the Golgi apparatus (Veit et al., 1993), IQ mediated disruption of AMF-R tubules is independent of the ability of IQ to depolymerize microtubules.

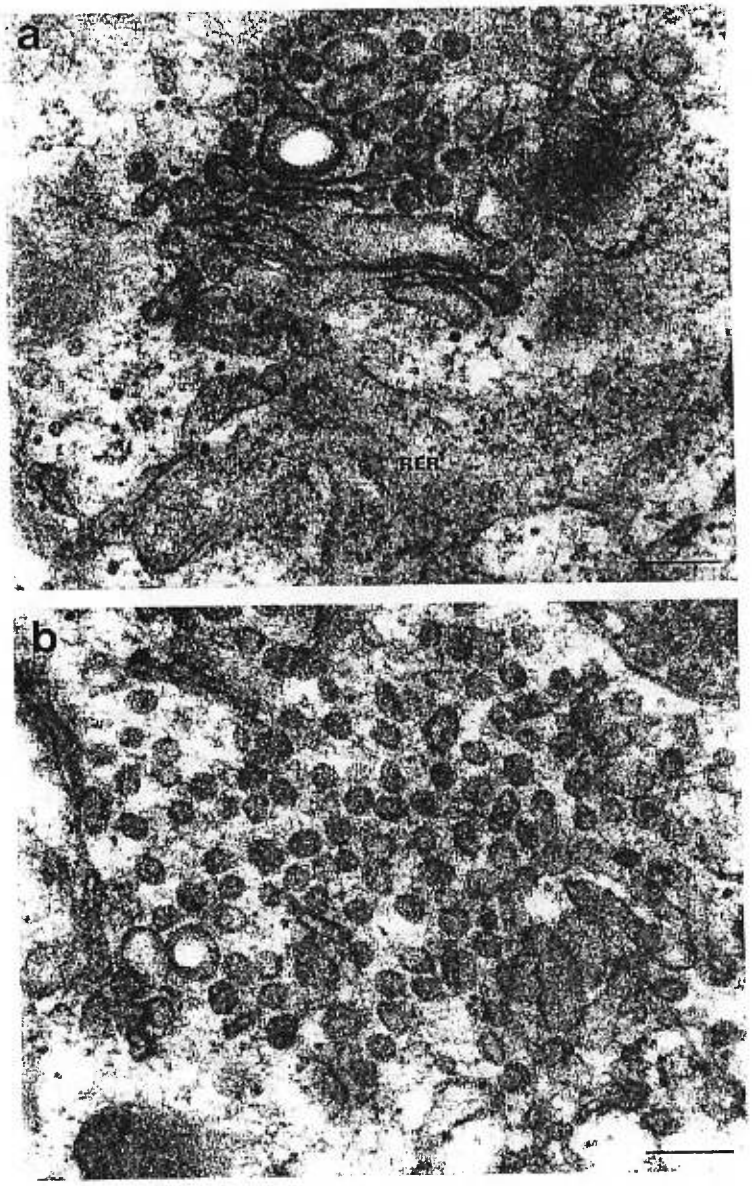

Fig. 4. IQ mediates Golgi membrane vesiculation in MDCK cells. Cells treated with 25 μM IQ for 30 minutes were visualized by electron microscopy. Intact Golgi saccules (G) could be observed in the vicinity of uniform 60-90 nm vesicles (a). Clusters of these vesicles were more frequently observed in the absence of Golgi saccules (b). RER: rough ER. Bars, 0.2 μM.

Ultrastructure of AMF-R tubules after IQ treatment

Based on our fluorescence microscopy observations, the maximal extent of IQ-mediated vesiculation of AMF-R tubules occurred between 30 and 45 minutes, depending on the experiment, and after 60 minutes larger clusters predominated (Fig. 1). Variability in the extent of disruption of both the Golgi apparatus and AMF-R tubules by IQ observed by immunofluorescence was confirmed by EM. After a 30 minute IQ treatment, vesiculation of the Golgi apparatus could be observed at various stages (Fig. 4). The products of Golgi vesiculation could be identified by the presence of intact Golgi saccules in the immediate vicinity of clusters of homogenous vesicles of 60-90 nm diameter (Fig. 4a). These vesicles are morphologically identical to those previously identified as Golgi-derived in NRK cells (Takizawa et al., 1993; Acharya et al., 1995a,b). Clusters of these vesicles were for the most part observed in the absence of Golgi saccules yet could be morphologically identified as Golgi-derived (Fig. 4b). Such vesicle clusters were not observed in untreated cells.

AMF-R tubules, similar to tubular lysosomes (Heuser, 1989), are labile structures which are stabilized by Ringer's solution and previous fixation protocols included a 15 minute incubation in Ringer's solution prior to fixation (Benlimame et al., 1995). In order to eliminate possible consequences of IQ washout during a 15 minute preincubation with Ringer's solution, the cells were only rinsed twice rapidly with Ringer's solution prior to fixation. Under these conditions AMF-R labeling is localized predominantly to smooth tubules with a minimum diameter of 40 nm (Fig. 5). As previously described, the post-embedding labeling is weak but specific (Benlimame et al., 1995). The labeled tubules are similar in morphology to those described following Ringer's treatment (Benlimame et

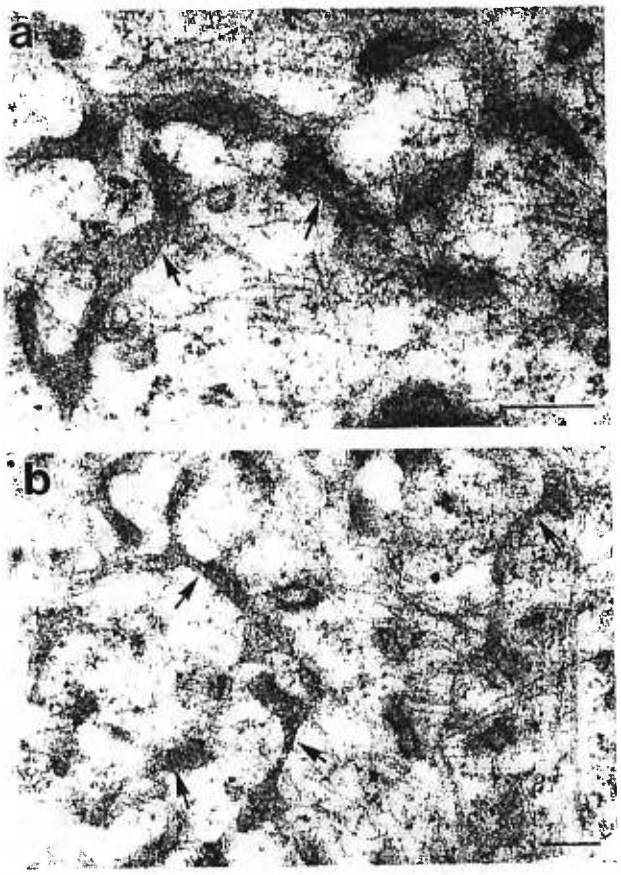

Fig. 5. Ultrastructure of AMF-R tubules by electron microscopy. MDCK cells were rinsed twice rapidly in Ringer's solution prior to fixation and post-embedding immunogold labeling for AMF-R. Labeled smooth AMF-R tubules are indicated by arrows. Bars, 0.2 μM.

al., 1995) but extended tubular networks are not present to the same extent.

AMF-R labeling of IQ treated cells is localized to highly fenestrated networks of narrow elongated tubular membranes which are observed only in IQ treated samples (Fig. 6a,b). These tubular networks represent the majority of AMF-R labeled structures, can be distinguished from the Golgi-derived vesicles and are morphologically equivalent to smooth ER. The majority of structures labeled for AMF-R were tubular networks identical to those in Fig. 6a and regions of complete vesiculation, as seen for the Golgi (Fig. 5b), were not observed. Numerous caveolae were also observed in IQ treated cells (not shown). The tubular networks may thus represent the final product of the effect of IQ on AMF-R tubules. In both untreated and treated cells, AMF-R tubules consist of broader regions of variable diameter connected by narrower tubules. In untreated cells, the diameter of double membrane tubular connections, essentially the narrowest portion of the tubule, is 30.5±9.0 nm (range: 19-54 nm) while following IQ treatment the connecting tubules are significantly narrower measuring 18.9±6.4 nm (range: 10-38 nm) (Fig. 6a,b). The tubular networks are highly fenestrated and contain distinct membrane bounded fenestrations both within individual tubules (arrowheads) and between tubules. As described for the Golgi, incompletely fragmented AMF-R tubules of larger diameter are also present in cells treated with IQ for 30 minutes (Fig. 6c). Both intratubular fenestrations (arrowheads) as well as larger fenestrations within the tubular network are present. Fenestrations of AMF-R tubules are never observed in untreated cells.

AMF-R tubules are a smooth ER subdomain distinct from ERGIC

As previously demonstrated for control MDCK cells (Benlimame et al., 1995), in IQ treated cells AMF-R labels rough ER tubules as well as tubules which exhibit both rough and smooth portions (Fig. 7). Of particular interest is the fact that while IQ does not affect the rough portion of the tubule, the smooth portion presents a morphology of interconnected narrow tubules morphologically identical to the tubular clusters described in Fig. 6. Direct connections between the smooth tubulated portion of the AMF-R tubule and the rough ER are evident (Fig. 7b, arrow). Smooth AMF-R tubules are therefore a subdomain of the ER and only the smooth portion of part rough/part smooth tubules is susceptible to IQ-mediated fragmentation. As previously described (Takizawa et al., 1993; Veit et al., 1993), both mitochondria (M) and rough ER tubules (RER) remained morphologically intact after IQ treatment (Figs 5, 6 and 7).

AMF-R tubules are therefore smooth tubular extensions of the rough ER. AMF-R labeling of structures similar to transitional ER elements, classically considered to be exit sites of newly synthesized proteins from the ER en route to the Golgi apparatus (Palade, 1975), suggests that AMF-R tubules might be equivalent to ERGIC. β-COP has been described as a marker for the *cis*-Golgi and ERGIC in exocrine pancreatic cells (Oprins et al., 1993) and confocal microscopy has shown that AMF-R tubules do not colocalize with β-COP in MSV transformed MSCK cells (Nabi et al., 1997). To further establish the relationship between AMF-R tubules and ERGIC, we performed double IF labeling for AMF-R and ERGIC-53, a well-defined marker for ERGIC (Schweizer et al., 1988) (Fig. 8). ERGIC-53 is predominantly localized to the perinuclear region where AMF-R tubules also accumulate in HeLa cells. While it is difficult to distinguish the two labels in this region, AMF-R labeled tubular structures are clearly defined in this region compared to the more diffuse labeling of ERGIC-53. Furthermore, AMF-R tubules extending to the periphery of the cell are unlabeled for ERGIC-53 and we have never observed an AMF-R tubule to be labeled for ERGIC-53. AMF-R tubules are therefore a smooth ER compartment distinct from ERGIC.

