Distinct caveolae-mediated endocytic pathways target the Golgi apparatus and the endoplasmic reticulum

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Summary

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

Key words: Endocytosis, Caveolae, Glycolipid rafts, Cholera toxin, Endoplasmic reticulum, Autocrine motility factor

Introduction

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

However, in hippocampal neurons, CTX bound to GM1 is found in detergent-insoluble membranes at the cell surface, but CTX endocytosis was blocked by inhibitors of clathrin-dependent endocytosis and not by filipin or mβCD (Shogomori and Futerman, 2001a). MβCD rather blocks the cholesterol-dependent delivery of CTX from endosomes to the Golgi apparatus (Shogomori and Futerman, 2001b), as previously reported for GPI-anchored folate receptor (Mayor et al., 1998). These studies therefore describe a clathrin-dependent, endosome-mediated pathway for CTX to the Golgi. They further indicate that cholesterol sensitivity is not necessarily an indicator of caveolae- or raft-mediated internalization to the Golgi.

Both clathrin- and caveolae/raft-dependent endocytosis are dynamin dependent (Damke et al., 1994; Henley et al., 1998; Herskovits et al., 1993; Le et al., 2002; Oh et al., 1998; van der Bliek et al., 1993). The fact that expression of mutant dynK44A inhibited only 40-50% of CTX uptake in HeLa cells and that neither filipin nor caveolin-1 overexpression inhibited CTX internalization in confluent CaCo-2 cells led to the suggestion that CTX also follows the dynamin-insensitive non-clathrin pathway (Torgersen et al., 2001). However, in human skin fibroblasts and in subconfluent CaCo-2 cells, cholesterol-disrupting agents inhibit CTX internalization whereas agents that inhibit clathrin-dependent endocytosis, such as chlorpromazine and potassium depletion, do not affect CTX endocytosis (Orlandi and Fishman, 1998; Puri et al., 2001). Depletion of membrane cholesterol prevents CTX entry into the cells, transport to the Golgi and induction of chloride secretion (Wolf et al., 2002). Furthermore, inhibition of dynamin function by either dynK44A expression or microinjection of anti-dynamin antibodies has been shown to qualitatively inhibit CTX endocytosis (Henley et al., 1998; Oh et al., 1998). These data from multiple studies in multiple cell types argue strongly for a role of caveolae in the internalization

of CTX. However, a single study in a single cell type demonstrating the clathrin-independent, dynamin-dependent, cholesterol-sensitive, caveolae-mediated endocytosis of CTX has yet to be performed.

CTX delivery to the Golgi apparatus in Cos-7 cells is filipin sensitive but is not blocked by inhibition of clathrin-dependent endocytosis with the eps15 mutant or inhibition of endosome function with a rab5 mutant. Therefore, a cholesterol-sensitive non-endosomal pathway is used for CTX delivery to the Golgi apparatus (Nichols et al., 2001). CTX is delivered instead to a caveolin-1–GFP-positive transferrin receptor (TfR)-negative endocytic intermediate prior to delivery to the Golgi, implicating caveolae in this pathway (Nichols, 2002). Similarly, SV40 has previously been shown to be internalized to the ER via a caveolin-1–GFP-positive intermediate, named the caveosome (Pelkmans et al., 2001). SV40 delivery via caveolae to the caveosome is associated with tyrosine kinase activation, the breakdown of actin filaments and the recruitment of dynamin II, whereas passage from the caveosome to the ER is blocked by treatment of the cells with nocodazole and is therefore microtubule dependent (Pelkmans et al., 2001; Pelkmans et al., 2002). The recent demonstration that BFA (brefeldin A) and a 20°C temperature block, as well as mutants of arf1 and sar1 and antibodies to β-cop, block SV40 internalization and infectivity as well as the internalization of CTX suggests that both caveolar ligands follow a similar internalization route (Norkin et al., 2002; Richards et al., 2002). Although a role for the Golgi apparatus in SV40 delivery to the ER remains to be established (Kartenbeck et al., 1989; Norkin et al., 2002; Pelkmans et al., 2001; Richards et al., 2002), SV40 and CTX appear to follow a similar, if not identical, pathway via the caveosome to the ER.

We have previously proposed the caveolae-mediated endocytosis of AMF to the ER on the basis of its sensitivity to mβCD, its inhibition by the dynamin-1 K44A mutant (dynK44A) and its negative regulation by overexpression of caveolin-1 (Benlimame et al., 1998; Le et al., 2000; Le et al., 2002). AMF-R is localized to a smooth ER subdomain, and we have no evidence from multiple EM studies that AMF-R is localized to the Golgi or that endocytosed AMF traverses the Golgi (Accola et al., 2002; Benlimame et al., 1998; Benlimame et al., 1995; Le et al., 2002; Wang et al., 1997; Wang et al., 2000). In order to compare the caveolae-mediated endocytosis of AMF and the retrograde pathway of CTX via the Golgi to the ER, we first undertook to define the endocytic pathway of CTX in NIH-3T3 fibroblasts. We show here that in NIH-3T3 cells, 80% of CTX uptake is blocked by adenoviral expression of the dynK44A mutant, but inhibition of clathrin-dependent endocytosis with the dominant-negative hub fragment of the clathrin heavy chain has only a limited effect on CTX endocytosis. MβCD treatment and overexpression of caveolin-1 selectively reduce CTX endocytosis to the Golgi apparatus but not to endosomes. In NIH-3T3 fibroblasts, the majority of CTX is therefore internalized via a caveolae-dependent mechanism to the Golgi with a minor part targeted via a clathrin-dependent mechanism to the endosome. Of particular interest is the fact that although BFA, nocodazole or a 20°C temperature block inhibit CTX delivery to the Golgi apparatus, they do not affect AMF delivery to the ER. Furthermore, although genistein inhibits the caveolae/raft-mediated endocytosis of both CTX and AMF to the Golgi and smooth ER, respectively, after five minutes of endocytosis in the presence of the clathrin hub the two ligands do not cointernalize and AMF is targeted, apparently directly, to the AMF-R-labeled smooth ER. The caveolae-mediated endocytic pathway of AMF is therefore distinct from that of CTX and SV40. Two caveolae-mediated endocytic pathways therefore exist that target either the Golgi or the ER from the plasma membrane.

Materials and Methods

Antibodies, reagents and cells

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

An NIH-3T3 fibroblast clone (Benlimame et al., 1998) was grown in complete medium consisting of DME supplemented with 10% calf serum, non-essential amino acids, vitamins, glutamine and a penicillin-streptomycin antibiotic mixture (Invitrogen Canada Inc., Burlington, ON).

