Ganglioside GM1 levels are a determinant of the extent of caveolae/raft-dependent endocytosis of cholera toxin to the Golgi apparatus

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Summary

Cholera toxin is associated with caveolae and raft domains in various cell types and previous studies have shown that cholera toxin can be internalized by caveolae/raft-dependent endocytosis as well as by other pathways. We undertook the study of cholera toxin endocytosis in CaCo-2 and HeLa cells. CaCo-2 cells do not express detectable levels of caveolin and, relative to HeLa cells, also present significantly reduced expression of ganglioside GM1, the cholera toxin receptor, that remains Triton X-100 insoluble. Amongst the HeLa cell population, caveolin expression is constant, however, GM1 expression is highly variable. Cholera toxin is internalized to the Golgi apparatus via a caveolae/raft-dependent pathway sensitive to methyl-β-cyclodextrin and genistein in high-GM1-expressing HeLa cells but not in low-GM1 HeLa cells or in CaCo-2 cells. Limited cholera toxin endocytosis to endosomes sensitive to neither methyl-β-cyclodextrin nor genistein is also observed in all cells and corresponds to a non-caveolae/raft endocytic pathway. Increasing cell-associated GM1 by adding GM1 to the cell media of both HeLa and CaCo-2 cells selectively enhances the methyl-β-cyclodextrin-, genistein-sensitive delivery of cholera toxin to the Golgi apparatus but not to endosomes. GM1 expression levels are therefore a selective determinant of caveolae/raft-dependent endocytosis of cholera toxin to the Golgi apparatus and variable expression of GM1 between cells can impact on the endocytosis and choice of pathway followed by cholera toxin.

Key words: Endocytosis, Caveolin, Glycolipid rafts, Epithelial cells

Introduction

Cholera toxin (CTX), which is produced by *Vibrio cholerae*, is an enterotoxin composed of A and B subunits. The A subunit comprises two peptides, A1 and A2, connected by a disulphide bond. The A2 peptide binds to a pentamer of B subunits that recognizes with high affinity, the cell surface GM1 ganglioside (Middlebrook and Dorland, 1984; Spangler, 1992). Binding of the B subunit to GM1 enables CTX endocytosis to the Golgi apparatus and subsequent retrograde transport of the holotoxin to the endoplasmic reticulum; the A1 peptide is subsequently translocated to the cytoplasm where it activates adenylate cyclase, inducing the production of cAMP (Lencer et al., 1995; Lencer et al., 1993; Majoul et al., 1996; Nambiar et al., 1993; Orlandi, 1997; Orlandi et al., 1993).

The cholera toxin receptor, the ganglioside GM1, has been extensively used as a marker for glycolipid raft domains (Harder et al., 1998; Janes et al., 1999; Kenworthy and Edidin, 1998). It is also associated with caveolae and on the basis of morphological studies has been considered to be internalized via caveolae (Montesano et al., 1982; Parton et al., 1994; Thyberg, 2000; Tran et al., 1987). Prevention of CTX internalization by cholesterol depletion and inhibition of dynamin function but not by inhibition of clathrin-dependent endocytosis, has supported a role for caveolae/raft domains in this process (Henley et al., 1998; Le and Nabi, 2003; Oh et al., 1998; Orlandi and Fishman, 1998; Puri et al., 2001; Schnitzer et al., 1996; Wolf et al., 2002). Recent data has further defined a non-endosomal, caveolae/raft-mediated endocytic pathway for cholera toxin to the Golgi apparatus (Le and Nabi, 2003; Nichols et al., 2001; Wolf et al., 2002). This pathway includes a caveolin-positive endosomal intermediate (Nichols, 2002; Parton et al., 1994) that is potentially equivalent to the caveosome defined for SV40 internalization (Pelkmans et al., 2001).

However, in neurons, although CTX binds to GM1 and GM1 remains raft associated, CTX is internalized via a clathrin-dependent pathway (Shogomori and Futerman, 2001). Using various cell lines, Torgerson et al. (Torgerson et al., 2001) suggested that CTX could be internalized via multiple mechanisms including the caveolae/raft- and clathrin-dependent pathways as well as the dynamin-independent non-clathrin or constitutive pinocytotic pathway (Torgersen et al., 2001). The data of Torgerson et al. (Torgerson et al., 2001) proved to be particularly contentious (Fishman and Orlandi, 2003; Sandvig and Van Deurs, 2003) as their inability to detect cholesterol-dependent or caveolin-1-regulated endocytosis in CaCo-2 cells conflicted with a previous report describing the filipin-sensitive endocytosis of CTX in CaCo-2 cells (Orlandi and Fishman, 1998). In addition, limited caveolae/raft-dependent endocytosis of CTX has been reported in HeLa cells (Singh et al., 2003).

Our previous studies using NIH-3T3 cells confirmed that CTX could be internalized by both clathrin- and caveolae/raft-dependent pathways (Le and Nabi, 2003). Indeed, by

determining the organelle targeted by internalized CTX, we were able to show that the cholesterol-sensitive, dynamin-dependent, genistein-inhibited caveolae/raft-dependent pathway specifically targeted the Golgi apparatus while the clathrin-dependent pathway targeted transferrin receptor (TfR)-positive endosomes. We therefore undertook to determine whether we could detect caveolae/raft-dependent delivery of CTX to the Golgi in cell models such as CaCo-2 and HeLa that had previously been reported to present limited endocytosis of CTX via the caveolae/raft-dependent pathway (Singh et al., 2003; Torgersen et al., 2001). We show here that total cell lysates of CaCo-2 cells show significantly reduced expression of GM1 relative to HeLa cells and that significant differences in GM1 expression, but not caveolin, are detected in the HeLa cell population. Methyl-β-cyclodextrin (mβCD)- and genistein-sensitive caveolae/raft-dependent endocytosis of CTX to the Golgi was detected only in HeLa cells expressing high GM1 levels. In low-GM1-expressing HeLa cells and in CaCo-2 cells, minimal internalization of CTX to both the Golgi and to TfR-positive endosomes was observed and in neither case was the endocytosis sensitive to mβCD or genistein. Increasing cell-associated GM1 by addition of GM1 to the cell media resulted in the selective enhancement of the caveolae/raft-dependent delivery of CTX to the Golgi in both HeLa and CaCo-2 cells.

Materials and Methods

Antibodies, reagents and cells

Mouse anti-β-actin antibody was purchased from Sigma (Oakville, ON). Mouse anti-GM130 and rabbit anti-caveolin antibody were purchased from Transduction Laboratories (Mississauga, ON), rabbit anti-TfR was from Chemicon (Temecula, CA) and HRP anti-mouse and anti-rabbit secondary antibodies from Santa Cruz Biotechnology (Santa Cruz, CA). Alexa568- and Alexa647-conjugated secondary antibodies were purchased from Molecular Probes (Eugene, OR). MβCD, genistein and the FITC- and HRP-conjugated B-subunits of CTX were purchased from Sigma. Ganglioside GM1 was purchased from EMD Biosciences (San Diego, CA).