DISCUSSION

IQ targets another organelle: the AMF-R tubule

The dictyoceratid sea sponge metabolite ilimaquinone induces breakdown of Golgi membranes to very small vesicles uniformly distributed throughout the cytoplasm, inhibiting protein transport between Golgi stacks and protein secretion (Takizawa et al., 1993). We show here that IQ activity is not Golgi specific and that the AMF-R tubule is another target for IQ. After IQ treatment, the tubular AMF-R labeling visualized

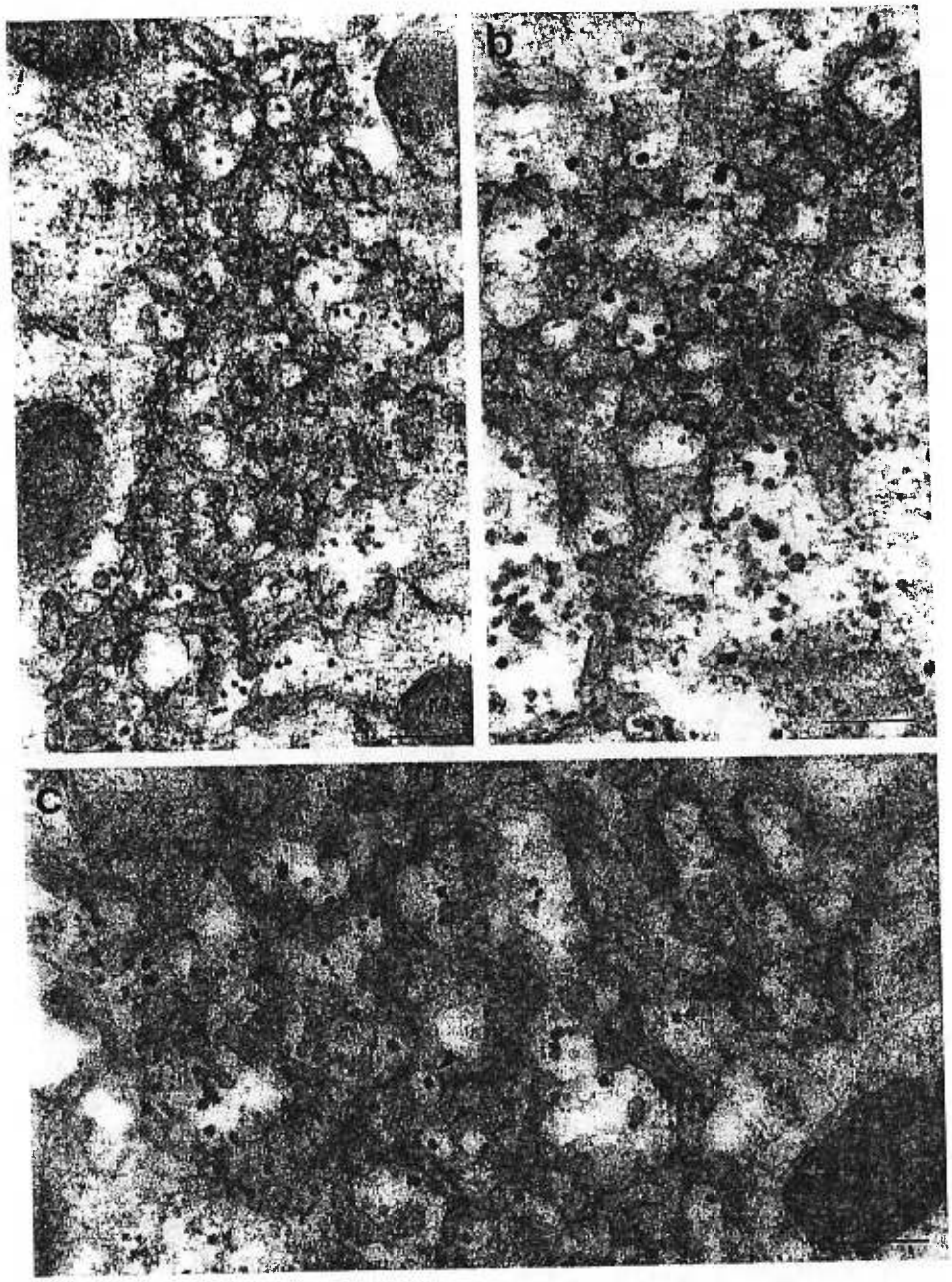

Fig. 6. AMF-R is localized to tubular networks in IQ treated cells. By postembedding immunoelectron microscopy, AMF-R is localized to networks of narrow interconnected tubules in MDCK cells treated with 25 μM IQ for 30 minutes (a,b). Incompletely fragmented AMF-R labeled tubules of larger diameter are also present in treated cells (c). Distinct membrane bounded fenestrations are present within tubules of larger diameter (arrowheads) as well as within the tubular networks. Bars, 0.2 μM.

by immunofluorescence is lost and AMF-R exhibits a punctate distribution throughout the cytoplasm. AMF-R distribution following IQ treatment resembles that of the Golgi apparatus but the products of IQ-mediated vesiculation of the TGN can be distinguished by confocal microscopy from those derived from AMF-R tubules. By EM, IQ treatment of AMF-R tubules gives rise to fenestrated networks of short narrow tubules labeled for AMF-R which can be distinguished from the uniform vesicles of 60-90 nm derived from Golgi fragmentation (Takizawa et al., 1993; Acharya et al., 1995b). The tubular networks derived from IQ-mediated fragmentation of AMF-R tubules are morphologically equivalent to smooth ER. While we cannot be certain that the effect of IQ was complete, the majority (~80%) of identifiable Golgi derived structures in the treated cells were completely vesiculated (Fig. 5). Similarly, tubular networks (Fig. 6a) represent the majority of AMF-R labeled structures and complete vesiculation of AMF-R labeled structures was not observed. The interconnected tubular clusters may thus represent the final stage of IQ-mediated fragmentation of the AMF-R tubule.

The half-life of IQ activity in culture medium is less than 60 minutes and the effect of IQ on the Golgi apparatus has been shown to be reversible (Takizawa et al., 1993). By immunofluorescence, we observe the formation of larger structures

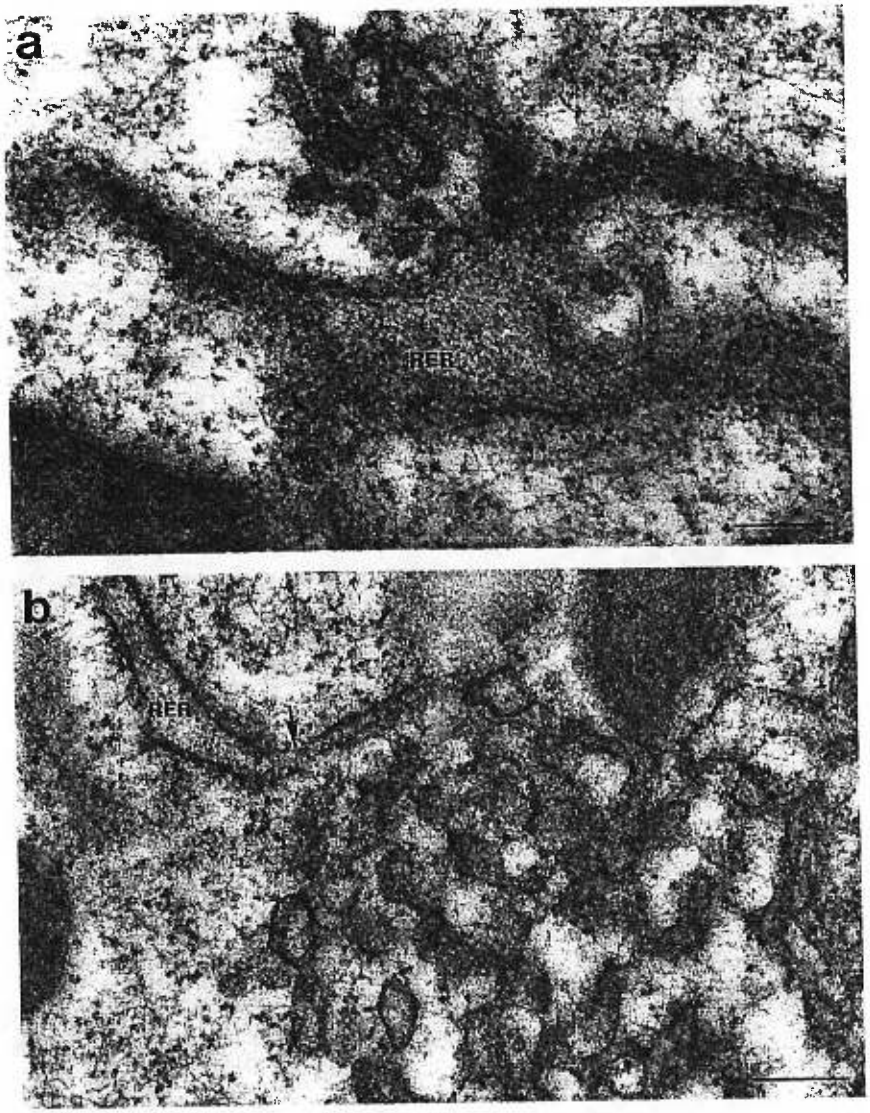

Fig. 7. IQ selectively targets smooth extensions of the ER. Only the smooth portion of AMF-R labeled part smooth/part rough ER membranes is fragmented in IQ treated cells (a,b). Connection between a ribosome studded rough ER cisterna and smooth ER networks is indicated by the arrow (b). Both rough ER cisternae (RER) and mitochondria (M) are intact in IQ-treated cells. Bars, 0.2 μM.

after 60 minutes of IQ treatment (Fig. 1e). By electron microscopy, these correspond to larger fenestrated clusters, however 4 hours after IQ treatment only unfenestrated tubules are present demonstrating that the effect of IQ is reversible (not shown). IQ also disrupts the integrity of microtubules and both the Golgi and AMF-R tubules are microtubule-associated organelles. Disruption of the microtubule cytoskeleton fragments the Golgi apparatus but does not disrupt the stacked cisternal morphology of the Golgi (Thyberg and Moskalewski, 1985). Similarly, disruption of the microtubule cytoskeleton results in a loss of the tubular extension and peripheral orientation of AMF-R tubules but not of their tubular morphology (Benlimame et al., 1995). For both organelles, the action of IQ is independent of the state of the microtubule cytoskeleton and the addition of IQ after nocodazole or taxol treatment induces a further fragmentation of both organelles to smaller structures (Fig. 3; Veit et al., 1993). The action of IQ on the two organelles is therefore similar in that it is not microtubule-dependent, is reversible, and does not occur at 4°C or in the presence of azide (Takizawa et al., 1993) (data not shown) suggesting that the molecular target of IQ in both organelles is the same. However, while IQ clearly induces Golgi vesiculation, the tubular network of AMF-R tubules formed following IQ treatment suggests that IQ does not stimulate vesicle budding.

The AMF-R tubule is a highly labile organelle and optimal fixation requires a rapid freezing fixation method in which methanol/acetone precooled to −80°C is added rapidly to cells. As described for the tubular lysosome, also a highly labile tubular organelle (Robinson et al., 1986), the tubular morphology of the AMF-R tubule is stabilized by Ringer's solution (Heuser, 1989; Racoosin and Swanson, 1993) and a protocol based on Ringer's solution permitted visualization of the AMF-R tubule by conventional postembedding EM procedures (Benlimame et al., 1995). Pretreatment of cells with Ringer's solution for 15 minutes results in the identification of extended smooth tubular networks (Benlimame et al., 1995) which are not present to the same extent if the cells are only rinsed rapidly

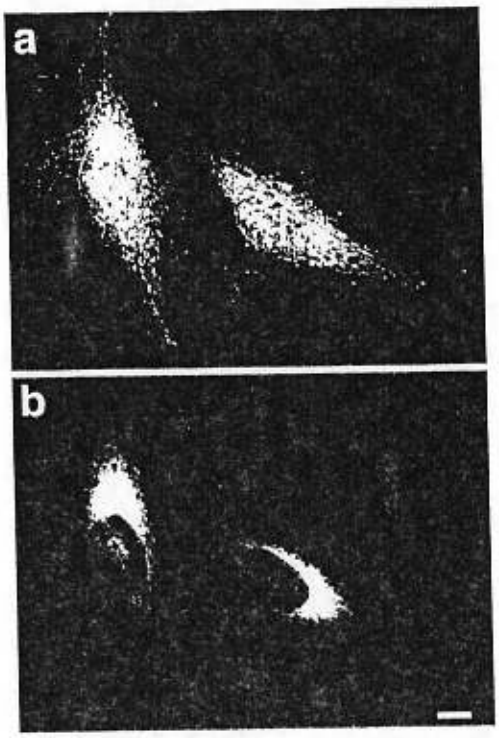

Fig. 8. AMF-R labeled tubules do not colocalize with the intermediate compartment labeled for ERGIC-53. HeLa cells were double labeled with antibodies to AMF-R (a) and to ERGIC-53 (b) and three adjacent confocal 1 μM sections summated. Distinct AMF-R labeled tubular structures can be visualized in the perinuclear region compared to the diffuse ERGIC-53 label and AMF-R tubules in the cell periphery are not labeled for ERGIC-53. Bar, 20 μM.

with Ringer's solution prior to fixation (Fig. 4). The presence of immunofluorescently labeled tubular structures in cells rapidly fixed by the addition of precooled (−80°C) methanol/acetone indicates that AMF-R tubules are present in viable cells and not a consequence of the EM fixation procedure.