Viral infection

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

Immunofluorescence

NIH-3T3 cells were incubated with 5 μg/ml FITC-CTX alone or coincubated with 5 μg/ml FITC-CTX and with 15 μg/ml Rh-Tf for

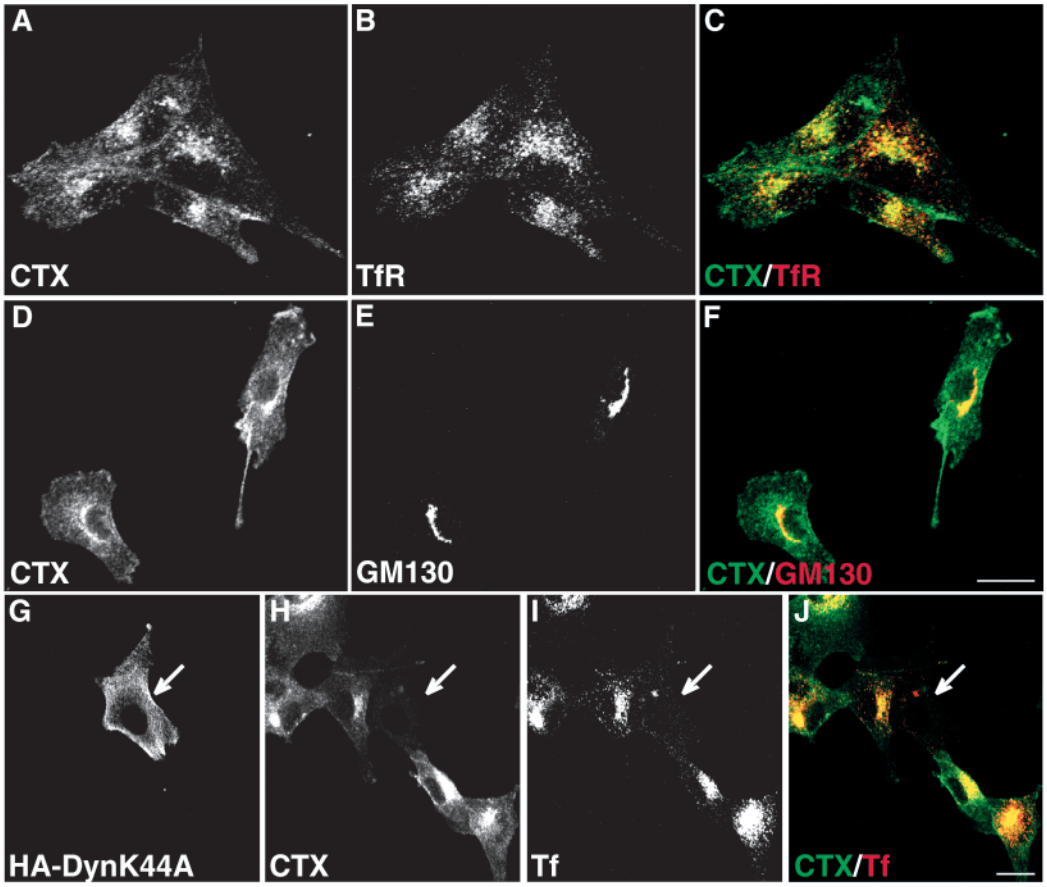

Fig. 1. DynK44A inhibits CTX internalization into endosomes and the Golgi apparatus. FITC-CTX internalized for 30 minutes at 37°C (A,D) is localized to both endosomes labeled for TfR (B) and the Golgi apparatus labeled for GM130 (E). The merged confocal images present FITC-CTX in green (C,F) and TfR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

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

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

Results

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

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

Quantitative analysis shows that dynK44A mutant expression significantly inhibits (by >80%) both FITC-CTX and Rh-Tf endocytosis into the perinuclear region, whereas the dynamin wildtype exhibits no effect (Fig. 2A). Clathrin hub expression inhibits >75% of Rh-Tf endocytosis and <25% of

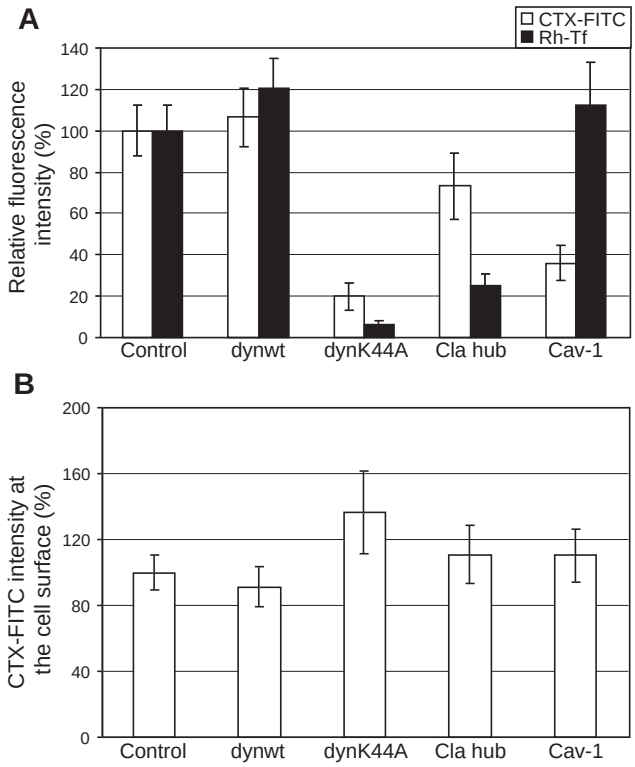

Fig. 2. Caveolae mediate CTX endocytosis. FITC-CTX (empty bars) and Rh-Tf (filled bars) were endocytosed for 30 minutes at 37°C in cells infected with wild-type dynamin, dynK44A, clathrin hub and caveolin-1 adenoviruses. Endocytosis into the perinuclear region was quantified in uninfected cells and in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

FITC-CTX endocytosis, whereas caveolin-1 overexpression does not affect Rh-Tf endocytosis but inhibits >60% of the endocytosis of FITC-CTX. To ensure that infection of NIH-3T3 cells with the various adenoviruses did not affect FITC-CTX binding to cell-surface GM1, cell-surface binding of FITC-CTX at 4°C was quantified in non-infected and infected cells. As shown in Fig. 2B, cells infected with the various adenoviruses did not present a reduction of cell-surface FITC-CTX labeling (Fig. 2B), indicating that infection with the various adenoviruses did not affect accessibility of exogenous FITC-CTX to cell-surface GM1. A significant increase