HeLa cells obtained from Eric Cohen (Department of Microbiology and Immunology, Université de Montréal) and CaCo-2 cells from Clifford Stanners (McGill Cancer Center, McGill University) were cultured in DMEM (Dulbecco's modified Eagle's medium) supplemented with 10% fetal bovine serum (FBS) (Immunocorp, Laval, Quebec), non-essential amino acids, vitamins, glutamine and a penicillin-streptomycin antibiotic mixture (Invitrogen Canada Inc., Burlington, ON) under a 5% CO_2 atmosphere at 37°C. To increase cell-associated GM1 levels, cells were incubated with 5 mM GM1 added to the cell medium for 4 hours and then chased for 4 hours in regular medium.

Cell lysis and fractionation

HeLa and CaCo-2 cells were grown to near confluence, scraped from the Petri dish and washed three times with precooled PBS buffer (pH 7.4). The harvested cell pellets were resuspended in 0.5 ml of ice-cold Tris-buffered saline (TBS) (140 mM NaCl, 20 mM Tris, 1 mM EDTA, pH 7.4), 0.5% Triton X-100, 1× Complete Mini (Protease Inhibitor Cocktail Tablets) (Roche, Laval, Quebec). After 20 minutes incubation on ice, the lysates were sonicated and centrifuged at 800 ***g*** for 10 minutes. The postnuclear supernatant was further centrifuged at 100,000 ***g*** for 1 hour at 4°C to obtain Triton X-100-soluble and -insoluble fractions. The postnuclear supernatant or total cell lysate and the Triton X-100-soluble and -insoluble fractions were analyzed by immunoblotting for caveolin and β-actin and by dot blotting for GM1 expression.

Immunoblotting and dot blotting

To determine caveolin and β-actin expression levels in HeLa and CaCo-2 cells, lysates were separated on 12% polyacrylamide gel and transferred to Hybond Extra nitrocellulose membranes (Amersham Biosciences). Membranes were blocked with 5% skim milk in phosphate-buffered saline (PBS) containing 0.1% Tween 20. Primary (rabbit anti-caveolin diluted 1:2000; mouse anti-β-actin diluted 1:1000) and secondary antibodies (anti-rabbit-HRP and anti-mouse-HRP diluted 1:2000) were applied in the same buffer and labeling was revealed by chemiluminescence.

To determine cellular expression levels of GM1, 2 μl of diluted cell lysates of equivalent protein content were dot blotted on nitrocellulose filter strips, incubated with HRP-conjugated cholera toxin (210 ng/ml), and revealed by chemiluminescence.

Immunofluorescence labeling

HeLa and CaCo-2 cells were incubated with 10 μg/ml FITC-CTX for 30 minutes at 37°C, washed five times with culture medium, fixed with 3% paraformaldehyde and permeabilized with 0.2% Triton X-100. The cells were then labeled for GM130, TfR or caveolin with appropriate primary antibodies and Alexa568- or Alexa647-conjugated secondary antibodies, as indicated. Cell surface FITC-CTX labeling was performed by incubating the cells at 4°C with 10 μg/ml FITC-CTX in bicarbonate-free DMEM supplemented with 25 mM Hepes pH 7.3 and containing 0.5% BSA (DMEM/Hepes/BSA) for 30 minutes (Benlimame et al., 1998). The cells were then rinsed 3 times with cold DMEM/Hepes/BSA, fixed with 3% paraformaldehyde and permeabilized with 0.2% Triton X-100 prior to labeling for the primary anti-GM130 and Alexa647-conjugated secondary antibodies. Where indicated, cells were pretreated for 30 minutes at 37°C with 5 mM mβCD or 100 μg/ml genistein (Le and Nabi, 2003) and the drugs were maintained during incubation with CTX-FITC. Fluorescently labeled cells were visualized using the 63× or 100× Planapochromat objectives of a Leica TCS-SP1 confocal microscope equipped with Argon 488, Krypton 568 and HeNe 633 lasers.

Specific FITC-CTX internalization to GM130-labeled Golgi or TfR-labeled endosomes was quantified from triple-labeled confocal images (100× objective, zoom 2) using Northern Eclipse mask overlay software (Empix Imaging, Mississuaga, Ontario) as previously described (Wang et al., 2000). A mask region defined by either the GM130 labeling or TfR labeling was used to determine the intensity of FITC-CTX-labeled pixels located at the Golgi or endosomes, respectively, relative to total cellular FITC-CTX labeling. Owing to potential overlap between the organelles and with cell surface CTX labeling, the mask overlay assay provides a relative, and not absolute, indication of organelle-associated CTX-FITC. Each measurement represents the quantification of at least 10 cells from three distinct experiments. For merged triple-labeled images, pseudocolor presentation shows CTX-FITC in green, the GM130-labeled Golgi in red and TfR-labeled endosomes in blue.

Results

Expression of GM1 and caveolin in HeLa and CaCo-2 cells

Caveolin expression was detected by immunoblot in HeLa cells but not in lysates of CaCo-2 cells, even after overexposure of the blot (Fig. 1A). GM1 levels, determined by dot blot of cell lysates, were found to be significantly reduced in CaCo-2 cells relative to HeLa cells (Fig. 1B). The intensity of CTX labeling

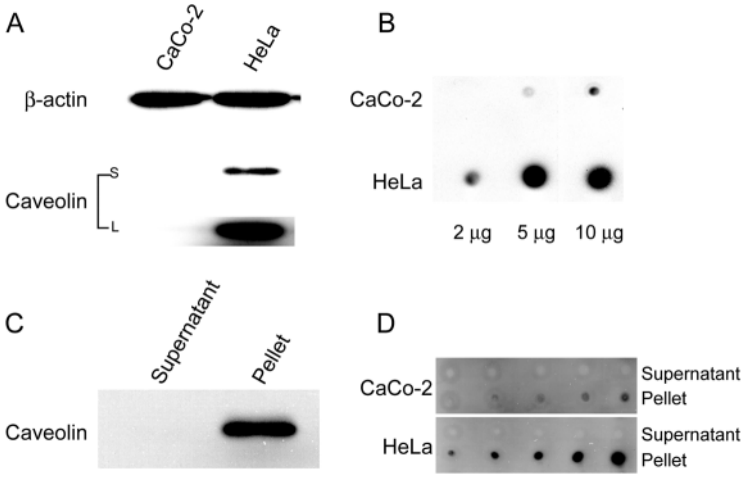


Fig. 1. Expression of caveolin and GM1 in HeLa and CaCo-2 cells. (A) Lysates of CaCo-2 and HeLa cells were separated by 12% SDS-PAGE and subjected to immunoblotting with polyclonal caveolin and β-actin antibodies. Caveolin expression was detected in HeLa cells using short (S) exposures but not in CaCo-2 cells even upon long (L) exposure of the film. (B) To detect ganglioside GM1, 2, 5 and 10 μg of total protein (in 2 μl) from lysates of HeLa and CaCo-2 cells were dot blotted onto nitrocellulose filter strips and then incubated with HRP-conjugated CTX B subunit and revealed by chemiluminescence. (C) Triton X-100 (TX-100) soluble (supernatant) and insoluble (pellet) fractions were prepared and caveolin was found to be present in the insoluble fraction of HeLa cells. (D) Dot blot analysis with HRP-CTX of dilutions of the TX-100 soluble (supernatant) and insoluble (pellet) fractions showed that ganglioside GM1 was found to be present in the TX-100 insoluble fraction in both HeLa and CaCo-2 cells.