The ability of IQ to fragment AMF-R tubules might be related to the labile nature of this organelle. It has been suggested that the structure of certain organelles might represent an equilibrium between homotypic fusion and periplasmic fusion, the fusion of internal membranes resulting in organelle vesiculation (Rothman and Warren, 1994). The AMF-R tubule exhibits a highly fluid morphology, contrary to the regular nature of rough ER tubules, that may permit the interaction of internal membranes necessary for periplasmic fusion (Benlimame et al., 1995; Benlimame and Nabi, 1997). In the presence of IQ AMF-R labeled tubules are narrower and present numerous fenestrations whose membranous limitations can be visualized in 80 nm ultrathin sections (Fig. 6). The disruption of AMF-R tubules by IQ as visualized by fluorescence microscopy (Figs 1, 2) would thus appear to be a consequence of the progressive fenestration of AMF-R tubules generating a fine meshwork of narrow interconnected tubules. These fenestrated networks resembles structures predicted by periplasmic fusion (Rothman and Warren, 1994). The selective effect of IQ on Golgi and AMF-R tubules may be due to the ability of those organelles to fenestrate (Rambourg and Clermont, 1990; Weidman et al., 1993) and the ability of IQ, by an as yet unknown mechanism, to favor this process.

The AMF-R tubule is a smooth subdomain of the ER

Quantitative postembedding labeling of MDCK cells reveals the predominant labeling of smooth tubules with a lesser labeling of rough ribosome-studded tubules (Benlimame et al., 1995). AMF-R tubules do not colocalize with the calnexin labeled rough ER, however, rare tubules can be identified which are labeled for both calnexin and AMF-R. Part smooth/part rough tubules, morphologically equivalent to transitional ER, are labeled for AMF-R and support the identity of the AMF-R tubule as a smooth ER-associated organelle. In MSV transformed MDCK cells, both AMF-R tubules and the calnexin labeled rough ER selectively associate with a subdomain of microtubules enriched in stabilized microtubules (Nabi et al., 1997). Evidence that AMF-R labeled tubules are equivalent to smooth ER is supported by the morphology of AMF-R tubules in IQ treated cells. The intermingled tubules observed after IQ treatment are smaller and narrower than those in untreated cells and resemble the smooth ER of hepatocytes (Fawcett, 1981). IQ does not affect rough ER and of particular interest is the selective fragmentation of the smooth portion of labeled tubules which extend from intact rough ER cisternae (Fig. 7). These images resemble the interface between smooth and rough ER in the hepatocyte in vivo (Fawcett, 1981) and following assembly from purified rat liver microsomes in the presence of ATP and GTP (Lavoie et al., 1996).

Transitional ER has been proposed to be the site of exit of newly synthesized proteins en route to the Golgi (Palade, 1975). The presence of marker proteins, ERGIC-53, p58 and rab2 have identified ERGIC as an organelle (compartment) distinct from the ER (Schweizer et al., 1988; Chavrier et al., 1990; Saraste and Svensson, 1991). However, some data support the idea that the ERGIC is continuous with the rough ER, and thus can be considered a smooth subdomain of the ER (Saraste and Svensson, 1991; Hauri and Schweizer, 1992; Krijnse-Locker et al., 1994). Degradation of newly synthesized proteins that are misfolded or fail to assemble into the appropriate oligomers takes place in the ER or in a related compartment, possibly the ERGIC (Bonifacino and Lippincott-Schwartz, 1991; Hauri and Schweizer, 1992; Klausner et al., 1992). Unassembled Rubella virus E1 glycoprotein is arrested in a smooth membranous tubular structure contiguous with the ER proximal to and distinct from ERGIC (Hobman et al., 1992). In thymic epithelial cells derived from transgenic mice deficient for the transporter associated with antigen presentation (TAP), misfolded major histocompatibility complex class I molecules accumulate in a degradative compartment consisting of an expanded network of tubular and fenestrated membranes which contains ERGIC-53; the morphology of this expanded compartment in plastic resins is remarkably similar to that of AMF-R tubules (Raposo et al., 1995).

Double immunofluorescence labeling of HeLa cells with antibodies to AMF-R and ERGIC-53 clearly demonstrate the presence of AMF-R tubules that do not contain ERGIC 53 indicating that the smooth ER-compartment labeled for AMF-R is not equivalent to ERGIC (Fig. 7). β-COP has been localized to ERGIC as well as to the *cis*-Golgi (Oprins et al., 1993; Krijnse-

Locker et al., 1994). β-COP does not label peripheral AMF-R tubules in MDCK cells and confocal studies of transformed MDCK cells showed that β-COP does not colocalize with AMF-R tubules (Benlimame et al., 1995; Nabi et al., 1997). Distinction between AMF-R tubules and ERGIC is further indicated by the sensitivity of AMF-R tubules to IQ. IQ blocks intraGolgi transport but not the delivery of newly synthesized Golgi proteins to *cis*-Golgi compartments. In the presence of IQ, newly synthesized VSV G protein does not acquire endo H sensitivity and thus does not reach medial Golgi compartments; it does however exhibit a slightly modified molecular mass consistent with partial glycosylation of the protein in *cis*-Golgi elements (Takizawa et al., 1993). This apparent functionality of ERGIC in the presence of IQ and the demonstrated IQ-sensitivity of AMF-R tubules discounts identity between these two organelles. We cannot, however, exclude the possibility that the AMF-R tubule can interact with other ER-associated subdomains or organelles, including ERGIC.

The endoplasmic reticulum is a continuous membrane network linked to the nuclear envelope and is composed of subcompartments morphologically identified as the rough ER, studded with ribosomes, and the smooth ER, which is devoid of ribosomes (Sitia and Meldolesi, 1992; Vertel et al., 1992). Transitional ER morphologically represents sites of interaction between rough and smooth subdomains of the ER, however, morphological similarity does not necessarily reflect functional identity. Distinct smooth ER subdomains have been proposed to be involved in the secretory pathway (Hobman et al., 1992) but they have also been shown to interact with organelles other than the Golgi apparatus and to be implicated in cellular functions other than ER to Golgi transport. The distinctive architecture of hippocampal neurons permitted the demonstration of the existence in axons and dendrites of smooth ER membranes distinct from ERGIC which are localized exclusively to the cell body (Krijnse-Locker et al., 1995). Autophagic vacuoles derive from ER membranes (Dunn, 1990a,b) and the transition of rough ER tubules into smooth ER tubules which subsequently fuse with lysosomes via a non-autophagic pathway has been described (Noda and Farquhar, 1992). Smooth ER has been implicated in calcium storage in a variety of cell types (Pozzan et al., 1994; Golovina and Blaustein, 1997). The expansion of smooth ER in specific cell types demonstrates the existence of specialized smooth ER compartments that can be exploited for specialized functions such as lipid biosynthesis or detoxification (Jones and Fawcett, 1966; Stäubli et al., 1969; Orci et al., 1984; Pathak et al., 1986). Induction of smooth ER in liver hepatocytes by phenobarbitol permitted the identification of epoxide hydrolase as a marker for liver smooth ER (Galteau et al., 1985). While AMF-R labels ribosome-studded tubules in MDCK cells, minimal overlap with the calnexin labeled rough ER is detected by immunofluorescence double labeling (Benlimame et al., 1995) and in NIH-3T3 and HeLa cells, AMF-R is localized almost exclusively to smooth tubules and labeling of rough ER is negligible (Benlimame and Nabi, unpublished results). AMF-R does not label a continuous network by immunofluorescence labeling suggesting that the AMF-R tubule is a subdomain of the smooth ER. The presence of AMF-R identifies a role for this subdomain of the ER in cell motility, perhaps as an internal pool of membrane which can be mobilized to the plasma membrane at the leading edge following a motile stimulus.

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3. SUPPLEMENTAL RESULTS

3. 1. Materials and methods

Antibodies and chemicals

Monoclonal antibody against AMF-R was used either in the form of ascites fluid or concentrated hybridoma supernatant (Nabi et al., 1990) Antibody to calreticulin was a gift from Dr. Luis Rokeach (Département de biochimie, Université de Montréal). Antibody to lysosomal associated membrane protein-2 (LAMP-2) was as previously described (Nabi et al., 1991; Nabi and Rodriguez-Boulan, 1993). Antibody to mitochondria (Mt-HSP-70) was purchased from Affinity Bioreagents, Inc (Golden, Colorado, U.S.). Secondary antibodies conjugated to either Texas red, or FITC were purchased from Jackson Laboratories (West Grove, PA). The fluorescent antibodies were designated 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.

Taxol was obtained from the National Products Branch of the National Cancer Institute (Bethesda, MD). Digitonin, ATP, GTP, nocodazole, N-ethylmaleimide (NEM) and dithiothreitol (DTT) were purchased from Sigma (Oakville, Ontario, Canada). Creatine kinase, creative phosphate and GTPγS were purchased from Boehringer Mannheim (Laval, Quebec, Canada).

Cells and cell culture

All cells were grown in an air-5% CO_2 incubator at constant humidity. MDCK, HeLa, and HT1080 cells were grown in Dulbecco's

modified Eagle medium (DMEM) supplemented with 5%-10% fetal calf serum, non-essential amino acids, vitamins, glutamine and a penicillin-streptomycin antibiotic mixture.

Immunofluorescence

Cells were plated (25,000-50,000 cells/35 mm dish) on glass cover slips for 2 days before each experiment. Cells were fixed by the addition of cold (-80°C) methanol/acetone (80%/20% v/v) directly to the coverslips, and then placed at -20°C for 15 minutes. After fixation, the cells were rinsed extensively with PBS (pH 7.4) supplemented with 0.1mM Ca^{++} and 1mM Mg^{++} (PBS/CM), and then incubated for 15 min with PBS/CM containing 0.5% BSA (PBS/CM/BSA) at room temperature to reduce nonspecific binding. All washings and incubations with both primary and secondary (FITC and Texas red conjugated) antibodies were done with PBS/CM/BSA. 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. Images were photographed using Kodak T-Max 400 film. Confocal analysis was performed with a Bio-Rad MRC 600 confocal microscope and printed with a Polaroid TX 1500 printer.

Permeabilization of MDCK cells with digitonin

50,000 MDCK cells were plated and incubated for 2 days in 35mm petri dishes in culture medium containing 10% fetal calf serum. The cells were pretreated with 10mM taxol for 30 minutes to stabilize the microtubules and then quickly washed twice with cytoskeleton stablizing buffer (CSB) (130mM Hepes, 2mM $MgCl_2$, 10mM EGTA PH 6.9) which had

been prewarmed to 20°C. The cells were then incubated with 10 mg/ml digitonin in Tris buffer (25mM Tris, 25mM KCl, 1mM DTT, PH 7.4) for one minute at 20°C. The cells then were washed with CSB and incubated in Tris buffer with ATP, GTP, ATP regenerating system and rat liver cytosol at 37°C as mentioned in the text. The cells were washed twice with CSB containing 0.2% BSA before fixation with 80%/20% (v/v) methanol/acetone.

Rat liver cytosol preparation

Cleaned rat liver was cut to small pieces by scissors in Tris buffer B (Tris buffer plus 85mM sucrose, pH 7.4) containing protease inhibitors. The cut-up liver tissue was homogenized with a Dounce homogenizer at 4°C in 1 volume of the Tris buffer B. The homogenate was centrifuged at 15,000 rpm for 20 minutes (Sorvall centrifuge, SS34 rotor), and the supernatant was recentrifuged at 100,000 g for 90 minutes at 4°C (Beckman, Ti 60 rotor). The supernatant was collected and assayed for protein concentration.

Calcium precipitated cytosol

Rat liver cytosol (70mg/ml) was adjusted to 10mM Ca^{++} on ice for 15 minutes and then centrifuged at 100,000g for 1 hour at 4°C (Sorvall, RP rotor). The supernatant is considered to be calcium-binding protein depleted cytosol (CBPDC) (Lin et al., 1992).

Electron microscopy

Cells grown on petri dishes were rapidly washed twice with Ringer's solution before fixing in the same solution containing 2% paraformaldehyde and 0.2% glutaraldehyde for 30 min at 37°C. The fixed cells were rinsed in

PBS/CM, scraped from the petri dishes 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, dehydrated and embedded in LR-White resin. Ultra-thin sections were blocked with 2% BSA in PBS/CM, and then incubated at room temperature with anti-AMF-R antibody followed by 12 nm gold conjugated anti-rat goat antibodies. The sections were stained with 5% uranyl acetate and visualized in a Philips 300 electron microscope.