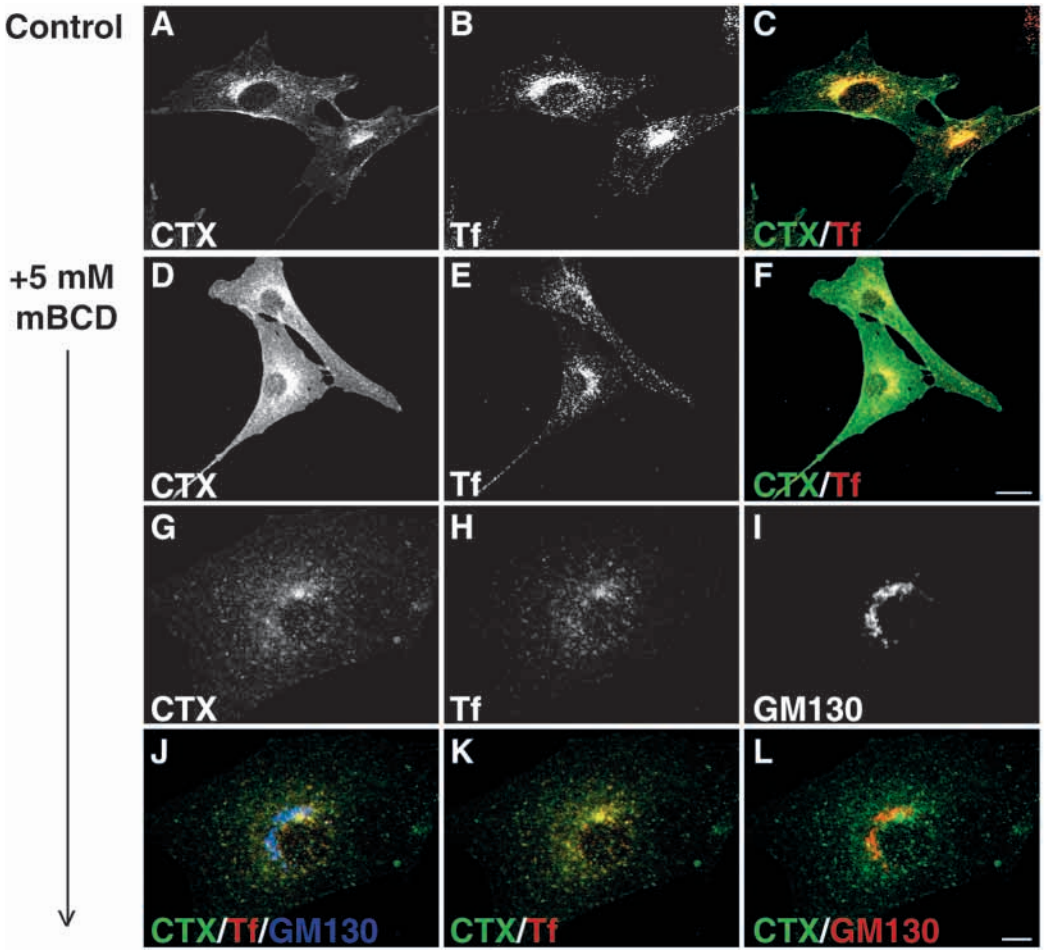

Fig. 3. MβCD blocks CTX delivery to the Golgi but not to endosomes. NIH-3T3 cells were either left untreated (A-C) or pretreated with 5 mM of mβCD (D-L) for 30 minutes then pulse labeled with FITC-CTX (A,D,G) and Rh-Tf (B,E,H) for 30 minutes at 37°C. Images presenting FITC-CTX internalization in the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

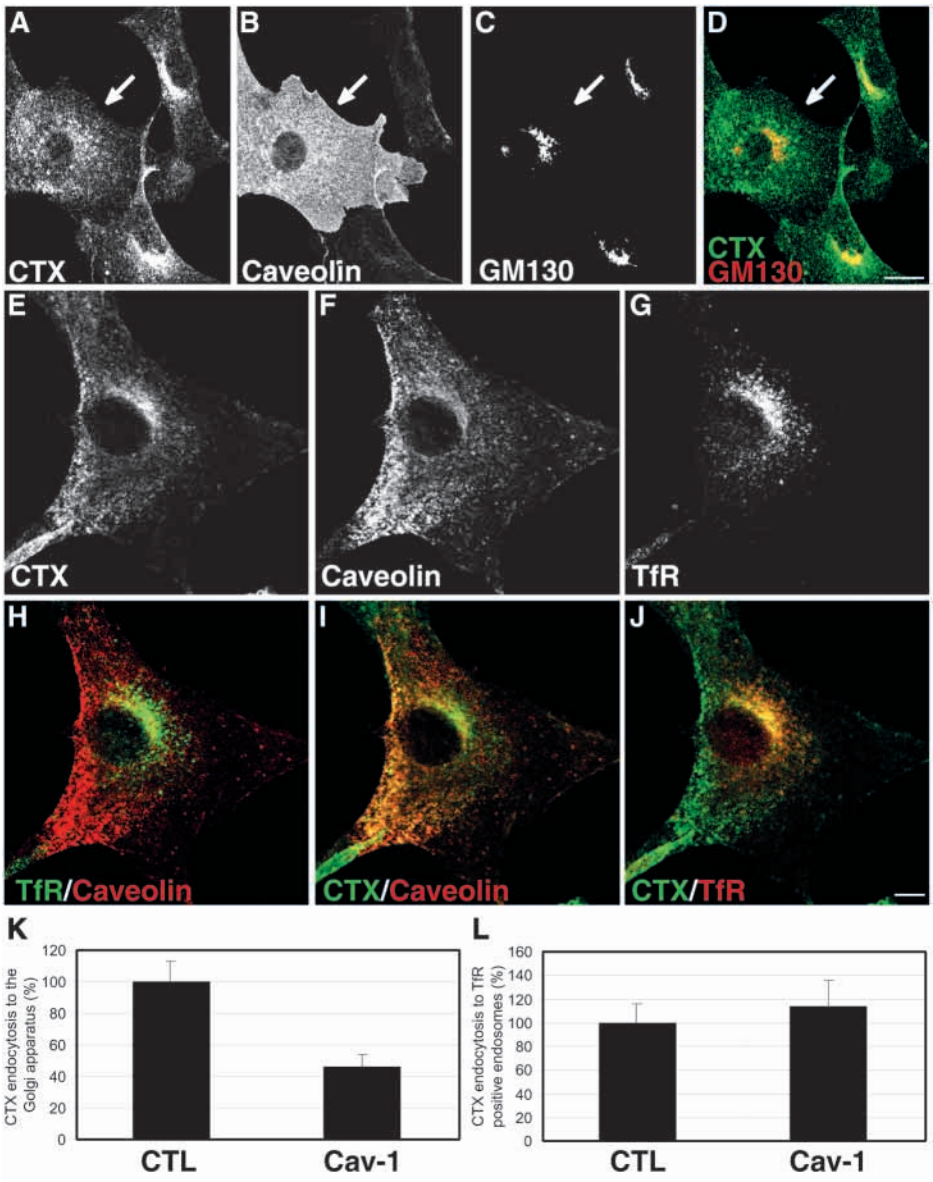

Fig. 4. Caveolin-1 overexpression negatively regulates CTX internalization to the Golgi apparatus but not to endosomes. NIH-3T3 cells were infected with the tTA and caveolin-1 adenoviruses and after 36 hours pulse labeled with FITC-CTX (A) for 30 minutes at 37°C prior to fixation. Cells were then triple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

(P<0.05) in cell-surface labeling was observed following infection with dynK44A, which is consistent with its ability to inhibit both the caveolae- and clathrin-mediated endocytosis of FITC-CTX. In NIH-3T3 cells, the majority of FITC-CTX is therefore internalized via a caveolin-dependent pathway and a minor portion via a clathrin-dependent pathway.