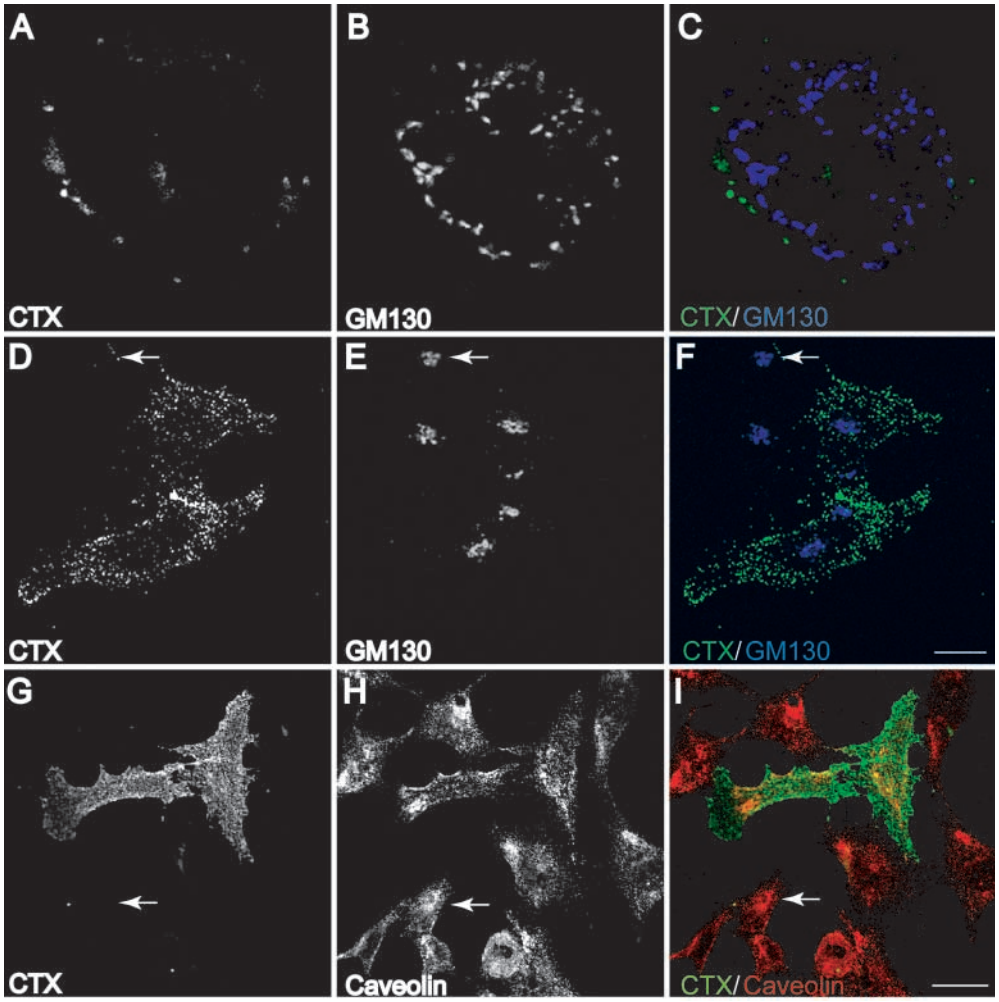


Fig. 2. Cell surface labeling of CTX-FITC. CaCo-2 (A-C) and HeLa (D-I) cells were incubated with CTX-FITC (A,D,G) for 30 minutes at 4°C prior to fixation. After fixation, the cells were labeled with either GM130 (B,E) or caveolin (H), as indicated. Merged confocal images present CTX-FITC in green (C,F,I), GM130 in blue (C,F) and caveolin in red (I). Arrows identify examples of HeLa cells that express low GM1 levels. Scale bars: 16 μm (A-F) and 20 μm (G-I).

of 10 μg HeLa cell lysate (14.5±2.0 intensity units) was comparable to that of 10 μg CaCo-2 cell lysate (13.7±1.7 intensity units) indicating that GM1 expression in HeLa cells was at least 5-fold greater than in CaCo-2 cells. To ensure that GM1 in the two cell types was raft-associated, cell lysates were extracted with Triton X-100 at 4°C and then centrifuged at >100,000 ***g*** for 60 minutes. As seen in Fig. 1C, caveolin was exclusively localized to the detergent-insoluble 100,000 ***g*** pellet in HeLa cells. In both HeLa and CaCo-2 cells, GM1 was also found in the detergent-insoluble fraction (Fig. 1D) indicating that it is localized to rafts in both these cell types.

The reduced expression of GM1 in CaCo-2 cells led us to address the cell surface binding of CTX in the two cell types. As seen in Fig. 2, even though GM1 levels were