Quantification of AMF-R tubule overlap with mitochondria

Confocal images of cells double labeled with anti-AMF-R (Texas red) and anti-Mt-HSP70 (FITC) were used to assess the extent of overlap of AMF-R tubule labeling with mitochondria, using Northern Eclipse software (Empix Imaging, Mississuage, Ontario). First, the non-specific AMF-R labeling of the nuclei was cut from the image and then total intensity and area of AMF-R labeling was determined. The mitochondria labeling was enhanced using a convolution filter and any AMF-R labeling which coincided with the enhanced mitochondrial labeling was cut from the image. The intensity and area of remaining AMF-R labeling was determined to generate a value for AMF-R labeling which did not codistribute with mitochondria. Enhancing the mitochondrial labeling with the convolution filter ensures that shifts between the FITC and Texas red images or variations in the acquisition of the mitochondrial image did not influence the quantification of the extent of AMF-R tubule overlap with mitochondria.

3. 2. Results

3. 2. 1. AMF-R tubules and mitochondria codistribute in MDCK cells

MDCK cells were fixed by cold methanol/acetone and double immunofluorescently labeled with anti-AMF-R and anti-Mt HSP70 (Green et al., 1995). Using Texas red conjugated antibody to reveal the AMF-R tubules and FITC conjugated antibody to visualize mitochondria (HSP70) by immunofluorescence microscopy (see Materials and Methods), we observed a significant degree of overlap between AMF-R tubules and mitochondria (Figure 2). The codistribution was not due to crossreaction as negative controls revealed that the secondary antibodies were species-specific.

Association between AMF-R tubules and mitochondria in MDCK cells is maintained following preincubation with Ringer's solution, a bicarbonate free buffer that has been used to study tubular lysosomal expression (Heuser, 1989; Racoosin and Swanson, 1993) and which induces the formation of highly elongated AMF-R tubules after an incubation of 15 to 30 minutes prior to fixation (Benlimame et al., 1995; Nabi et al., 1992). In MDCK cells pretreated with the microtubule depolymerizing drug nocodazole, AMF-R and mitochondria still colocalize, even though the linear morphology of the AMF-R tubules and mitochondria changed to curly and short tubules (Figure 2).

3. 2. 2. Overlap between AMF-R tubules and mitochondria in different cell types

To investigate whether the overlapping distribution of AMF-R tubules and mitochondria exists in different cell types, MDCK, HeLa

(transformed human cervix epitheloid carcinoma cells) and HT-1080 (transformed human fibrosarcoma cells) cells were fixed with cold methanol/acetone and double immunofluorescently labeled with anti-AMF-R antibody and anti-Mt-HSP70 to reveal AMF-R and mitochondria, respectively (Figure 3). AMF-R and mitochondria labeling also show a certain degree of overlap in HeLa cells, but in HT-1080 cells extensive AMF-R labeling could be observed which did not overlap with mitochondria.

To obtain quantitative information about the extent of AMF-R and mitochondria overlap, confocal images of cells double fluorescently labeled for AMF-R and mitochondria were analyzed with Northern Eclipse software to quantify AMF-R labeling which overlapped with mitochondrial labeling (see Materials and Methods). In HeLa and MDCK cells approximately 80% of AMF-R labeling overlapped with mitochondria, while in MDCK cells preincubated with Ringer's solution more than 90% of AMF-R tubules overlap with mitochondria (Figure 4). In HT-1080 cells there is only 40% overlap between AMF-R tubules and mitochondria.

3. 2. 3. AMF-R and mitochondria dissociate following incubation with PBS-CM

The high degree of overlap between AMF-R and mitochondria in MDCK cells can be disrupted by incubation of MDCK cells with PBS-CM. In MDCK cells preincubated with PBS-CM for 15 to 30 minutes before fixation with methanol/acetone and double labeled for AMF-R and mitochondria, AMF-R labeling is localized to diffuse, punctate structures and very short tubules rather than elongated tubules throughout the cytoplasm. Mitochondria labeling is localized to elongated tubules oriented towards to the cell periphery, even in cells preincubated with PBS-CM for 15 to 30

minutes (Figure 5). In MDCK cells preincubated for 120 minutes with PBS-CM, AMF-R labeling relocalizes to elongated tubular structures which colocalize with mitochondria of MDCK cells. The association of AMF-R tubules with mitochondria can therefore be transiently dissociated by incubation with PBS-CM.

3. 2. 4. Ultrastructure of AMF-R tubule and mitochondria association.

Following post-embedding immunoelectron microscopy labeling using anti-AMF-R antibody and 12nm gold conjugated anti-rat goat antibody (see material and methods), we have seen that some gold labeled AMF-R tubules or vesicles are very closely associated with mitochondria (Figure 6). Other labeled AMF-R tubules and vesicles are dispersed in the cytosol, and not associated with mitochondria in EM sections.

Postembedding labeling of AMF-R shows that some gold labeling is localized to mitochondria. To determine the specificity of AMF-R labeling to mitochondria, we have quantified the post-embedding AMF-R labeling of rough ER, smooth ER and mitochondria in MDCK, HeLa and NIH-3T3 cells (Table 1). In all cell types, the majority of AMF-R labeling is found on smooth ER. A lesser labeling is also found on RER defined by the presence of a linear array of membrane-bound ribosomes. Minor labeling associated with mitochondria has also been observed, which most likely represents background labeling (Table 1).

3. 2. 5. The association between AMF-R and mitochondria is dissociated by cytosol incubation and is not NEM sensitive

The relationship between AMF-R tubules and mitochondria was further studied *in vitro*, using digitonin at concentrations which selectively

permeabilize the plasma membrane keeping intracellular organelles membrane intact (semi-intact cell) (Azucena et al., 1994). We use 10mg/ml digitonin in Tris buffer and incubated the MDCK cells at 20° C for 1 minute (see material and methods).

To ensure that intracellular organelles remain intact, we used anti-keratin, anti-lysosome associated membrane protein-2 (LAMP-2) and anti-calreticulin antibodies to monitor permeabilization of the cells. Anti-LAMP-2 and anti-calreticulin bind only to the lumenal domain of the proteins within lysosomes and ER, respectively. If the plasma membrane of the cells is permeabilized and the intracellular organelles are intact, cells permeabilized with digitonin and incubated with antibody before fixation should be labeled by anti-keratin but not anti-calreticulin and anti-LAMP-2. As we show in Figure 7, permeabilized MDCK cells are labeled by anti-keratin, but not labeled by anti-calreticulin and anti-LAMP-2. Digitonin therefore selectively permeabilizes the plasma membrane (Figure 7).

Semi-permeabilized MDCK cells were incubated at 37°C with Tris buffer alone, or containing a combination of an ATP regenerating system (ARS) (2mM ATP, 37u/ml creatine kinase, 8mM creatine phosphate), 2mM GTP, and 5mg/ml rat liver cytosol for 120 minutes (see material and methods) (Figure 8). Semi-intact MDCK cells incubated with Tris buffer alone and double immunofluorescently labeled for AMF-R and mitochondria revealed significant overlap. The control cells have 1.17% area of non-overlap labeling, meaning that 98.83% of AMF-R tubule and mitochondria labeling overlap. Lesser overlap labeling was observed in cells incubated with ARS plus rat liver cytosol, or only rat liver cytosol. In cells incubated with rat liver cytosol, non-overlap labeling area increased to 18.5% (Figure 9). Thus rat liver cytosol can disrupt AMF-R and

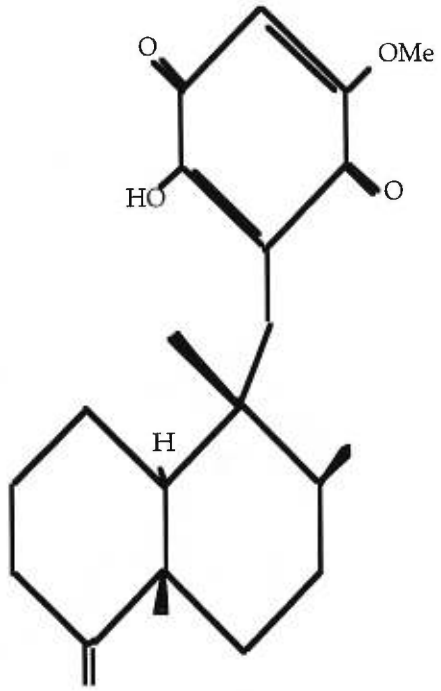
mitochondria association by a process which is not ATP dependent in semi-intact MDCK cells.

To determine if NSF is involved in these processes, rat liver cytosol was first treated with 0.5mM NEM for 15 minutes on ice and quenched with 25 mM DTT for 15 minutes on ice before incubation with semi-intact cells (Glick and Rothman, 1987). The overlap of AMF-R labeling and mitochondria in MDCK permeabilized cells incubated with NEM-treated cytosol is similar to cells incubated with rat liver cytosol. Cells incubated with NEM-treated cytosol have 14.7% area of non-overlap labeling. NSF or NSF like proteins and SNAPs are therefore unlikely to be involved in disruption of the association of AMF-R tubules and mitochondria (Figure 8). In permeabilized MDCK cells incubated with 2mM GTPγS and rat liver cytosol in Tris buffer, increased non-overlap labeling of AMF-R tubules with mitochondria was observed (28.6%) than in cells incubated with only rat liver cytosol (18.5%) (Figure 9).

3. 2. 6. Calcium-binding proteins are responsible for the dissociation of AMF-R tubules from mitochondria

To identify the proteins that are responsible for the association of AMF-R tubules and mitochondria, calcium-binding proteins depleted cytosol (CBPDC) was prepared by calcium precipitation. 5mg/ml rat liver cytosol was adjusted to 10mM calcium and incubated for 15 minutes on ice and the mixture was centrifuged at 100,000 g for 60 minutes at 4° C to deplete the calcium-binding proteins (Lin et al., 1992). Semi-intact MDCK cells were incubated at 37°C with CBPDC in Tris buffer for 120 minutes, and the cells were then fixed and double immunofluorescently labeled with anti-AMF-R and anti-Mt-HSP70 antibodies. A high degree of overlap

between AMF-R tubules and mitochondria was observed in cells incubated with CBPDC (3.6% dissociation), essentially equivalent to control cells (1.2%) (Figure 9) indicating that calcium-binding proteins present in cytosol might mediate the cytosol induced dissociation of AMF-R tubules and mitochondria.



Ilimaquinone (M. Wt. 350)

Figure 1. The chemical structure of ilimaquinone

Figure 2. AMF-R tubules condistribute with mitochondria in MDCK cells by immunofluoresecence labeling. MDCK cells were untreated (A, B), or preincubation with Ringer's solution for 15 minutes (C, D), nocodazole 20 mM for 60 minutes (E, F). Cells were double immunofluoresecently labeled with anti-AMF-R to reveal AMF-R (A, C, E), and with anti Mt-HSP70 antibody to reveal mitochondria (B, D, F). Bar=20mM.