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

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

We subsequently infected cells with the caveolin-1 adenovirus and assessed FITC-CTX delivery to either the GM130-labeled Golgi or TfR-labeled endosomes. In caveolin-1-overexpressing cells, identified with a polyclonal anti-caveolin antibody, delivery of FITC-CTX to the Golgi was significantly reduced (Fig. 4A-D). Perinuclear FITC-CTX was localized predominantly to TfR-positive endosomes that were not labeled for caveolin, whereas peripheral FITC-CTX labeling colocalized extensively with caveolin, apparently at the cell surface (Fig. 4E-J). Using mask overlay image analysis software (Wang et al., 2000), we quantified the delivery of FITC-CTX to either GM130-positive Golgi or TfR-positive endosomes in caveolin-1-infected and in -uninfected cells. Caveolin-1 overexpression resulted in a significant reduction (~50%) in the delivery of FITC-CTX to the Golgi but did not affect delivery of FITC-CTX to endosomes (Fig. 4K,L). The fact that caveolin-1 overexpression selectively reduces CTX delivery to the Golgi but not transferrin-positive endosomes provides direct evidence for a caveolae-mediated endocytic pathway to the Golgi apparatus.

To confirm that CTX internalization to the Golgi is caveolae-mediated, FITC-CTX was added to NIH-3T3 cells infected

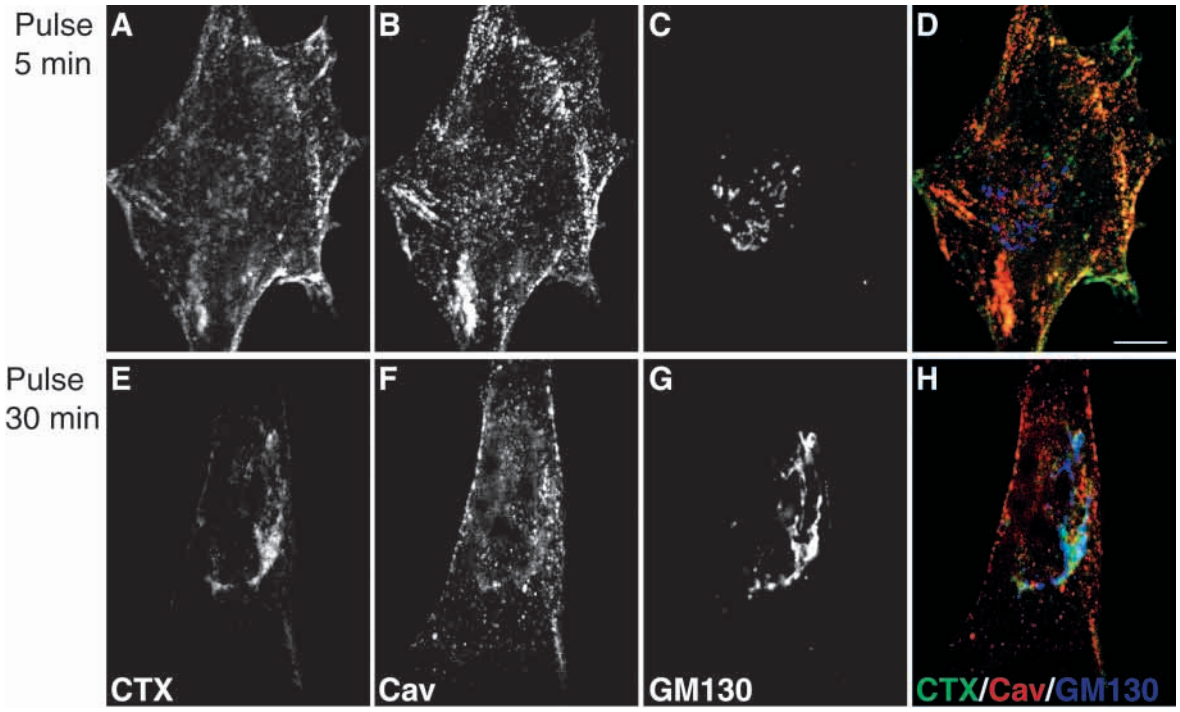

Fig. 5. CTX endocytosis in clathrin-hub-expressing cells. NIH-3T3 cells infected for 36 hours with the tTA and clathrin hub adenoviruses were incubated at 37°C with FITC-CTX for 5 (A-D) or 30 minutes (E-H). The distribution of FITC-CTX was visualized directly (A,E) and post-fixation labeling with anti-caveolin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

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

Caveolae mediate distinct endocytic pathways to the Golgi and ER

AMF-R is a marker for a mitochondria-associated smooth ER subdomain (Benlimame et al., 1998; Benlimame et al., 1995; Wang et al., 1997; Wang et al., 2000), and in NIH-3T3 cells, AMF-R is specifically localized to smooth and not rough ER tubules (Benlimame et al., 1998). We have previously reported, using quantitative electron microscopy, that caveolin-1 overexpression reduces the caveolae-mediated endocytosis of AMF to the ER in NIH-3T3 fibroblasts as well as in ras and abl-transformed NIH-3T3 fibroblasts that express little caveolin-1 (Le et al., 2002). Quantification using a mask overlay assay revealed a 42.0±2.4% reduction in AMF-FITC delivery to the AMF-R-labeled smooth ER in NIH-3T3 fibroblasts that overexpress Myc-tagged caveolin-1 (Fig. 6). These results are equivalent to those previously obtained by quantitative EM (Le et al., 2002). The tubular distribution of the AMF-R-labeled smooth ER, a reflection of its interaction with mitochondria, can be disrupted by ilimaquinone or low cytosolic calcium (Wang et al., 1997; Wang et al., 2000). In caveolin-1-infected cells, the AMF-R-labeled smooth ER exhibits a more diffuse distribution (Fig. 6B, arrow), suggesting that regulation of caveolae-mediated endocytosis to this ER subdomain may influence the extent of its association with mitochondria. The fact that overexpression of caveolin-1 reduces the internalization of AMF to the ER and of CTX to the Golgi demonstrates that they are both mediated by a similar caveolae-based endocytic mechanism.