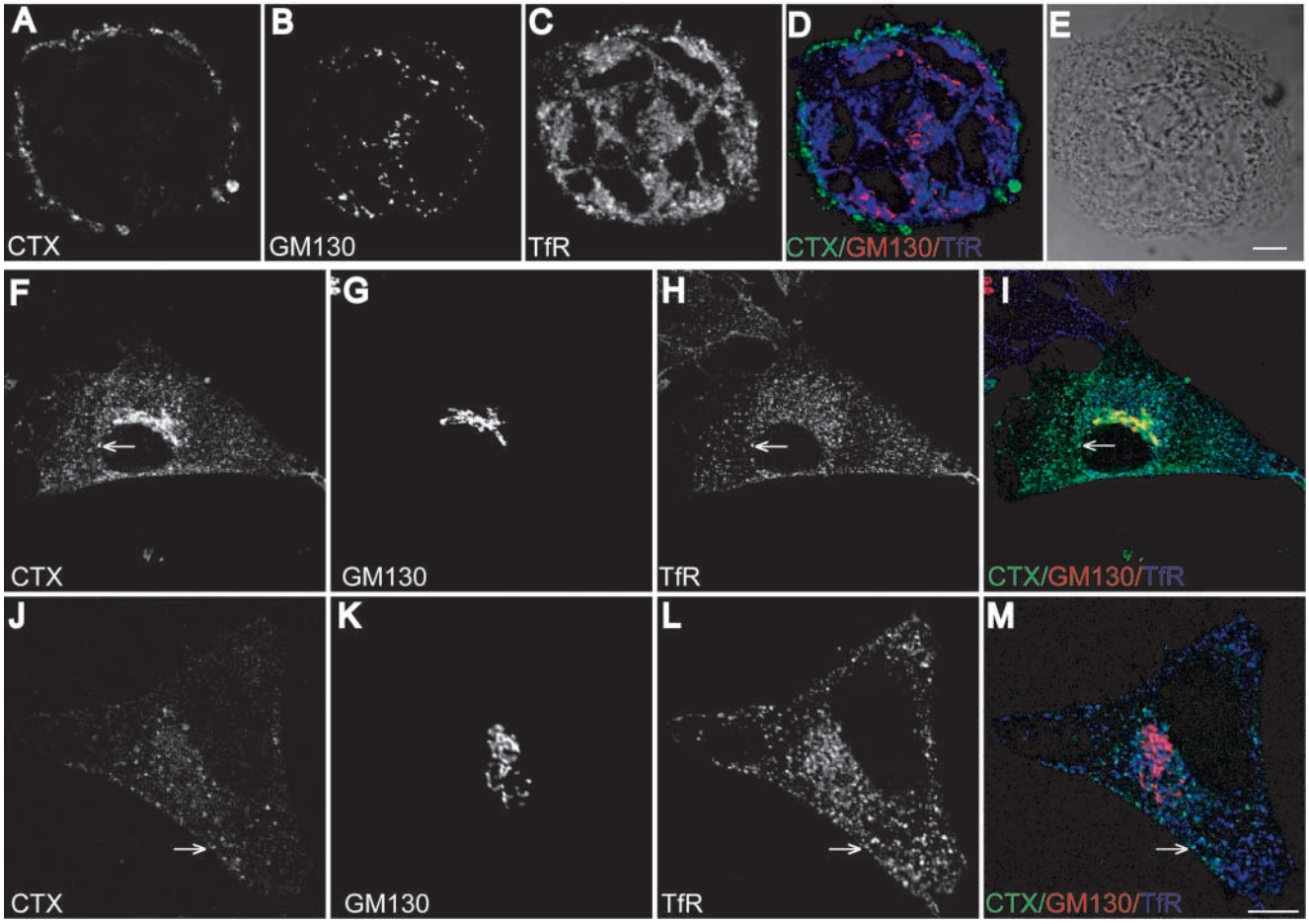


Fig. 3. Internalization of CTX in CaCo-2 and HeLa cells. CTX-FITC was internalized for 30 minutes at 37°C in CaCo-2 (A) and HeLa (F,J) cells and after fixation the Golgi apparatus was labeled for GM130 (B,G,K) and endosomes for TfR (C,H,L). Minimal internalization of CTX to either the Golgi apparatus or endosomes was detected in CaCo-2 cells (A-D). Internalized CTX was localized primarily to the Golgi in HeLa cells expressing high levels of GM1 (F-I). Increasing the sensitivity of confocal detection of cell-associated GM1 permitted the visualization of CTX in cells expressing low GM1 levels and overlap with the Golgi was not observed (J-M). The merged confocal images (D,I,M) present CTX-FITC in green, GM130 in red and TfR in blue with colocalization between CTX-FITC and GM130 in yellow and between CTX-FITC and TfR in cyan. (E) A DIC image of a CaCo-2 cell island. Arrows indicate colocalization of internalized CTX with TfR-positive endosomes. Scale bars: 8 μm (A-E) and 16 μm (F-M).

dramatically reduced, some CTX-FITC could still be detected to associate with CaCo-2 cells when added to cells at 4°C (Fig. 2A-C). The labeling was predominantly localized to the periphery of epithelial cell clusters. Binding to the cell surface of some HeLa cells (high-GM1 HeLa cells) was increased relative to CaCo-2 cells although other cells in the population (low-GM1 HeLa cells) exhibited minimal and even undetectable binding of CTX (Fig. 2D-F). In high-GM1 HeLa cells, there was a punctate pattern of cell surface CTX that partially colocalized with caveolin (Fig. 2G-I). Low-GM1 HeLa cells outnumbered high-GM1 HeLa cells such that the fivefold increase in GM1 expression in the total cell population (Fig. 1B) probably means that GM1 expression is more than tenfold greater in high-GM1 HeLa cells than in CaCo-2 cells. The low-GM1 HeLa cells still expressed equivalent caveolin levels relative to high-GM1 HeLa cells (Fig. 2G-I). The HeLa cell population is therefore mixed with respect to GM1 expression but not with respect to caveolin expression.

Reduced GM1 expression limits the caveolae/raft-dependent endocytosis of CTX to the Golgi

CaCo-2 cells incubated with CTX-FITC for 30 minutes showed very little colocalization with either the GM130-labeled Golgi or TfR-labeled endosomes (Fig. 3A-E). The majority of cell-associated CTX appeared to remain associated with the cell surface either at the periphery of the cell island or at regions of cell-cell contact. Addition of CTX to HeLa cells for 30 minutes at 37°C resulted in its internalization predominantly to the Golgi apparatus labeled with anti-GM130 but also to endosomes labeled for TfR (Fig. 3F-M). CTX delivery to the Golgi was particularly evident in HeLa cells expressing high amounts of GM1 (Fig. 3F-I). Increasing the sensitivity of confocal detection of cell-associated GM1 permitted the visualization of CTX that had been delivered to TfR-labeled endosomes but not to the Golgi apparatus in low-GM1 HeLa cells (Fig. 3J-M).

Treatment of high-GM1 HeLa cells with 5 mM mβCD, under conditions that selectively inhibit caveolae/raft-

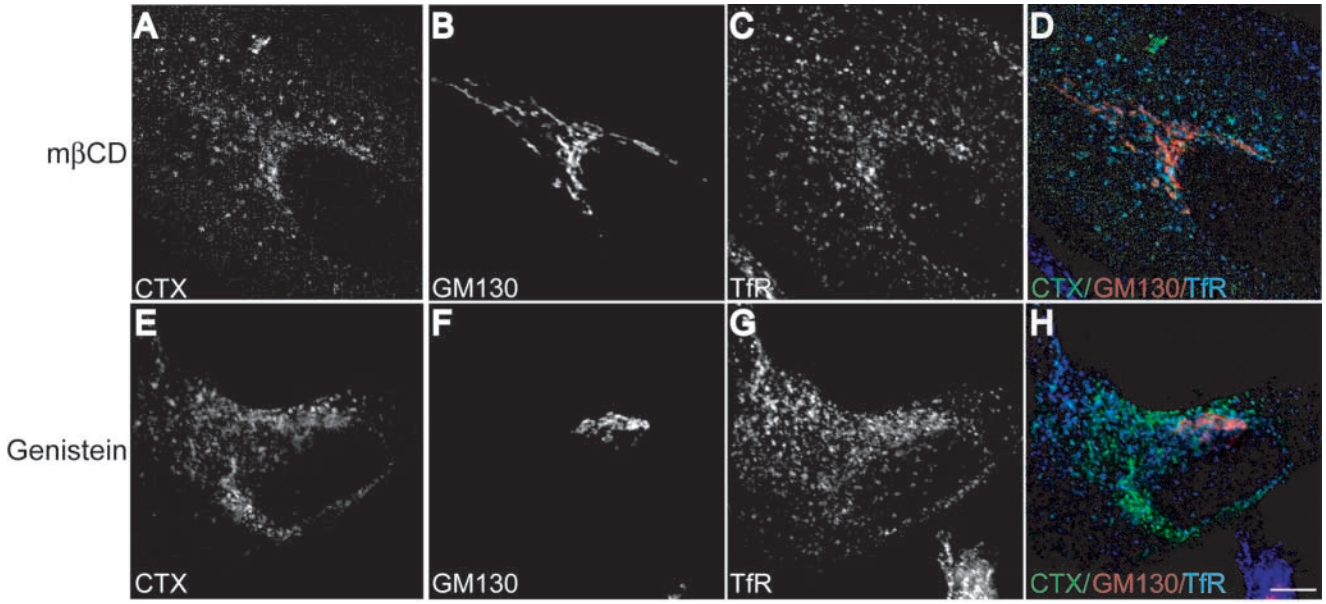
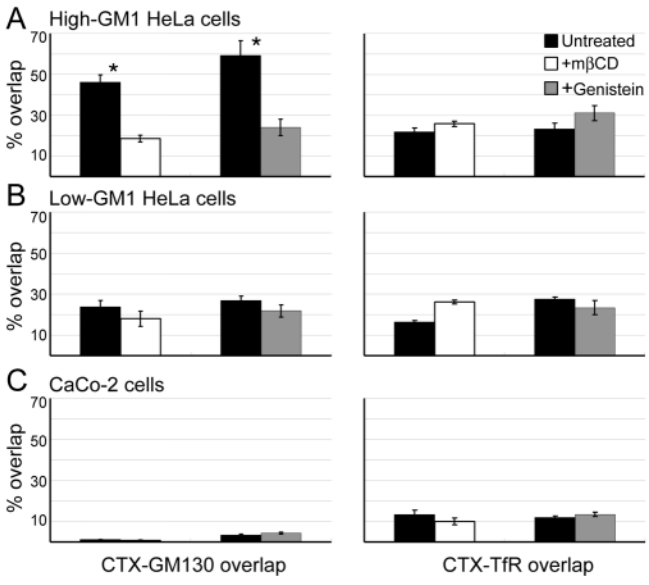