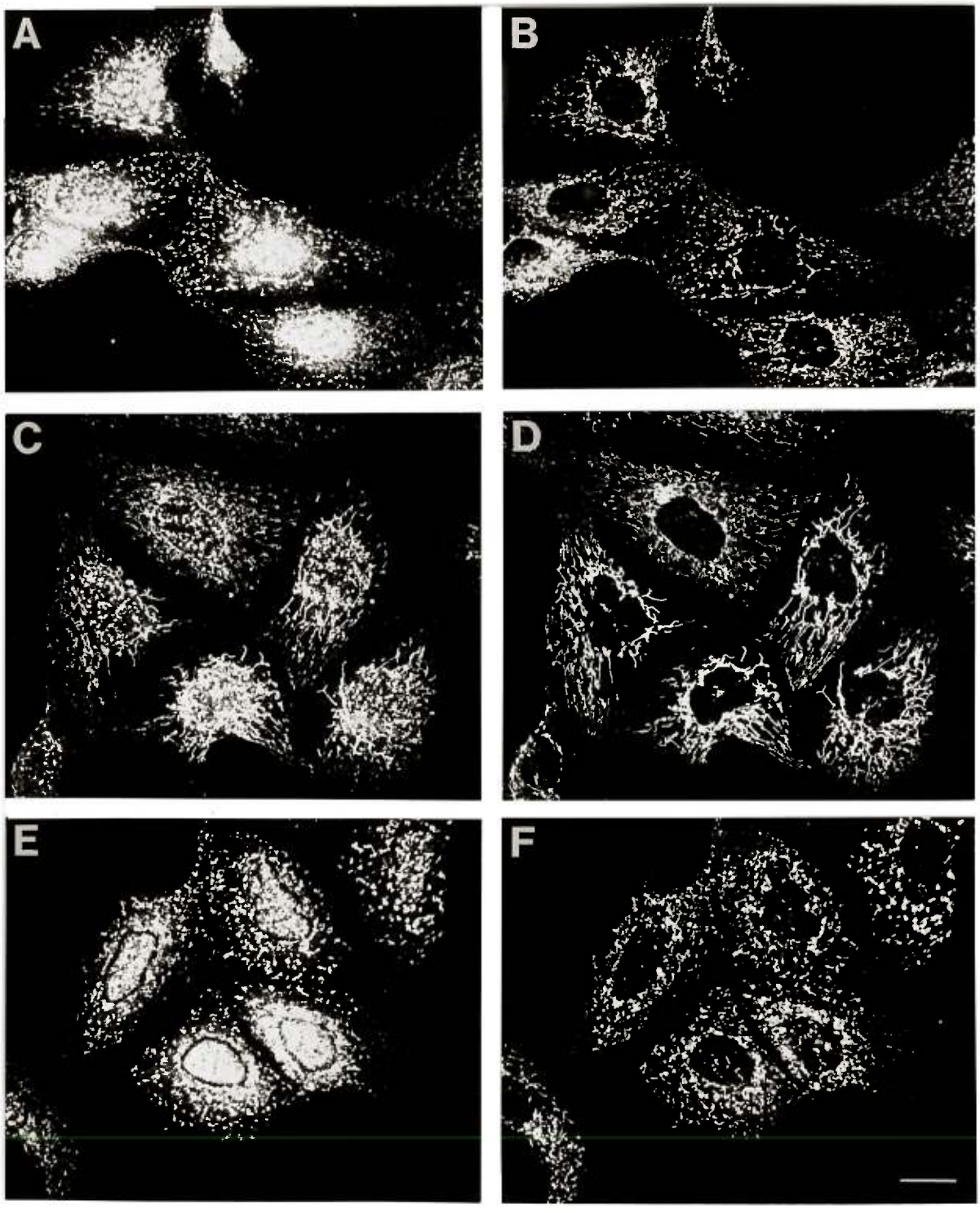

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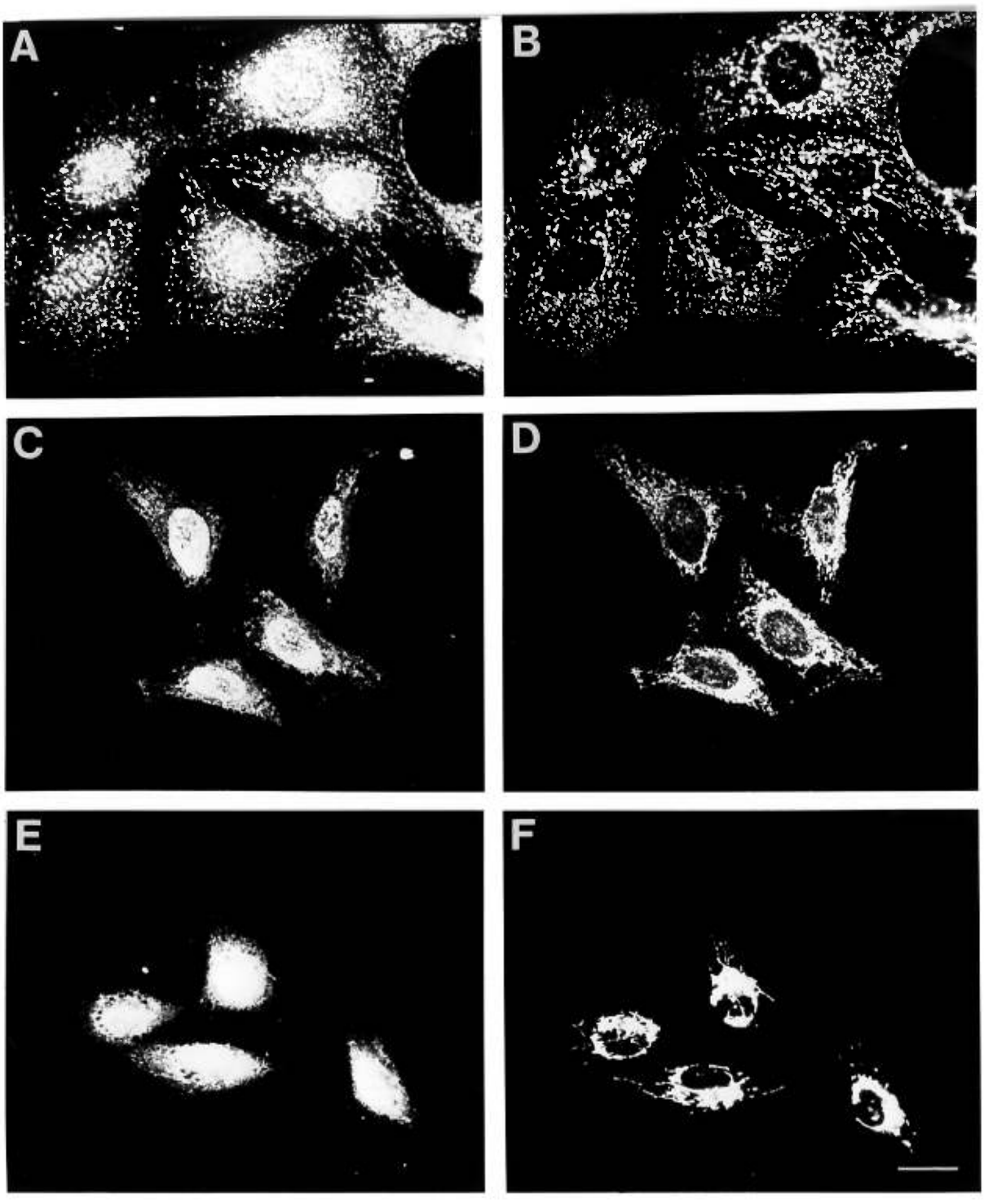
Figure 3. AMF-R tubule and mitochondria labeling do not colocolized in different cell types. MDCK cells (epithelial cells) (A, B), Hela cells (transformed human cervix epitheloid carcinoma cells) (C, D), and HT1080 (transformed human fibrosarcoma cells) (E, F) are fixed with methanol/acetone and double immunofluorescently labeled with anti-AMF-R antibody to reveal AMF-R (A, C, E), and anti Mt-HSP70 antibody to reveal mitochondria (B, D, F). Bar=20mM.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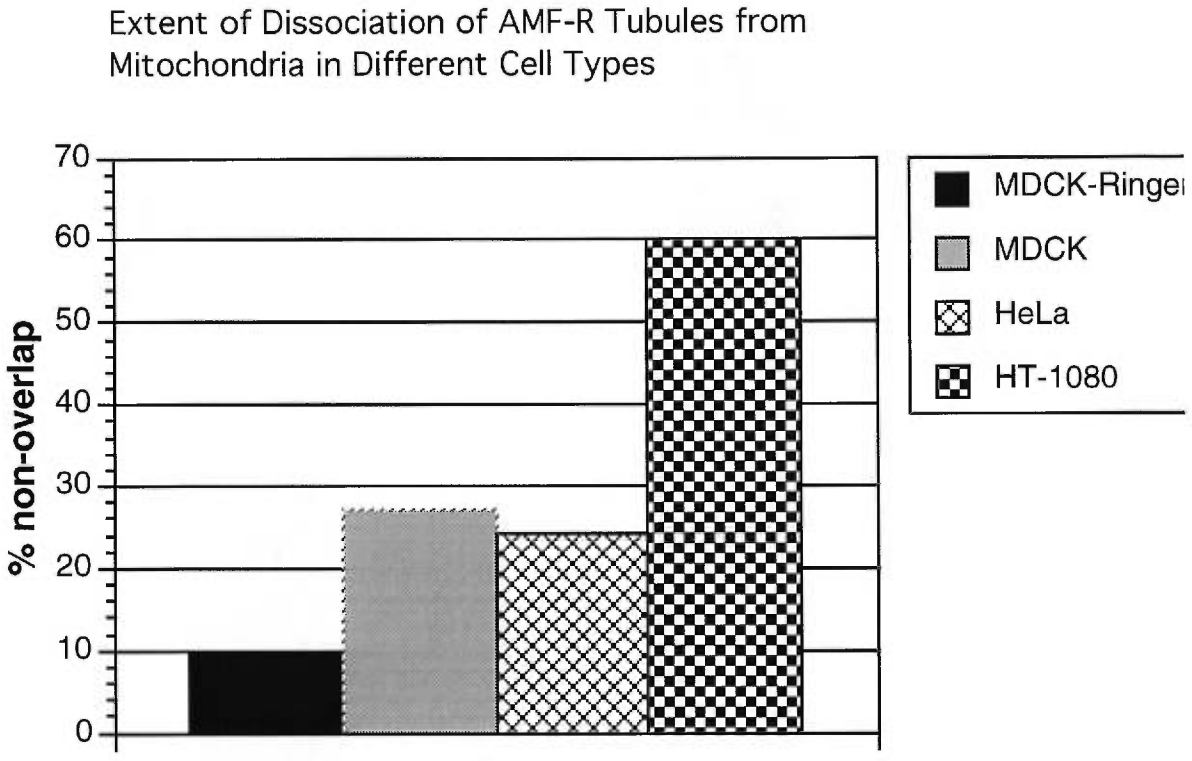

Figure 4. HeLa, HT-1080, MDCK cells and MDCK preincubated with Ringer's solution were double labeled for AMF-R and Mt-HSP70 and confocal images analyzed for non-overlap using Northern Eclipse software.

Figure 5. AMF-R tubules dissociate from mitochondria in MDCK cells treated with PBS/CM. MDCK cells were incubated with PBS-CM for 15 minutes (A, B), 30 minutes (C, D), and 120 minutes (E, F). Cells were double immunofluoresecently labeled with anti-AMF-R antibody to reveal AMF-R (A, C, E) and anti Mt-HSP antibody to reveal mitochondria (B, D, F). Bar=20 mM.

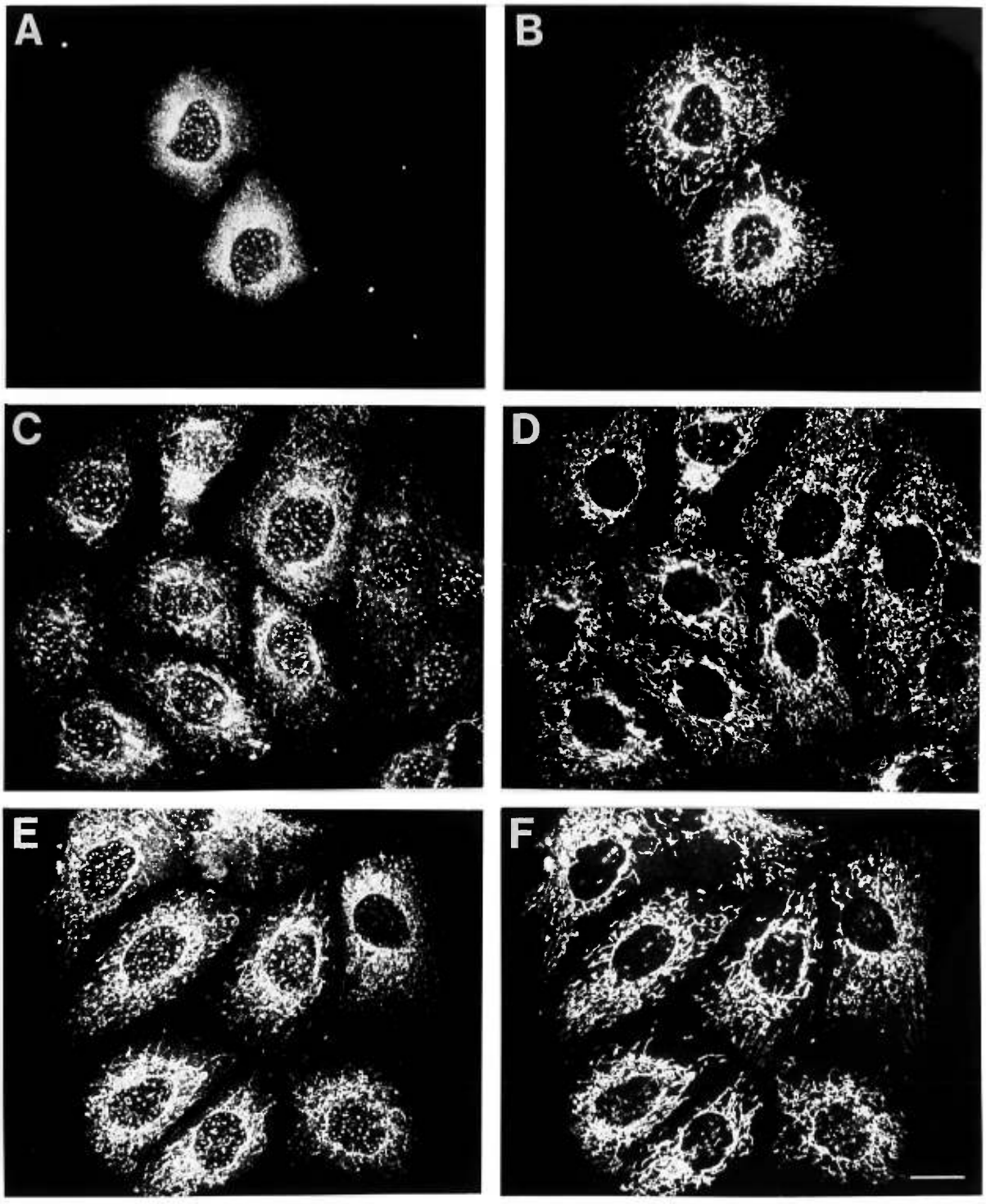
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Figure 6. AMF-R labeling is localized to smooth vesicles and tubules which can form elaborate networks in MDCK cells by electron microscopy. MDCK cells were rinsed twice rapidly in Ringer' s solution prior to fixation and post-embedding immunogold labeling for AMF-R. AMF-R labeled smooth tubules are indicated by arrows. Bar=0.2 mM.