Entry of CTX is sensitive to BFA (Donta et al., 1993; Lencer et al., 1993; Morinaga et al., 2001; Nambiar et al., 1993; Orlandi et al., 1993), and BFA has recently been shown to prevent delivery of CTX to the Golgi but not to endosomes (Richards et al., 2002). In cells treated with nocodazole, SV40 entry is blocked at the level of the caveosome, and it is not delivered to the ER (Pelkmans et al., 2001). Furthermore, a 20°C temperature block and BFA treatment inhibit early entry steps of SV40, preventing its delivery to the ER (Norkin et al., 2002; Richards et al., 2002). As seen in Fig. 7, BFA treatment of NIH-3T3 fibroblasts inhibits FITC-CTX delivery to the fragmented Golgi but not to endosomes (Fig. 7A-F). By contrast, BFA does not affect AMF-FITC delivery to the AMF-R-labeled smooth ER (Fig. 7G-I). In the presence of nocodazole, FITC-CTX is not present in the Golgi apparatus although extensive colocalization is observed with caveolin and with TfR (Fig. 8A-H). By contrast, AMF-FITC is delivered efficiently to the

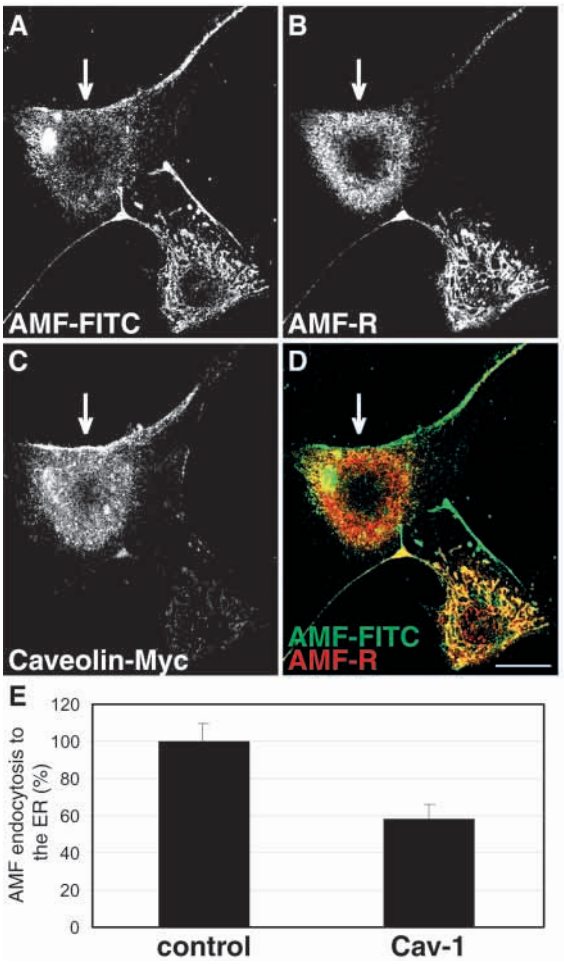

Fig. 6. Caveolin-1 overexpression reduces delivery of AMF-FITC to the smooth ER. NIH-3T3 cells were infected with the tTA and caveolin-1 adenoviruses and after 36 hours pulse labeled with AMF-FITC for 60 minutes at 37°C prior to fixation. AMF-FITC was revealed with rabbit anti-FITC followed by Alexa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

smooth ER in the presence of nocodazole as well as at 20°C (Fig. 8I-N).

The ability of BFA, nocodazole and a 20°C block to inhibit the targeting of CTX to the Golgi but not of AMF to the ER indicates that caveolae-internalized ligands can follow distinct intracellular targeting pathways. To determine whether the sorting of caveolae-internalized CTX and AMF occurred at the plasma membrane or intracellularly, clathrin-hub-infected NIH-3T3 cells were incubated with AMF-FITC and Alexa-594–CTX for only 5 minutes (Fig. 9A-F). Essentially no colocalization between the two caveolar ligands could be detected and, interestingly, internalized AMF colocalized with AMF-R-labeled smooth ER tubules. After 30 minutes, although CTX is colocalized predominantly to the Golgi apparatus, internalized AMF remains colocalized with the smooth ER and is excluded from the Golgi (Fig. 9G-J). AMF and CTX would therefore appear to be segregated at the plasma membrane into different caveolae-mediated internalization pathways. To reconfirm that caveolae truly mediate delivery of both CTX to the Golgi and AMF to the smooth ER, we treated the cells with genistein, a tyrosine kinase inhibitor that inhibits the caveolae-mediated endocytosis of SV40 and the transcytosis of albumin across the endothelial cell (Pelkmans et al., 2002; Tiruppathi et al., 1997). As seen in Fig. 10, in the presence of genistein, CTX delivery to the Golgi but not to TfR positive endosomes was inhibited, confirming that CTX delivery to the Golgi is caveolae mediated. Genistein also inhibited the delivery, detected after 5 minutes of incubation, of AMF-FITC to the smooth ER (Fig. 10G-L).

Discussion

Caveolae mediate CTX endocytosis to the Golgi apparatus

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

Treatment of NIH-3T3 cells with mβCD selectively inhibited CTX delivery to the Golgi but not to transferrin-positive endosomes (Fig. 3). Although these results could be explained by intracellular mβCD inhibition of CTX delivery to the Golgi from the endosome (Mayor et al., 1998; Shogomori and Futerman, 2001b), overexpression of caveolin-1 (Fig. 4) and treatment with genistein (Fig. 10), an inhibitor of caveolae-mediated endocytosis (Pelkmans et al., 2002; Tiruppathi et al., 1997), also selectively inhibit Golgi delivery but not endosomal delivery of CTX. Caveolin-1 overexpression affects neither the cell-surface expression of GM1 nor the endocytosis of CTX to endosomes and therefore does not reduce Golgi delivery of CTX by reducing clathrin-mediated endocytosis to the endosome. These data provide direct evidence for the existence of a caveolae-mediated endocytic route to the Golgi and are consistent with and support previous studies describing the caveolae-mediated endocytosis of CTX to the Golgi (Nichols, 2002; Nichols et al., 2001). CTX can therefore be endocytosed via both caveolae- and clathrin-mediated pathways. Differential rates of clathrin- or caveolae-mediated endocytosis and differential affinities of GM1 for endocytosis-competent raft domains may