Fig. 4. mβCD and genistein block CTX delivery to the Golgi but not to endosomes. HeLa cells were pretreated with either 5 mM mβCD for 30 minutes (A-D) or 100 μg/ml genistein for 30 minutes (E-H), then incubated with CTX-FITC (A,E) for 30 minutes at 37°C. The Golgi apparatus of a high-GM1-expressing HeLa cell is labeled with GM130 (B,F) and endosomes with TfR (C,G). Merged confocal images (D,H) present CTX-FITC in green, GM130 in red and TfR in blue with colocalization between CTX-FITC and GM130 in yellow and between CTX-FITC and TfR in cyan. Scale bar: 8 μm.

dependent and not clathrin-dependent endocytosis (Le et al., 2002; Le and Nabi, 2003), inhibited CTX delivery to the Golgi and not to endosomes. Similarly, the tyrosine kinase inhibitor genistein inhibited CTX delivery to the Golgi and not to endosomes (Fig. 4). Quantification of the extent of delivery of CTX to the Golgi or endosomes using a mask overlay assay showed clearly that in high-GM1 HeLa cells CTX was internalized to the Golgi in an mβCD- and genistein-sensitive manner (Fig. 5A). Endocytosis to TfR-positive endosomes was not altered in the presence of the two inhibitors, confirming that CTX is delivered to the Golgi and not to endosomes via a caveolae/raft-dependent pathway (Fig. 5A) (Le and Nabi, 2003). In low-GM1 HeLa cells, mβCD and genistein did not affect delivery to either the Golgi or endosomes (Fig. 5B). While we were able to detect CTX in TfR-positive endosomes in the low-GM1 HeLa cells, Golgi labeling was not evident (Fig. 3) and the CTX overlap with the Golgi apparatus in these cells may represent non-specific labeling caused by the high gain levels used to acquire the images. Similarly, the spread distribution of TfR-labeled endosomes throughout the cells may overlap with non-endosomal CTX labeling and the values obtained for CTX delivery to endosomes appear to overestimate the extent of CTX endocytosis via this pathway. In CaCo-2 cells, the limited overlap of CTX labeling with the Golgi apparatus and endosomes that was measured was not affected by either mβCD or genistein (Fig. 5C), corresponding to the minimal endocytosis of CTX detected by immunofluorescence in this cell line (Fig. 3A-E).



Addition of GM1 selectively favors the caveolae/raft-dependent endocytosis of CTX to the Golgi apparatus

In order to determine whether GM1 levels are a determinant

Fig. 5. Quantification of CTX internalization in HeLa and CaCo-2 cells. CTX-FITC internalization to the GM130-positive Golgi (CTX-GM130 overlap; left panels) or to TfR-positive endosomes (CTX-TfR overlap; right panels) was quantified in untreated cells (black bars), and in the presence of mβCD (white bars) or genistein (grey bars) in high-GM1 HeLa cells (A), low-GM1 HeLa cells (B) and CaCo-2 cells (C), as indicated. Inhibition of CTX delivery to the Golgi with mβCD and genistein was significant (*P<0.05) in high-GM1 cells (A) but not in low GM1 (B) or CaCo-2 (C) cells.

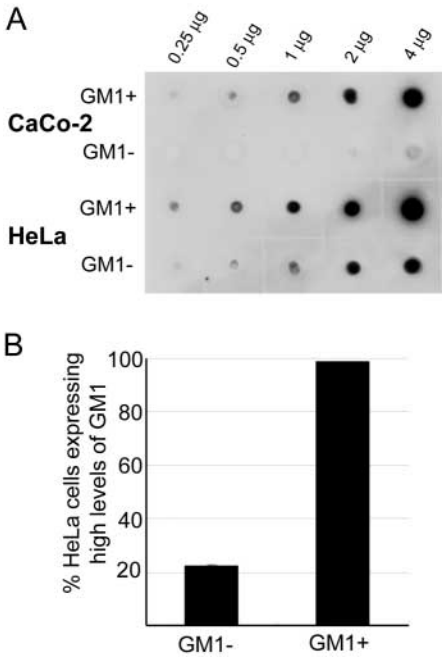


Fig. 6. Increased GM1 expression levels following addition of GM1 to the cell medium. (A) Dot blot analysis of GM1 expression in 0.25, 0.5, 1, 2 and 4 µg lysates from CaCo-2 and HeLa cells grown in the absence (GM1–) or presence (GM1+) of 5 mM GM1 in the medium for 4 hours followed by a 4-hour chase in regular medium. (B) The number of cells expressing high levels of GM1, as determined by CTX labeling, was counted in HeLa cells grown in regular medium (GM–) and GM1 supplemented medium (GM+).

of the endocytosis of CTX and more specifically which endocytic pathway is followed by CTX, cells were cultured with 5 mM GM1 for 4 hours followed by a 4-hour chase to facilitate GM1 integration into cellular membranes. As seen in Fig. 6, both CaCo-2 and HeLa cells grown in the presence of exogenous GM1 showed dramatically increased cell-associated GM1, as determined by dot blot. Following GM1 treatment, essentially all HeLa cells exhibited high levels of CTX labeling and we were no longer able to identify differential CTX labeling in the HeLa cell population (Fig. 6B). Low-GM1 HeLa cells therefore incorporate more GM1 than high-GM1 HeLa cells, indicating that GM1