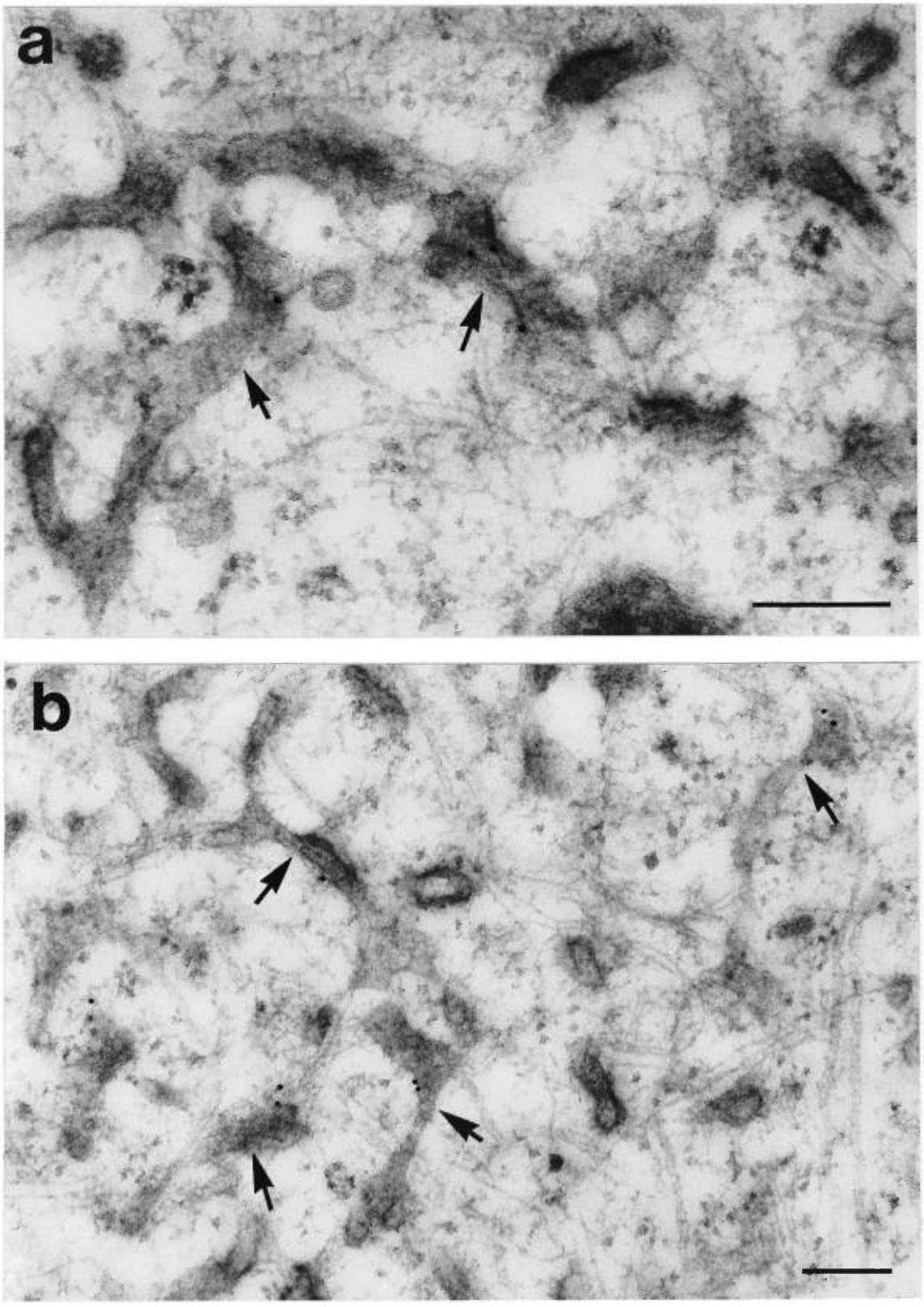
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Figure 7. Lysosomes and ER remain intact after permeabilization of MDCK cells by digitonin. MDCK cells are preincubated with 10mg/ml digitonin for 1 minute at 20°C and incubated with anti-keratin, anti-LAMP-2 and anti-calreticulin (ER protein antibody, against lumenal domain) at 4° C before (A, B, E, F) and after (C, D, G, H) fixation and permeabilization with saponin. Indirect immunofluorescence labeling reveal keratin (A, C, E, G), LAMP-2 (B, D), and calreticulin (F, H). Cells incubated with antibody before fixation are labeled by keratin , but not labeled when recogenise lumenal protein domain of lysosomal and ER proteins respectively.

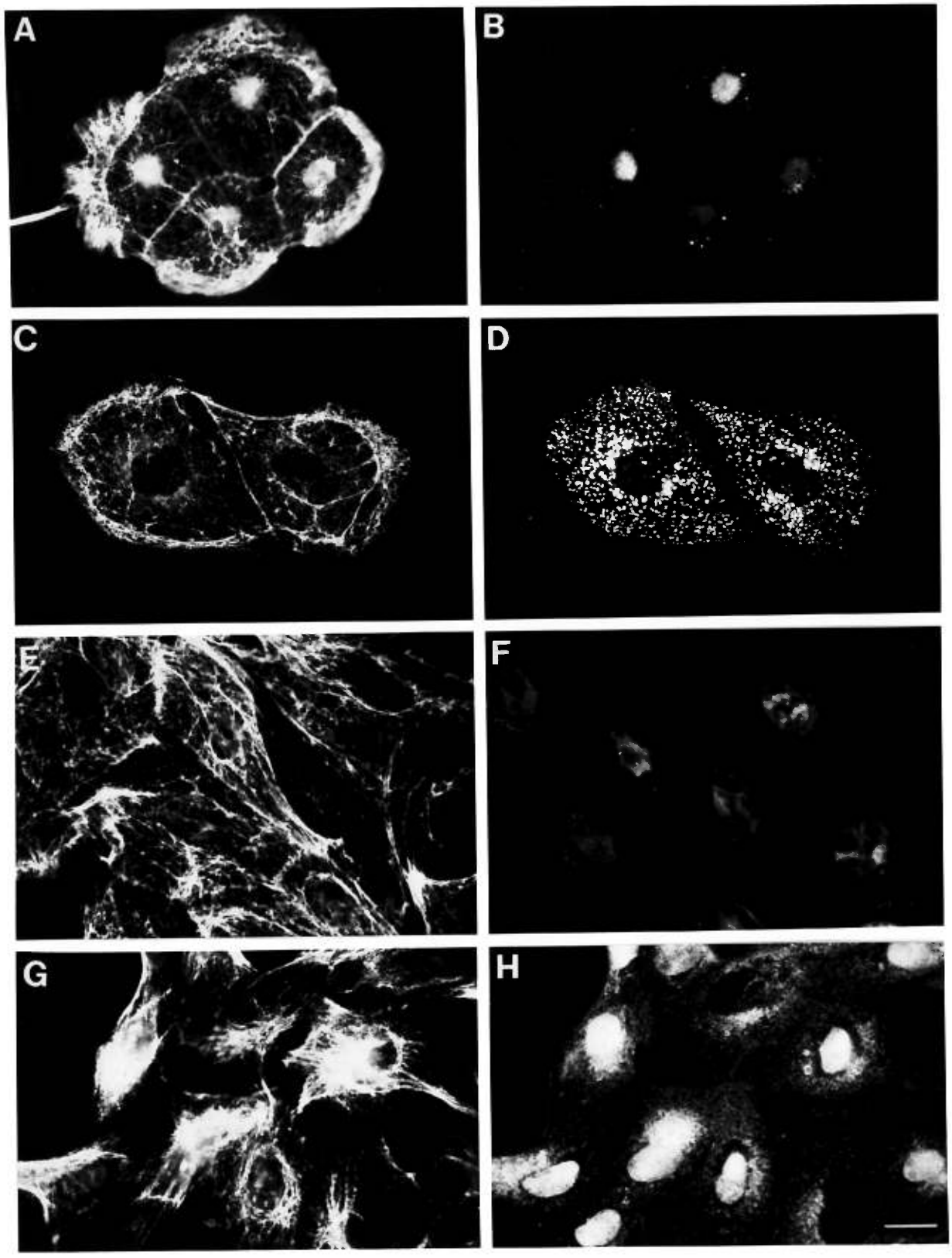
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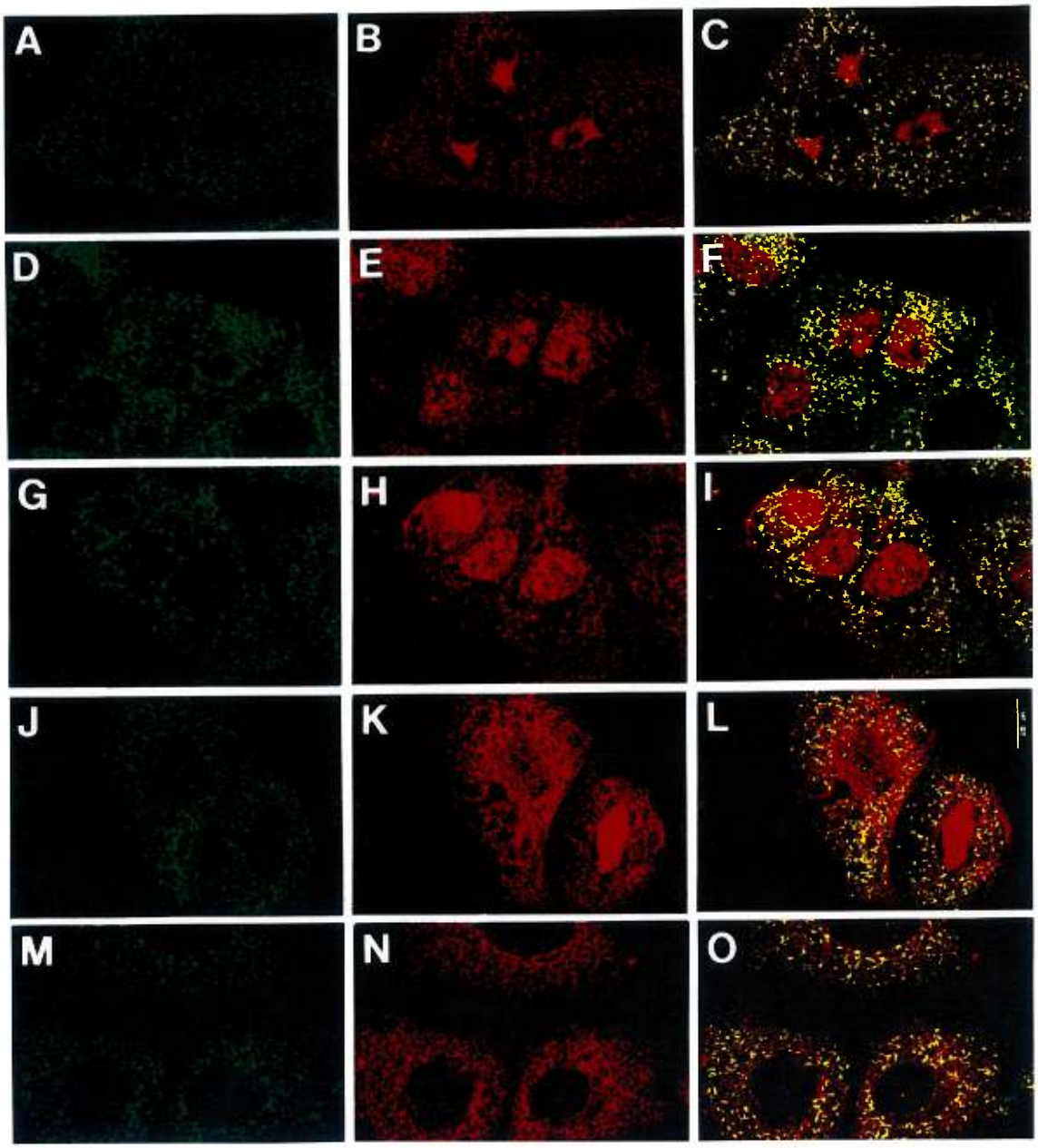
Figure 8. Regulation of the interaction between AMF-R tubules and mitochondria in semi-digitonin permeabilized MDCK cells. MDCK cells are permeabilized by digitonin (see material and method), and incubated with Tris buffer (A, B, C), ARS (ATP regeneration system: ATP, creatine kinase, creatine phosphate)(D, E, F), ARS+cytosol (G, H, I), cytosol (J, K, L) and NEM treated cytosol (M, N, O) for 1 hour at 37 °C. Cells were immunofluoresecently labeled with anti-AMF-R antibody to reveal AMF-R (A, D, G, J, M) and anti-HSP70 (B, E, H, K, N) to reveal mitochondria. Dual color merged confocal images revealed that AMF-R and mitochondria colocalization (C, F, I, L, O). AMF-R (red), mitochondria (green). In the presence of cytosol AMF-R labeling could be clearly identified which was not associated with mitochondria. Bar=20mM.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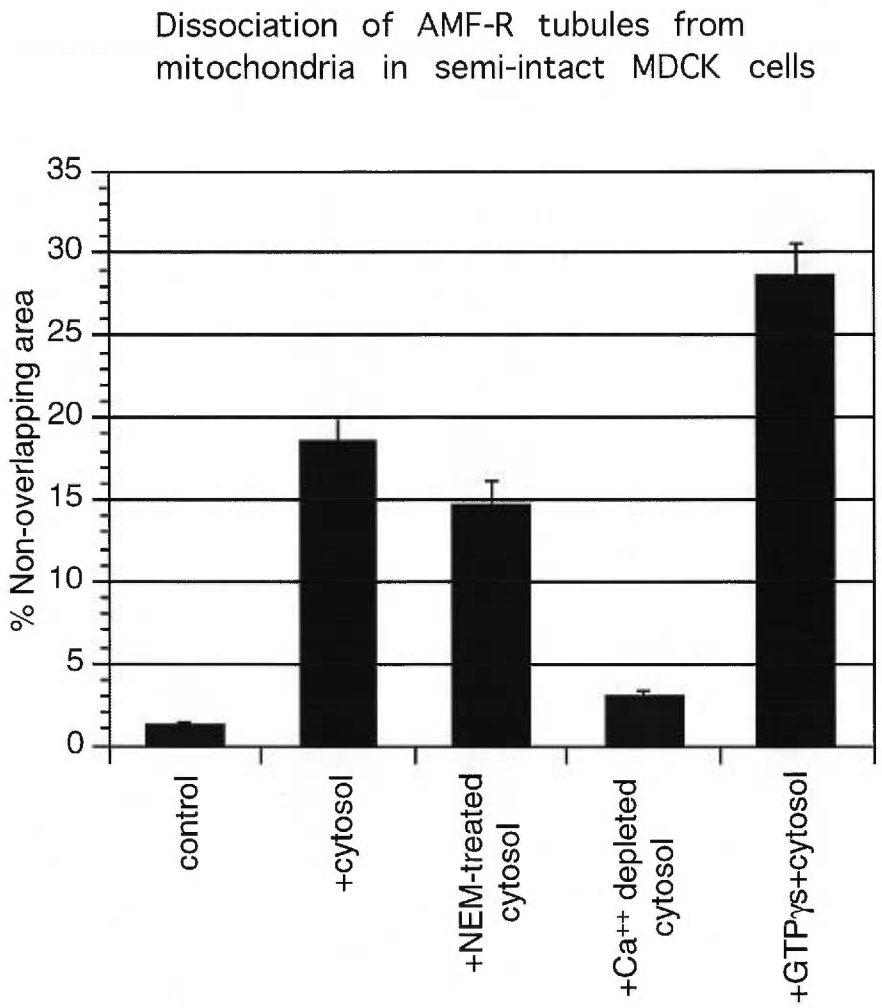