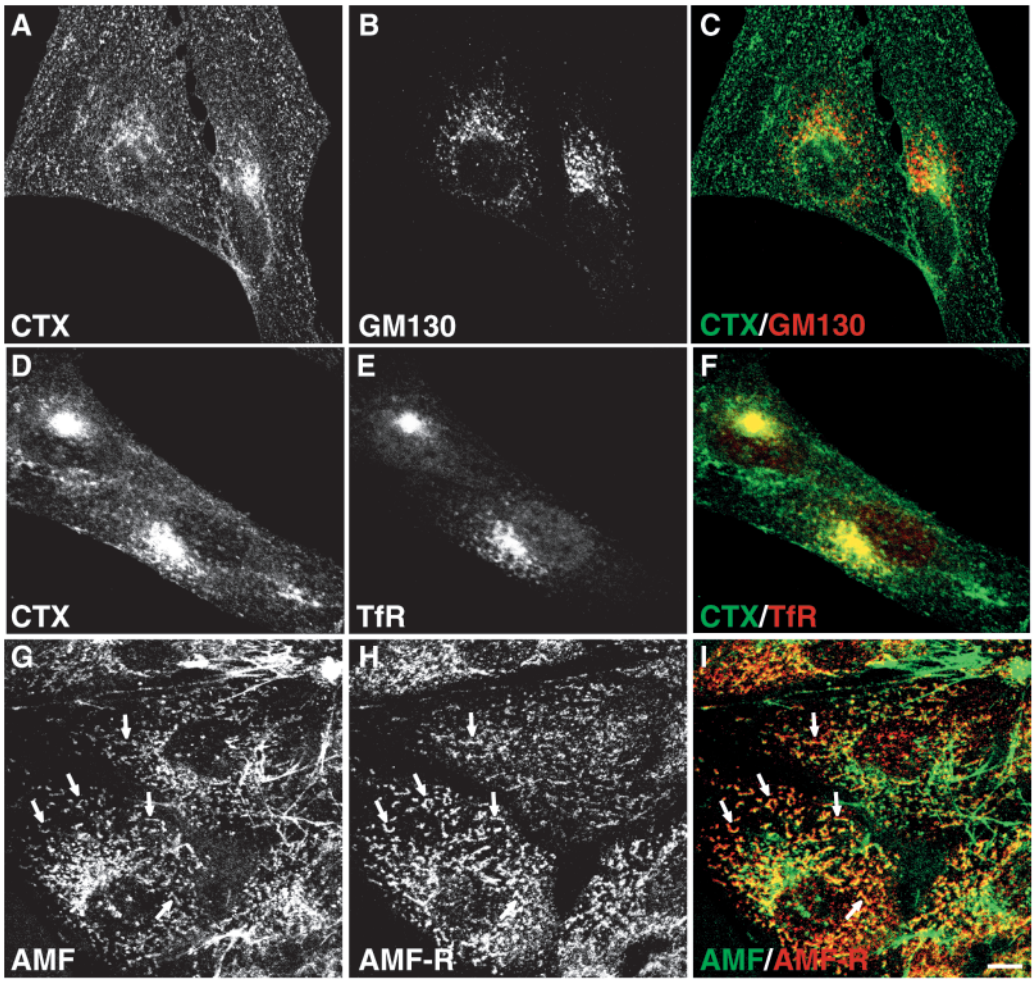

Fig. 7. BFA treatment inhibits the caveolae-mediated endocytosis of CTX to the Golgi apparatus but not of AMF to the smooth ER. NIH-3T3 cells were pretreated with 10 μg/ml BFA for 30 minutes at 37°C and then incubated in the presence of BFA with 5 μg/ml FITC-CTX for 30 minutes at 37°C (A-F) or with 50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

influence the choice of endocytic pathway of CTX in different cell types.

Caveolin-1- and caveolae/raft-mediated endocytosis

In cells expressing caveolin-1–GFP, a caveolin-positive endocytic intermediate, the caveosome, has been implicated in caveolae-mediated endocytosis, including that of both CTX and SV40 (Mundy et al., 2002; Nichols, 2002; Parton et al., 1994; Pelkmans et al., 2001). In our study, expression of perinuclear caveolin-positive, TfR-negative vesicular structures was observed, particularly after caveolin-1 overexpression, and some were positive for internalized CTX. Cell-associated CTX exhibited significant colocalization with caveolin-1, primarily to peripheral cell-surface regions but also to some perinuclear structures (Figs 4, 5). It was, however, difficult to ascertain whether these caveolin-positive structures are indeed intracellular endocytic vesicles and equivalent to caveosomes.

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

The role of caveolin-1 as a negative regulator of caveolae/raft-mediated endocytosis is consistent with recent reports describing the immobilization of caveolin-1 at the cell surface (Pelkmans et al., 2002; Thomsen et al., 2002). Caveolin-1 expression may serve to stabilize the mobility of raft domains in and out of the plane of the membrane. The role of caveolin-1 as a regulator and not as an essential component of the caveolae/raft endocytic machinery does not question its importance in defining this endocytic pathway. Caveolin-1 remains a critical determinant of caveolae/raft-mediated endocytosis as a negative regulator of caveolae budding from the plasma membrane, a recruiter of cargo to endocytic caveolar domains or as a component of caveolae-specific endosomal intermediates.