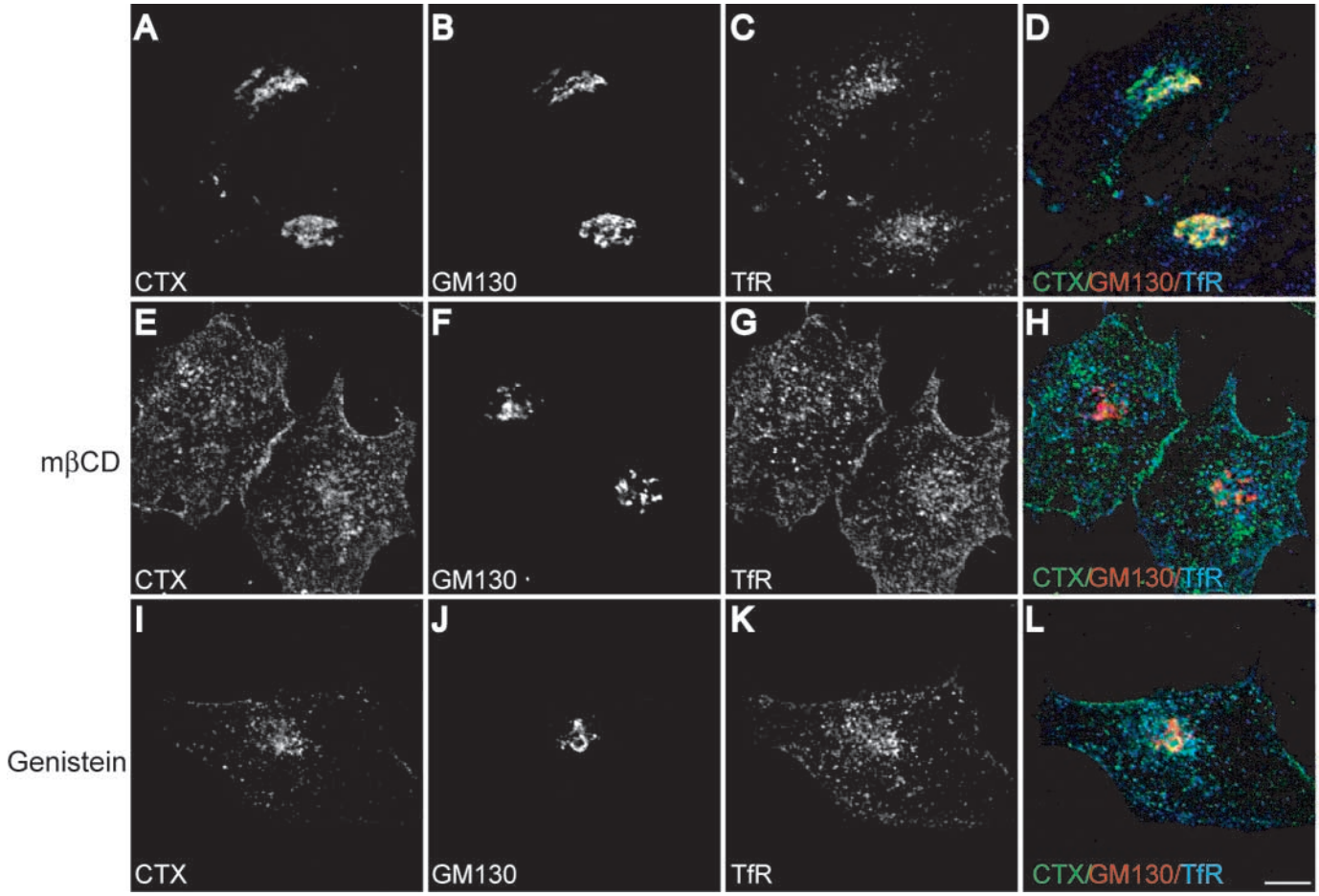


Fig. 7. mβCD and genistein block CTX delivery to the Golgi but not to endosomes in GM1-supplemented HeLa cells. HeLa cells grown in the presence of 5 mM GM1 for 4 hours followed by a 4-hour chase in regular medium were incubated with CTX-FITC for 30 minutes at 37°C (A,E,I) in regular medium (A-D) or in the presence of mβCD (E-H) or genistein (I-L). The Golgi apparatus was labeled with GM130 (B,F,J) and endosomes with TfR (C,G,K). Merged confocal images (D,H,L) show CTX-FITC in green, GM130 in red and TfR in blue with colocalization between CTX-FITC and GM130 in yellow and between CTX-FITC and TfR in cyan. Scale bar: 8 µm.