Figure 9. Digitonin permeabilized MDCK cells were incubated in Tris buffer pH7.4, plus cytosol, plus NEM-treated cytosol, plus Ca++-binding proteins depleted cytosol or plus GTPgs and cytosol, as indicated. The cells were then fixed and labeled for AMF-R and Mt-HSP70 and the extent of non-over lap between the two organelles determined from confocal microscopy.

Post-embedding AMF-R labeling of smooth ER, rough ER and mitochondria in HeLa, NIH-3T3 and MDCK cells

		Smooth ER	Rough ER	Mitochondria
HeLa	Surface Area (μ^2)	10.9	6.4	10.0
	Gold particles	428	47	49
	Gold particles/μ^2	**39.2**	**7.3**	**4.90**
NIH-3T3	Surface Area (μ^2)	19.5	7.9	15.2
	Gold particles	425	41	76
	Gold particles/μ^2	**21.8**	**5.2**	**5.0**
MDCK	Surface Area (μ^2)	10.4	2.4	5.7
	Gold particles	656	76	54
	Gold particles/μ^2	**62.9**	**32.0**	**9.5**

4. DISCUSSION

4. 1. Overlap of AMF-R tubules and mitochondria

4. 1. 1. Immunofluorescence microscopy shows overlap of AMF-R tubules and mitochondria

Using cultured MDCK cells or semi-permeabilized MDCK cells, double immunofluorescence labeling with anti-AMF-R and anti-Mt-HSP70 antibodies revealed significant overlap between AMF-R tubules and mitochondria. By immunofluorescent labeling, the overlap between these two organelles is maintained in MDCK cells preincubated with Ringer's solution, which elongates the AMF-R tubules (Heuser, 1989; Nabi et al., 1992; Racoosin and Swanson, 1993) or pretreated with nocodazole that disrupts the extension of AMF-R tubules (Benlimame et al., 1995). However, the degree of the overlap between AMF-R and mitochondria varies between different cell types, such as MDCK, HeLa and HT-1080 cells (Figure 3). In HeLa and MDCK cells, about 80% of AMF-R labeling are colocalized with mitochondria, while in HT-1080 cells only 40% of AMF-R labeling is colocalized with mitochondria. In semi-intact MDCK cells (incubated with Tris buffer) increased overlap between these two organelles was observed compared to intact cultured cells (see Figure 4 and Figure 9).

The colocalization of the AMF-R tubules and mitochondria can be disrupted either by preincubation the MDCK cells with PBS-CM buffer for a short time before fixation (Figure 5), or by incubating the semi-intact MDCK cells with rat liver cytosol (Figure 8). We also investigated and quantified the labeling of smooth tubules, rough ER and mitochondria by using

postembedding immunoelectron microscopy labeling with anti-AMF-R antibody and 12nm gold conjugated anti-rat secondary antibody in Hela, NIH-3T3 and MDCK cells (Table 1). The AMF-R labeling is predominantly located to smooth tubules compare to rough ER and mitochondria in these three cell types. These results indicate that the AMF-R tubules and mitochondria are two different organelles, and that mitochondria do not express proteins or antigens that are recognized by anti-AMF-R antibody. AMF-R tubules might be intimately associated with mitochondria.

The resolution of IF is limited for analysing the detailed organization of the membrane compartments involved in the biosynthetic or endocytic routes and there are substantial problems in relating the immunofluorescence image to the cellular structure carrying the antigen. This becomes especially apparent when the localization of an antigen at the levels of both immunofluorescence light microscopy and electron microscopy are compared (Griffiths et al., 1993). By EM, some AMF-R tubules are very closely associated with mitochondria, but others are dispersed in the cytosol (Wang et al., 1997). The colocalization of AMF-R tubules with mitochondria by EM is not as extensive as that observed by immunofluorescence microscopy. This difference by EM and IF may due to low resolution of IF.

4. 1. 2. The relationship between AMF-R tubules and mitochondria

4. 1. 2. 1. The AMF-R tubule is a smooth subdomain of the ER

Smooth AMF-R labeled tubules exhibit continuity with the rough ER cisternae and part smooth /part rough tubules morphologically equivalent to transitional ER, are labeled for AMF-R, supporting the identity of the

AMF-R tubule as a smooth ER-associated organelle (Benlimame et al., 1995). Transitional ER has been proposed to be the site of exit of newly synthesized proteins en route to the Golgi (Palade, 1975). The presence of marker proteins, ERGIC-53, p58 and rab2 have identified ERGIC as a compartment distinct from the ER (Chavrier et al., 1990; Saraste and Svensson, 1991; Schweizer et al., 1988). However, some data support the idea that the ERGIC is continuous with the rough ER, and thus can be considered a smooth subdomain of the ER, perhaps transitional ER (Hauri and Schweizer, 1992; Krijnse-Locker et al., 1994; Saraste and Svensson, 1991). Double immunofluorescence labeling of HeLa cells with antibodies to AMF-R and ERGIC-53 clearly demonstrate the presence of AMF-R tubules that do not contain ERGIC indicating that the smooth ER compartment labeled for AMF-R is not equivalent to ERGIC (Wang et al., 1997)(Section 2).

IQ selectively fragments the smooth portion of AMF-R labeled tubules which extend from rough ER cisternae (Wang et al., 1997). These images resemble the interface between smooth and rough ER in the hepatocyte in vivo (Fawcett, 1981) and following assembly from purified rat liver microsome in the presence of ATP and GTP (Lavoie et al., 1996). Thus the AMF-R tubule is a smooth ilimaquinone-sensitive subdomain of the ER (Wang et al., 1997).

4. 1. 2. 2. ER and mitochondria relationship

A close relationship between ER and mitochondria has been described. In hepatocytes and many other cell types, mitochondria are located very close to cisternae of the endoplasmic reticulum and nuclear envelope (Dallner et al., 1968; Loud, 1968;Ruby et al., 1969). Previously

experiments found that the outer mitochondrial membrane is continuous with ER membranes, predominantly smooth ER membranes, although occasionally a few ribosomes were observed attached to membrane connections between ER and mitochondria (Werner and Kartenbeck, 1971). Tubules continuous with the outer mitochondrial membrane which form connections between two neighbouring mitochondria or between mitochondria and ER have been visualized from electron microscopy examination of thin sections and negative staining of hepatocytes (Morré et al., 1971; Werner and Kartenbeck, 1971). Serial section analysis of intact rat liver indicated that RER saccules fit over mitochondria like caps providing broad areas of contact with the mitochondrion (Montisano et al., 1982). It has also been found that on sedimentation of liver homogenates a fraction of the reticulum remains bound to mitochondria (Lewis and Tata, 1973) and can only be completely removed by mechanical shearing or digitonin extraction (Cascarano et al., 1982; Pickett et al., 1981), procedures which strip off the mitochondrial outer membrane. Zonal centrifugation and sedimentation equilibrium centrifugation show very close association between RER and mitochondria (Montisano et al., 1982).

Bcl-2 is a oncoprotein that is expressed in nuclear envelope, ER and mitochondria (Krajewski et al., 1993; Lithgow et al., 1994). Subcellular fractionation analysis of a lymphoma cell line revealed the presence of bcl-2 protein in nuclear heavy membranes and a light membrane fraction. Further fractionation of light membrane fractions using discontinuous sucrose gradients revealed association of bcl-2 protein primarily with light-density microsomes derived from the smooth ER as opposed to heavy-density microsomes derived from the rough ER. These results have shown

that proteins associated with smooth ER can also associate with mitochondria.