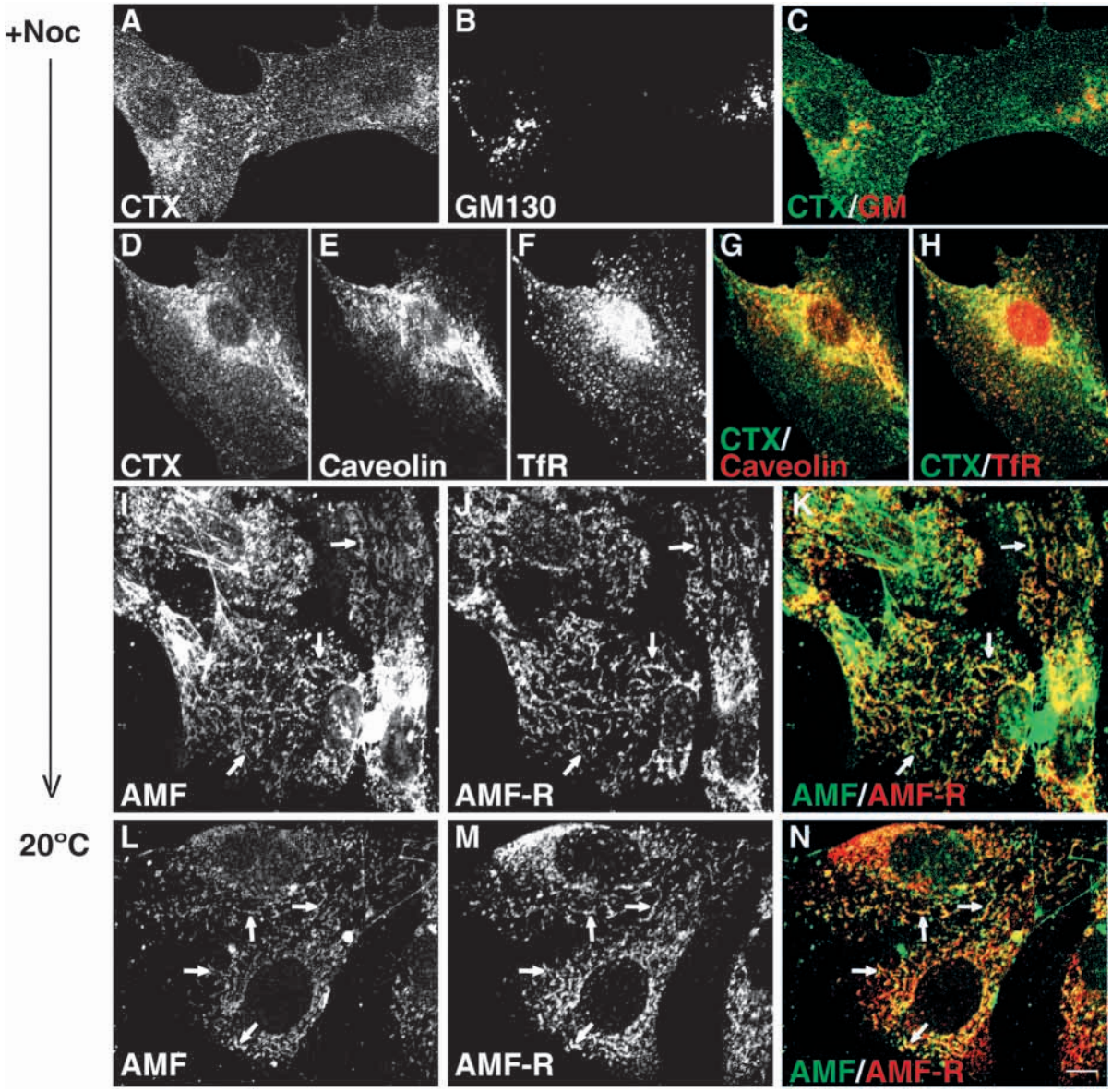

Fig. 8. Nocodazole treatment and a 20°C temperature block inhibit the caveolae-mediated endocytosis of CTX to the Golgi but not of AMF to the smooth ER. NIH-3T3 fibroblasts pretreated with 10 μM nocodazole at 37°C (A-K) were pulse labeled with either 5 μg/ml FITC-CTX for 30 minutes at 37°C (A-H) or with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

Existence of two distinct caveolae/raft-mediated pathways

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

In contrast to CTX endocytosis to the Golgi and SV40 delivery to the ER, BFA, nocodazole and a 20°C incubation did not prevent the caveolae-mediated delivery of AMF to the ER (Figs 7, 8). Furthermore, following incubations of both 5 and 30 minutes in the presence of the clathrin hub, the vast majority of AMF and CTX did not colocalize (Fig. 9). AMF is therefore internalized via a caveolae-mediated endocytic route distinct from that of either CTX or SV40. The fact that AMF could be detected in AMF-R-positive smooth ER tubules after only 5