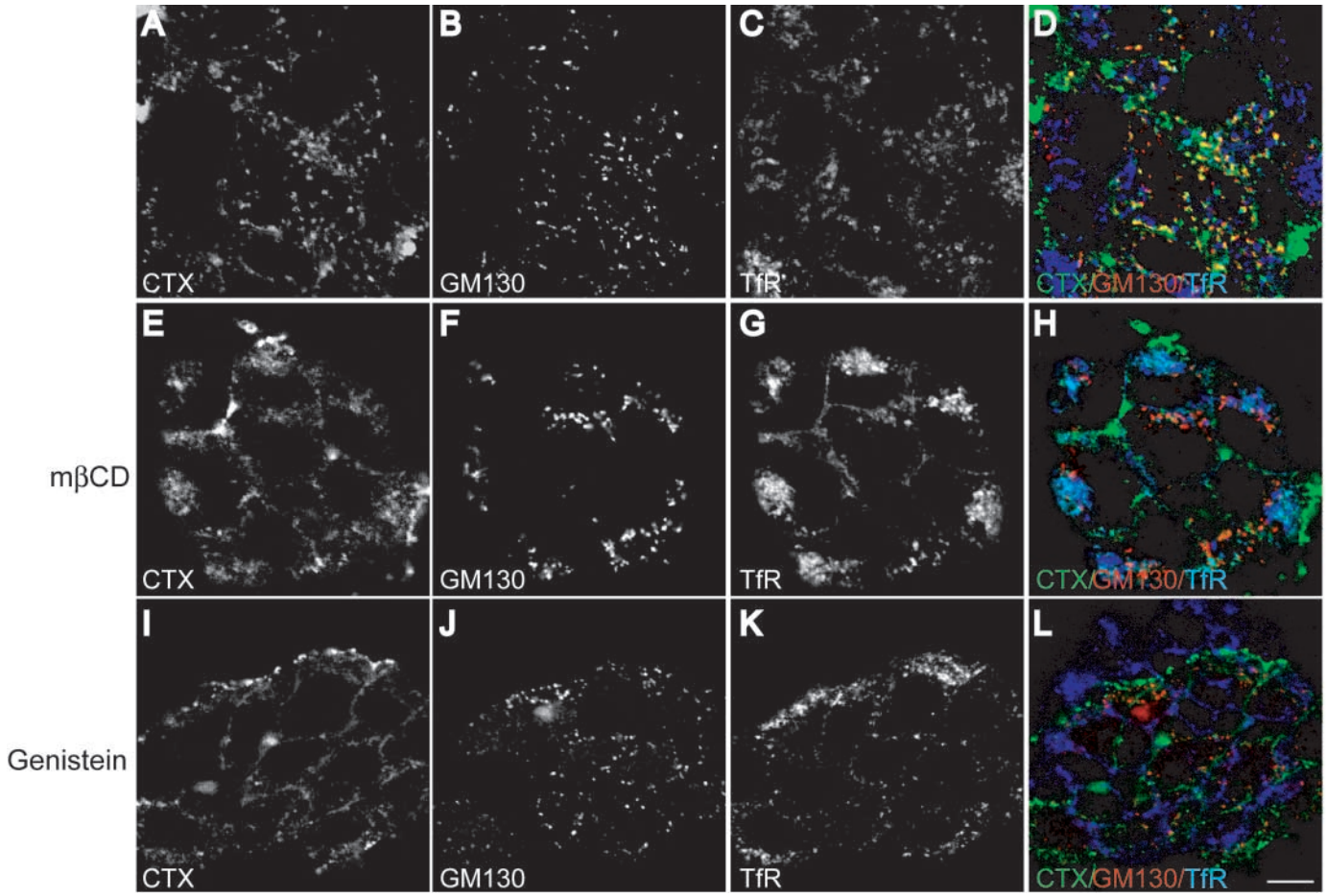


Fig. 8. mβCD, genistein-sensitive uptake of CTX to the Golgi apparatus in CaCo-2 cells supplemented with GM1. CaCo-2 cells grown in the presence of 5 mM GM1 for 4 hours followed by a 4-hour chase in regular medium were incubated with CTX-FITC for 30 minutes at 37°C (A,E,I) in regular medium (A-D) or in the presence of mβCD (E-H) or genistein (I-L). The Golgi apparatus was labeled with GM130 (B,F,J) and endosomes with TfR (C,G,K). Merged confocal images (D,H,L) present CTX-FITC in green, GM130 in red and TfR in blue with colocalization between CTX-FITC and GM130 in yellow and between CTX-FITC and TfR in cyan. Distinct colocalization of internalized CTX with Golgi elements can be seen in untreated cells (A-D). Scale bar: 8 μm.

incorporation into cell membranes is specific and saturating. Growth in GM1-containing medium resulted in the dramatic uptake of CTX to the Golgi that was inhibited by mβCD and genistein in the complete HeLa cell population (Fig. 7). Growth of CaCo-2 cells in GM1-containing medium and increased GM1 expression also enabled CTX endocytosis to elements of the Golgi apparatus that was mβCD and genistein sensitive (Fig. 8).

Quantification showed that the extent of Golgi and endosomal delivery of CTX in the total population of GM1-treated HeLa cells was essentially equivalent to that observed in high-GM1 cells in the original HeLa cell population (Fig. 9A). In CaCo-2 cells, delivery of CTX to the Golgi, but not to endosomes, was quantitatively enhanced relative to untreated cells and inhibited by mβCD and genistein (Fig. 9B). Disruption of raft domains by mβCD did however result in a significant increase in CTX delivery to endosomes in CaCo-2 cells grown in GM1-containing medium (Fig. 9B). GM1 expression levels are therefore a selective determinant of the caveolae/raft-dependent endocytosis of CTX to the Golgi apparatus.

Discussion

CTX endocytosis in HeLa and CaCo-2 cells

Discrepancy concerning the role of caveolae/rafts in CTX entry into CaCo-2 cells (Fishman and Orlandi, 2003; Sandvig and Van Deurs, 2003) led us to determine whether, as we had previously reported in NIH-3T3 cells (Le and Nabi, 2003), we could detect caveolae/raft-dependent delivery of CTX to the Golgi apparatus in CaCo-2 cells. We were unable to detect significant amounts of CTX internalized over 30 minutes at 37°C to either the Golgi apparatus or endosomes with the majority of CTX remaining at the cell surface. These results would appear to support the limited extent of caveolae-mediated endocytosis of CTX in CaCo-2 cells reported by Torgenson et al. (Torgenson et al., 2001). However, previously, filipin and mβCD had been convincingly shown to reduce both the internalization and cytotoxicity of CTX in CaCo-2 cells as well as other cell lines that do not express caveolin (Orlandi and Fishman, 1998). Our demonstration that the majority of CTX follows a cholesterol-sensitive endocytic pathway to the Golgi in a HeLa cell subpopulation expressing high GM1 levels also contrasts with a recent report that the majority of

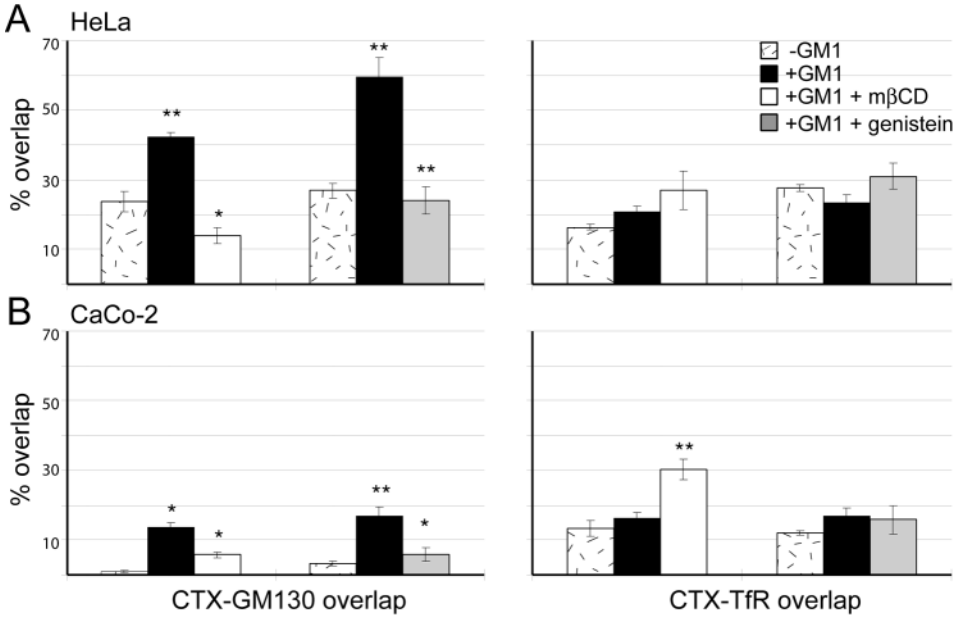


Fig. 9. Quantification of CTX-FITC internalization in HeLa and CaCo-2 cells after incubation with GM1. CTX-FITC internalization to the GM130-positive Golgi (CTX-GM130 overlay; left panels) or to TfR-positive endosomes (CTX-TfR overlay; right panels) was quantified using a mask overlay assay in HeLa (A) and CaCo-2 (B) cells grown either in regular medium (GM–; speckled bars) or in the presence of 5 mM GM1 for 4 hours followed by a 4-hour chase in regular medium (GM+) and left untreated (black bars) or treated with mβCD (white bars) or genistein (grey bars), as indicated. Data for cells grown in regular medium (GM–) was taken from Fig. 5 for both the mβCD and genistein series of experiments. The increase in CTX uptake to the Golgi in GM1-supplemented HeLa and CaCo-2 cells and its inhibition by mβCD and genistein is significant as is the increased delivery of CTX to endosomes in mβCD treated CaCo-2 cells (*P<0.01; **P<0.05 relative to bar to the left).