4. 2. What is the basis for the intimate association between AMF-R tubules and mitochondria?

4. 2. 1. Mitochondria are the major energy maker of the cell

Mitochondria occupy a substantial portion of the cytoplasmic volume of eucaryotic cells and are present in virtually all eucaryotic cells. The membrane of the mitochondrion plays a crucial role in the function of these energy converting organelles by providing a framework for electron transport processes. Each mitochondrion is bounded by two highly specialized membranes: an inner membrane folded into numerous cristae, and an outer membrane. The inner membrane contains three types of functions: they carry out the oxidation reactions of the respiratory chain, synthesize ATP in the matrix by an ATP synthase enzyme complex and regulate the passage of metabolites into and out of the matrix by specific transport proteins. The outer membrane contains a large channel-forming protein, porin, and is therefore permeable to all molecules of 5000 daltons or less, as well as enzymes involved in mitochondrial lipid synthesis that convert lipid substrates into forms that are subsequently metabolized in the matrix. These two membranes play a crucial part in mitochondrial function and together they create two separate mitochondrial compartments (Alberts et al., 1994).

The metabolism of sugars is completed in mitochondria: the pyruvate is imported from the cytosol into the mitochondrion and oxidized

by molecular oxygen (O_2) to CO_2 and H_2O. The energy released is equivalent to about 30 molecules of ATP for each molecule of glucose oxidized. By contrast, only 2 molecules of ATP are generated by glycolysis which is the ubiquitous metabolic pathway in the cytosol by which sugars are incompletely degraded with production of ATP alone (Alberts et al., 1994). Mitochondria are therefore the major ATP producer in the cell.

The energy from the oxidation of foodstuffs is used to drive membrane-bound proton pumps (H^+ pumps) that transfer H^+ from one side of the membrane to the other. These pumps generate an electrochemical proton gradient across the membrane, which is used to drive various energy requiring reactions when the protons flow back downhill through membrane-embedded protein machines. Foremost among these machines is the enzyme ATP synthase, which uses the energy of the H^+ flow to synthesize ATP from ADP and Pi. Other proteins couple the H^+ flow to the transport of specific metabolites into and out of the mitochondria (Alberts et al., 1994).

4. 2. 2 AMF-R tubules and mitochondria are both microtubule-associated organelles

AMF-R tubules and mitochondria are both microtubule-associated organelles. Their linear morphology and orientation are dependent on the integrity of microtubules (Benlimame et al., 1995; Beretter-Hahn and Vöth, 1994). The movement of mitochondria in the cytoplasm along microtubules may determine the orientation and distribution of mitochondria in different cell types (Beretter-Hahn and Vöth, 1994). In cells treated with nocodazole, which depolymerizes microtubules or taxol which stabilizing microtubules, AMF-R tubules lose their linear extension and present short and curly

tubular morphology (Benlimame et al., 1995). The relationship of both organelles with microtubules may be responsible for their close association.

It is also possible that AMF-R tubules are intimately associated with mitochondria for some other reasons.

4. 2. 3. Cell motility and the association of AMF-R tubules and mitochondria

The presence of AMF-R within a smooth ER associated tubule implicates this organelle in the motile process. Membrane ruffling at the cell periphery precedes lamellipod extension, the fundamental aspect of ameoboid cellular movement. The establishment of stable contact with the substrate results in the displacement of the cell in the direction of pseudopodial extension. Cell movement therefore requires that both membrane and cell adhesion molecules be targeted to the leading edge of the cell prior to pseudopodial extension. The role of targeted vesicular traffic in the establishment of polarity in some cell types, in particular epithelial cells and neurones, has been well-characterized (Rodriguez-Boulan and Powell, 1992). Cell motility might require an internal pool of membrane which can be mobilized to the leading edge following a motile stimulus.

It might be possible that when the cell is stimulated by AMF, intracellular AMF-R tubules are transported along the microtubular network to a region near the nucleus and subsequently toward the leading edge where they eventually fuse with plasma membrane, resulting in pseudopodial extension and increase migration of responsive cells.

The association of AMF-R tubules and mitochondria varies in different cell types such as MDCK, HeLa and HT-1080 cells (Figure 3).

MDCK is a polarized epithelial cell line and HeLa is a transformed human cervix epitheloid carcinoma cell. Both of these cell lines are low motile. HT-1080, a transformed human fibrosarcoma cell line exibit high cell motility and metastasis ability (Hay et al., 1992). In MDCK and HeLa cells, approximately 80% of AMF-R labeling overlaps with mitochondria, while in HT-1080 cells 40% of AMF-R labeling are colocalized with mitochondria. The extent of colocalization between AMF-R tubules and mitochondria might therefore be related to cell motility. In low motile cells, the intracellular AMF-R tubules may be less labile such that AMF-R tubules and mitochondria are intimately associated. In very motile cells, such as HT-1080, intracellular AMF-R tubules may be required for transport to the leading edge and dissociation of AMF-R tubules from mitochondria may facilitate their movement to the cell periphery and the leading edge. In order to verify this hypothesis we will have to study the association between AMF-R tubules and mitochondria in cell lines which exhibit differential cell motility.

4. 3. The role of calcium concentration in dissociation of AMF-R tubules and mitochondria

Ca^{++} is an intracellular signal molecule and the resting concentration of the Ca^{++} in the cytosol must be kept low ($\leq 10^{-7}$). The Na^{+}-Ca^{++} ATPase in the plasma membrane and membrane of the ER is responsible for pumping the Ca^{++} to the extracellular fluid or into the ER. Intracellular organelles play major roles in the regulation of cytosolic Ca^{++} concentration due to their ability to sequester and to release it following cell activation. In addition, various molecules in the cytosol bind free Ca^{++}, such as

calmodulin. Together Na^{+}-Ca^{++} ATPase and calcium binding proteins maintain the stable concentration of cytosolic Ca^{++} in the cell.

Increasing the concentration of Ca^{++} following incubation of the cells with PBS-CM (extracellular calcium concentration of 10^{-4}), might induce a transient increase in intracellular Ca^{++} that activates calcium-dependent proteins which, by an unknown mechanism, disrupt the association of AMF-R tubules and mitochondria. After an increase in cytosolic Ca^{++}, the Na^{+} -Ca^{++} ATPase on the plasma membrane and in the ER pumps the Ca^{++} out of the cytosol and calcium binding molecules bind to cytosolic Ca^{++}, such that the concentration of free Ca^{++} in the cytosol becomes normal. Calcium dependent proteins are no longer activated and AMF-R tubules and mitochondria can reassociate as seen in the time course experiment with PBS-CM incubation (Figure 5).

Further evidence in support of this hypothesis was obtained with the permeabilized MDCK cells. In semi-intact MDCK cells incubated with Tris buffer, the percentage of colocalization of AMF-R tubules and mitochondria is 98.8%. When the semi-intact MDCK cells are incubated with rat liver cytosol, the percentage of colocalization is reduced to 81.5%. Pretreatment of cytosol with NEM, results in a colocalization of is 85.7%, indicating that NSF is not involved in this process. However, when the semi-intact cells are incubated with calcium depleted cytosol, the colocalization labeling of the AMF-R tubules and mitochondria is 96.4%. Calcium-dependent proteins are therefore involved in the process of AMF-R tubule and mitochondria dissociation.

4. 4. Why do AMF-R tubules associate with or dissociate from mitochondria?

The association of AMF-R tubules and mitochondria also can be disrupted by GTPγS. In semi-intact MDCK cells incubated with cytosol and GTPγS, the percentage of the colocalization between AMF-R tubules and mitochondria is further reduced to 71.4%, a further degree of dissociation than in cells incubated with cytosol alone (81.5%). GTPγS inhibits the process of homotypic fusion, an ATP-dependent process by which a membrane compartment fuses with copies of itself. It has been demonstrated that GTPγS inhibits homotypic fusion of endosomes (Lenhard et al., 1992) and ER membranes (Latterich and Schekman, 1994) derived from mammalian tissue culture cells.

It has been suggested that the structure of certain organelles might represent an equilibrium between homotypic fusion and periplasmic fusion, the fusion of internal membrane resulting in organelle vesiculation (Rothman and Warren, 1994). Periplasmic fusion is proposed to be stochastic, ATP-independent, and initiated by contact on the lumenal side of membrane s of the same organelle (Rothman and Warren, 1994). It may be possible that GTPγS also inhibits the homotypic fusion betweeen AMF-R tubules, thereby promoting periplasmic fusion within the AMF-R tubule. The AMF-R tubule exhibits a highly fluid morphology, contrary to the regular nature of rough tubules (Benlimame et al., 1995) that may permit the interaction of internal membrane necessary for periplasmic fusion (Rothman and Warren, 1994). Such a process may be involved in dissociation of AMF-R tubules from mitochondria.

IQ causes Golgi membranes to break down completely into very small vesicles which are dispersed throughout the cytoplasm in NRK cells. IQ inhibits protein transport between Golgi membranes and further transport along the secretory pathway by specially inhibiting vesicle

formation (Takizawa et al., 1993). This effect of IQ is not Golgi specific and it also fragments AMF-R tubules; fragmented Golgi apparatus and AMF-R tubules do not colocalize (Wang et al., 1997) (section 2). IQ also depolymerizes microtubules (Veit et al., 1993). AMF-R tubules and mitochondria are both microtubule-associated organelles, but IQ fragments AMF-R tubules and the GA independent of microtubule integrity (Veit et al., 1993; Wang et al., 1997) (section 2). In MDCK cells treated with IQ, the AMF-R labeling exhibits a punctate distribution throughout the cytoplasm (Wang et al., 1997), while mitochondria are present as short tubules which do not colocalize with AMF-R tubules. Thus IQ is an another factor that disrupts the association of the AMF-R tubules and mitochondria.

How does IQ fragment AMF-R tubules? The ability of IQ to fragment AMF-R tubules might be related to the labile nature of this organelle. Both IQ and GTPγS disrupt the association of AMF-R tubules and mitochondria. By EM observation of IQ treated cells, AMF-R is distributed to fenestrated networks of narrow interconnected tubules (Wang et al., 1997) (section 2). The fenestrated AMF-R tubules of MDCK cells treated with IQ may represent intermediates in the periplasmic fusion fragmentation process.

Mitochondria are the major ATP producer of the cell, and homotypic fusion requires ATP. The association of AMF-R tubule with ATP-rich mitochondria may promote homotypic fusion between AMF-R tubules. Following dissociation of AMF-R tubules from mitochondria, the increased distance of AMF-R tubules from mitochondria, that provide ATP for homotypic fusion, may promote periplasmic fusion within AMF-R tubules. It is not known yet whether the ultrastructure of AMF-R tubules in semi-intact MDCK cells treated by cytosol and GTPγS compares with the ultrastructure of IQ treated cells.

In HT-1080 cells, a tumor cell line with high motility and metastasis ability, AMF-R labeling is localized to punctate structures which do not associate with mitochondria. The basis for AMF-R tubules dissociation from mitochondria in HT-1080 cells is not clear. Further experiments will determine whether the balance between homotypic and periplasmic fusion regulates the association between AMF-R tubules and mitochondria.

5. CONCLUSION

AMF-R is a marker for a distinct smooth membranous tubule. Smooth AMF-R labeled tubules exhibit continuity with rough ER cisternae and IQ selectively targets smooth and not rough ER. AMF-R tubules can be distinguished from the intermediate compartment labeled for ERGIC-53 by confocal microscopy and thus constitute a distinct IQ-sensitive subdomain of the smooth ER.

AMF-R labeled smooth ER is intimately asssociated with mitochondria in both cultured cells and semi-intact MDCK cells, however, this association varies in different cell types. The association of AMF-R tubules and mitochondria can be disrupted by PBS-CM preincubation in culture cells and in semi-intact cells incubated with cytosol and GTPγS. The effect of cytosol is not NEM sensitive and does not requires ATP, indicating that NSF and SNAPs are not involved in this process. The cytosol can be inactivated by depleting calcium-binding proteins, suggesting that calcium-binding proteins are responsible for the dissociaton of AMF-R tubules and mitochondria. We have shown that the AMF-R tubule is a distinct IQ-sensitive subdomain of the ER and is intimately associated with mitochondria.

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