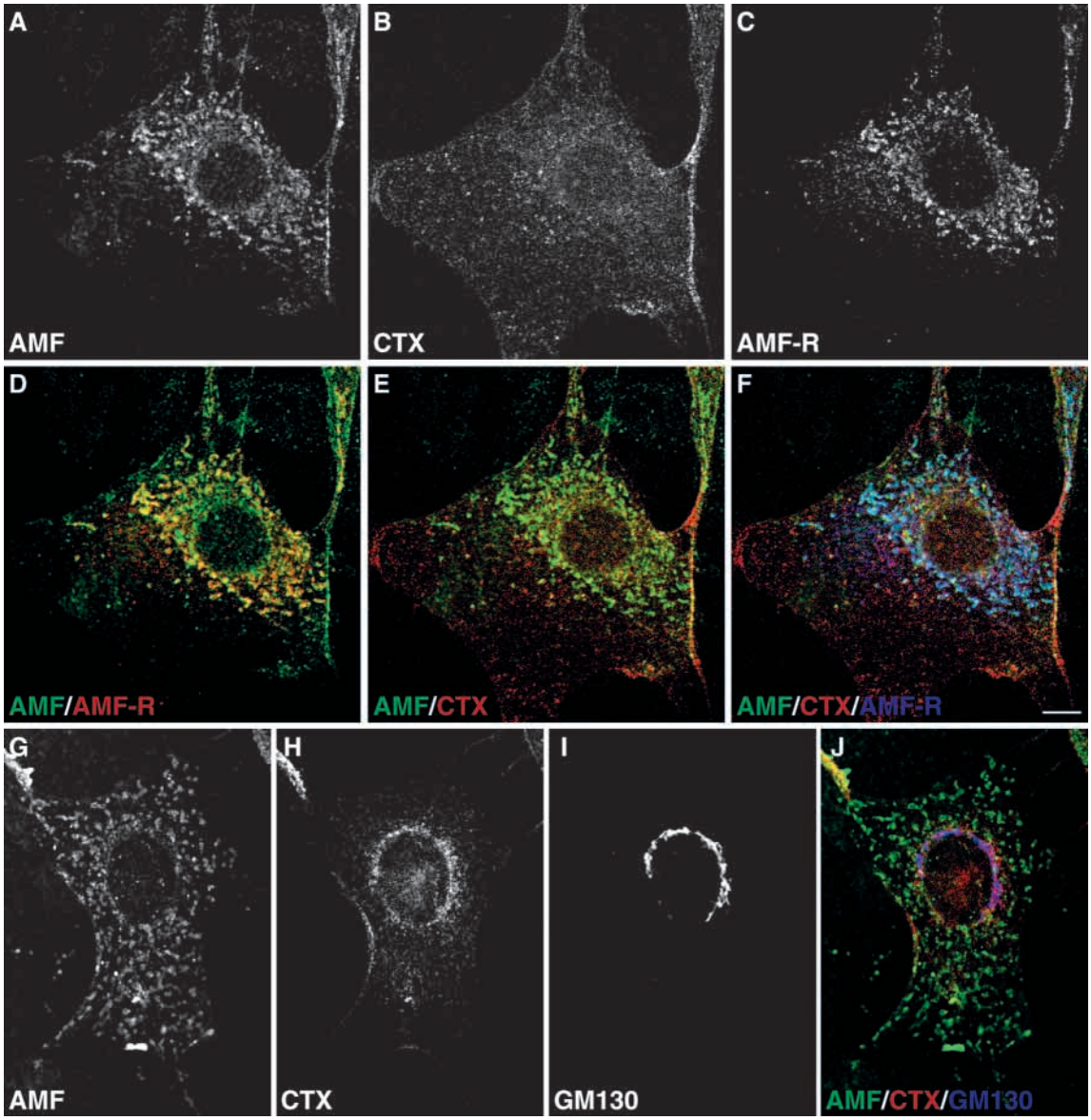

Fig. 9. AMF and CTX do not cointernalize. NIH-3T3 cells were coincubated with 50 μg/ml of AMF-FITC and 5 μg/ml of Alexa 594-CTX at 37°C for 5 minutes (A-F) or 30 minutes (G-J) prior to fixation with precooled methanol/acetone. The distributions of AMF-FITC, revealed with rabbit anti-FITC followed by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

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

The functional significance of an alternative, apparently direct, endocytic pathway to the ER remains to be determined. Such a pathway could be involved in the recovery of Golgi or ER proteins mistargeted to the plasma membrane or in the targeting of proteins for degradation in the ER. Caveolin redistributes to the ER in the presence of cholesterol oxidase (Conrad et al., 1995), and cholesteryl ester is transported to the ER from the plasma membrane (Uittenbogaard et al., 2002); however, these pathways appear to be non-vesicular and distinct from the endocytic pathway of AMF-R. AMF-R is localized to a mitochondria-associated subdomain of the smooth ER, whose association with mitochondria is calcium

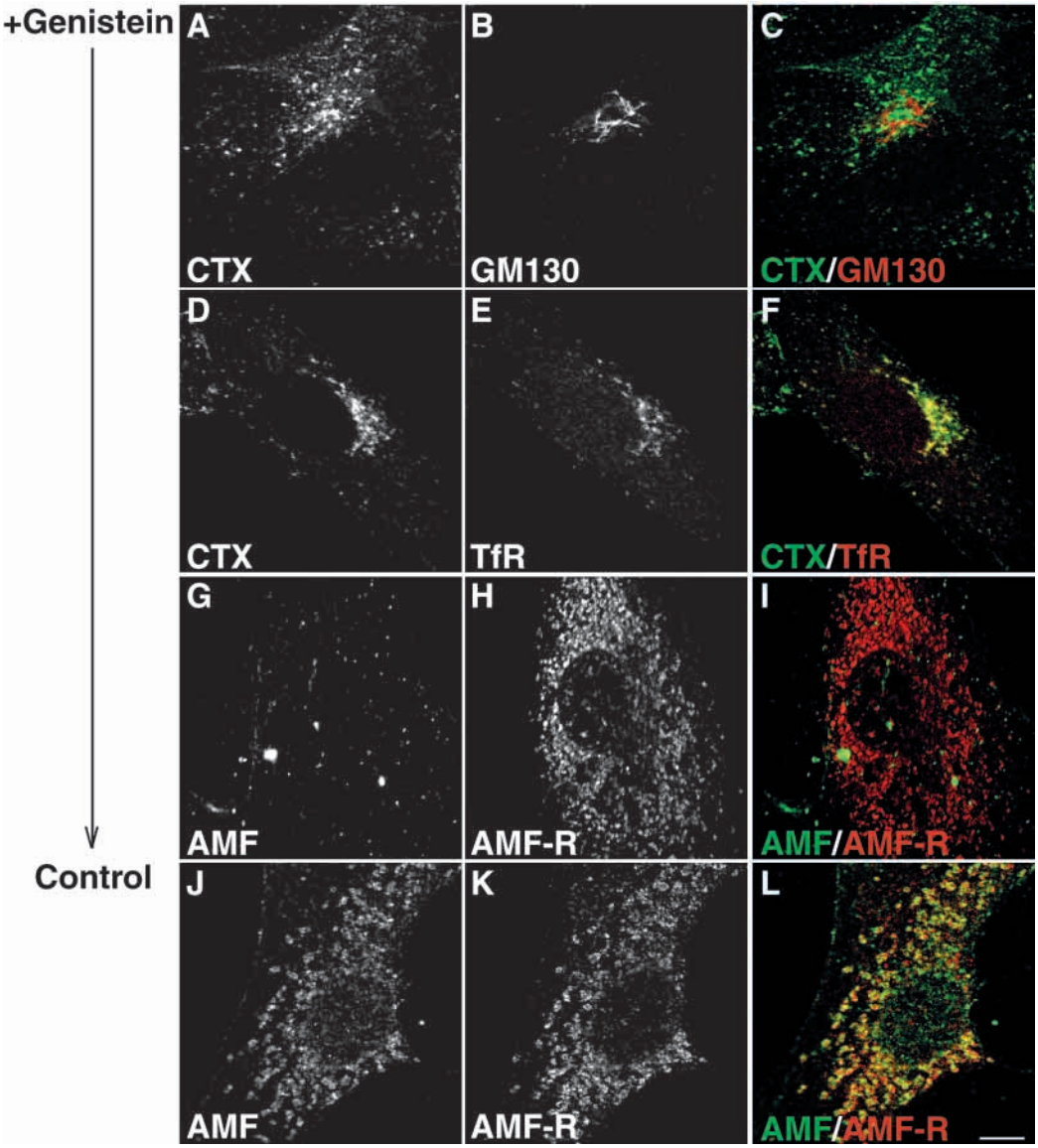

Fig. 10. Targeting of AMF to the ER and CTX to the Golgi apparatus is tyrosine kinase dependent. NIH-3T3 cells were pretreated with genistein (100 μg/ml) for 30 minutes during ligand incubation (A-I) or left untreated (J-L). Cells were pulse labeled with FITC-CTX for 30 minutes at 37°C (A,D) and labeled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

dependent (Wang et al., 2000). A direct endocytic pathway to this smooth ER subdomain may function to maintain its integrity and functionality. The IP3R is also localized to caveolae and smooth ER (Fujimoto et al., 1992; Ross et al., 1989; Sharp et al., 1992), implicating these two organelles and any interaction between them in the regulation of calcium homeostasis.

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

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

induction of caveolae invagination and internalization (Pelkmans et al., 2002). Although common denominators of caveolae/raft-mediated endocytosis include cholesterol sensitivity, dynamin-mediated internalization, tyrosine kinase activation and regulation by caveolin-1, other factors may sort cargo to diverse plasma membrane caveolae/raft domains, segregate endocytic cargo within caveolae/raft domains and regulate the invagination and intracellular targeting of caveolae/raft-derived caveolar vesicles.

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