CTX internalized in HeLa cells followed a clathrin-dependent pathway that was nystatin insensitive (Singh et al., 2003). Lack of caveolin expression in the HeLa cell population led to the conclusion that caveolin expression was required for caveolae/raft-dependent CTX uptake, as had been demonstrated for lactosylceramide (Singh et al., 2003). In contrast to the lack of caveolin expression in CaCo-2 cells, the HeLa cells used for our studies did express caveolin (Fig. 1). Furthermore, differential mβCD-, genistein-sensitive uptake of CTX in the HeLa cell population was related not to caveolin expression, which was equivalent amongst the cells, but rather to GM1 expression levels. Similarly, the reduced uptake of CTX to the Golgi apparatus of CaCo-2 was associated with minimal expression of GM1 in these cells. In cells expressing reduced levels of GM1 and consequently limited caveolae/raft-dependent endocytosis, basal levels of endocytosis via non-caveolae/raft pathways may represent a significant proportion of the CTX that enters the cell.

The extensive binding of CTX to the cell periphery and minimal intracellular labeling that we observed for CaCo-2 cells (Fig. 3) contrasts with images of CTX uptake in CaCo-2 cells presented by Orlandi and Fishman (Orlandi and Fishman, 1998). The tremendously varied expression of GM1 that we describe in HeLa cells demonstrates that GM1 expression levels are not constant within a cell population and could have potentially been lost through cloning. CaCo-2 cells have been reported not to express caveolin (Breuza et al., 2002; Mirre et al., 1996; Torgersen et al., 2001; Vogel et al., 1998) although other reports have described caveolin expression by CaCo-2 cells (Field et al., 1998; Orlandi and Fishman, 1998). Similarly, differential expression of caveolin in HeLa cells has been reported (Shack et al., 2003; Singh et al., 2003; Skretting et al., 1999). We have previously reported the presence of caveolae in the HeLa cell population used in these studies (Benlimame et al., 1998). Variations in GM1 and caveolin expression between different cell populations may explain many of the discrepancies in the extent of caveolae/raft-dependent CTX uptake reported from different labs.

Recent studies have demonstrated that caveolin-1-GFP is essentially immobile at the cell surface and that only a minor fraction of caveolin-1 actually internalizes (Mundy et al., 2002; Pelkmans et al., 2001; Thomsen et al., 2002). At the same time, caveolin-1 overexpression has been shown to negatively regulate the internalization of caveolae/raft ligands, including CTX, to the Golgi apparatus (Le et al., 2002; Le and Nabi, 2003). Multiple evidence argues that caveolae and rafts mediate essentially the same endocytic pathway (Nabi and Le, 2003). Indeed, caveolae/raft-dependent endocytosis independent of caveolin expression has been reported for AMF and the IL-2 receptor (Lamaze et al., 2001; Le et al., 2002) and CTX uptake to the Golgi has been shown to occur in CaCo-2 cells that do not express caveolin as well as following caveolin-1 depletion with RNAi (Nichols, 2002; Orlandi and Fishman, 1998). Absence of caveolin expression cannot therefore be considered evidence for the absence of a caveolae/raft-dependent pathway (Nabi and Le, 2003). Caveolin expression may be required for the caveolae/raft-dependent uptake of select ligands, such as albumin or lactosylceramide (Razani et al., 2001; Singh et al., 2003), but not for others, such as CTX, GPI-anchored proteins and the AMF and IL-2 receptors (Deckert et al., 1996; Lamaze et al., 2001; Le et al., 2002; Orlandi and Fishman, 1998). Multiple caveolae/raft-dependent endocytic pathways can therefore exist and multiple, varied parameters may serve to recruit ligands to these domains for endocytosis (Nabi and Le, 2003).

GM1 expression levels selectively regulate the caveolae/raft-dependent endocytosis of CTX to the Golgi apparatus

The dramatically increased caveolae/raft-dependent endocytosis of CTX to the Golgi in a select population of HeLa

cells expressing high levels of GM1 led us to determine whether we could induce this pathway by increasing GM1 levels. Increased GM1 expression in both HeLa and CaCo-2 cells resulted in increased uptake of CTX to the Golgi apparatus via a mβCD-, genistein-sensitive pathway (Figs 7-9). Delivery to TfR-positive endosomes was not affected, indicating that increased GM1 levels preferentially recruit CTX for endocytosis via the caveolae/raft-dependent pathway to the Golgi. The increased endocytosis of CTX to endosomes in CaCo-2 cells in the presence of excess GM1 and mβCD does nevertheless indicate that GM1 can recruit CTX for endocytosis via non-caveolae/raft-dependent pathways under conditions where the caveolae/raft pathway is disrupted.

GM1 association with raft domains has been shown to be critical to elicit a toxin-induced cAMP-dependent Cl^- secretory response (Wolf et al., 1998). The reduced caveolae/raft-dependent endocytosis of CTX in both low-GM1 HeLa cells and CaCo-2 cells is apparently not due to the inability to recruit CTX to raft domains by GM1, as the low levels of GM1 are still associated with Triton X-100 insoluble raft domains in CaCo-2 cells. In neurons, CTX associates with rafts yet is internalized via clathrin-dependent endocytosis (Shogomori and Futerman, 2001). CTX-labeled GM1-containing lipid rafts are depleted within clathrin-rich cell surface regions (Nichols, 2003) and how raft-associated GM1 is recruited for clathrin-dependent endocytosis remains unclear. In HeLa cells that show majority uptake of CTX via a clathrin-dependent pathway, Bodipy-GM1 is still internalized via a clathrin-independent, nystatin-sensitive pathway (Singh et al., 2003). Each molecule of CTX binds five molecules of GM1 (Lencer et al., 1999) and GM1 density within rafts may potentially influence the nature and stability of the raft domains (Kenworthy, 2002; Subczynski and Kusumi, 2003) with which CTX-bound GM1 is associated and thereby determine whether CTX is internalized via a caveolae/raft-dependent pathway or is directed for endocytosis via a clathrin-dependent pathway.

Caveolae and rafts represent an important route of entry for multiple ligands (Duncan et al., 2002; Johannes and Lamaze, 2002; Nichols and Lippincott-Schwartz, 2001; Pelkmans and Helenius, 2002). This pathway is highly complex and caveolae/raft cell surface domains may exhibit differential endocytic capabilities and even differential intracellular targeting upon endocytosis (Nabi and Le, 2003; van Deurs et al., 2003). However, it is becoming increasingly clear that caveolae/raft-dependent ligands can internalize via alternate endocytic pathways and that raft association is not necessarily a determinant of caveolae/raft-dependent endocytosis (Abrami et al., 2003; Shogomori and Futerman, 2001). CTX represents such a ligand and studies of the endocytic mechanisms by which CTX is internalized have revealed that entry of this toxin into the cell can follow multiple endocytic pathways even in the same cell (Le and Nabi, 2003; Shogomori and Futerman, 2001; Torgersen et al., 2001). The mechanism of entry of CTX is cell-dependent and can vary between almost complete clathrin-dependence in neurons to almost complete caveolae/raft-dependence in various other cell types (Orlandi and Fishman, 1998; Puri et al., 2001; Shogomori and Futerman, 2001; Singh et al., 2003). The demonstration here that GM1 levels are a determinant of recruitment of CTX for endocytosis via the caveolae/raft-dependent pathway suggests that receptor density within raft domains may represent a further element of complexity that determines not only ligand recruitment to rafts but also the endocytic potential and endocytic pathway followed by raft domains.

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