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Heterogeneity of detergent-insoluble membranes from human intestine containing caveolin-1 and ganglioside G_{M1}

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Abstract

In intestinal epithelia, cholera and related toxins elicit a cAMP-dependent chloride secretory response fundamental to the pathogenesis of toxigenic diarrhea. We recently proposed that specificity of cholera toxin (CT) action in model intestinal epithelia may depend on the toxin's cell surface receptor ganglioside G_{M1}. Binding G_{M1} enabled the toxin to elicit a response, but forcing the toxin to enter the cell by binding the closely related ganglioside G_{D1a} rendered the toxin inactive. The specificity of ganglioside function correlated with the ability of G_{M1} to partition CT into detergent-insoluble glycosphingolipid-rich membranes (DIGs). To test the biological plausibility of these hypotheses, we examined native human intestinal epithelia. We show that human small intestinal epithelia contain DIGs that distinguish between toxin bound to G_{M1} and G_{D1a}, thus providing a possible mechanism for enterotoxicity associated with CT. We find direct evidence for the presence of caveolin-1 in DIGs from human intestinal epithelia but find that these membranes are heterogeneous and that caveolin-1 is not a structural component of apical membrane DIGs that contain CT.

Keywords

caveolae; cholera toxin; toxigenic diarrhea

Cholera toxin (CT) produced by *Vibrio cholerae* is the virulence factor responsible for the massive secretory diarrhea seen in Asiatic cholera. CT and the closely related *Escherichia coli* heat-labile toxin type I (LT) are comprised of five identical B polypeptides (the B subunits or CTB), which primarily bind ganglioside G_{M1} at the cell surface, and a single enzymatically active A polypeptide (the A subunit or CTA), which activates adenylyl cyclase by catalyzing the ADP ribosylation of the regulatory GTPase G_sα (15,17,47). In the intestinal crypts, these

toxins elicit a cAMP-dependent chloride secretory response fundamental to the pathogenesis of secretory diarrhea.

To induce disease, the toxin must breach the intestinal barrier and enter the epithelial cell. This depends exclusively on interactions between CT and the target cell, as *V. cholerae* is not an enteroinvasive pathogen. Entry of CT into the epithelial cell occurs by receptor-mediated endocytosis and retrograde vesicular transport of toxin into Golgi cisternae or endoplasmic reticulum, where the A subunit likely translocates across the membrane (3,20-22,26,32,34). We recently found that engagement of CT into this complex pathway may depend on the specific structure of the toxin's cell surface receptor ganglioside G_{M1} . Binding G_{M1} enabled the toxin to elicit a response from sensitive cells, but forcing the toxin to enter the cell by binding the closely related ganglioside G_{D1a} rendered the toxin inactive (23,51). The specificity of ganglioside function in toxin action correlated closely with the ability of G_{M1} to partition CT into detergent-insoluble membranes that displayed some of the biochemical characteristics of caveolae (23,33,51). On the basis of these in vitro studies, we proposed that CT binding to G_{M1} at the cell surface represents a form of protein acylation that acts as a sorting motif critical to toxin function and is dependent on association with detergent-insoluble glycosphingolipid-rich membranes (23,51).

So-called detergent-insoluble glycosphingolipid-rich membranes [DIGs; (46)] or detergent-resistant membranes (7) can be isolated from essentially all mammalian cell types by virtue of their insolubility in nonionic detergents, such as Triton X-100, at 4°C and their light buoyant density (reviewed in Refs. 1 and 7). Although the terminology has been the subject of debate, plasma membranes that conform to the functional definition of DIGs can be distinguished in two general categories: classical caveolae that are only abundant in some cell types, such as endothelial cells and myofibroblasts, and exhibit a characteristic ultrastructural morphology and non-caveolar DIGs that can be isolated from essentially all cell types, the in situ morphology of which has been controversial (1,7). Caveolae are 50- to 80-nm plasmalemmal invaginations with a striated cytoplasmic coat containing caveolin-1 (36,39). Expression of caveolin-1 may be structurally necessary and possibly sufficient for the formation of caveolae (9,13,25). Caveolae have been implicated in a variety of key cellular functions, such as ligand-induced signal transduction, protein and lipid sorting, and vesicular trafficking (reviewed in Refs. 1,38,42, and 45). Caveolae-associated signal transduction may be mediated through the caveolin family of integral membrane proteins that organize specific signaling complexes at the plasma membrane (10,31). However, DIGs that do not contain caveolins have also been shown to function as organizing centers for signal transduction (18,40).

In many cell types, a large fraction of ganglioside G_{M1} is found in caveolae. In fact, ligand overlay with CT has been used for morphological identification of caveolae (35,43). The proximity of G_{M1} and caveolin-1 has also been suggested in studies in which a photoreactive analog of G_{M1} could be directly cross-linked to caveolin-1 (12). Although our recent studies and those of others have raised the possibility of an association between CT action and caveolae (33,51), the relationships between CT, its cell surface receptor G_{M1} , and caveolin-1 in intestinal epithelia remain unknown.

To test the biological plausibility of our hypotheses regarding CT action that are based on observations made in the T84 cell model, we examined DIGs isolated from native human intestinal epithelial cells. These data show that epithelial cells isolated from normal human jejunum, like cultured T84 cells, contain DIGs that exhibit the ability to distinguish between toxin bound to gangliosides G_{M1} or G_{D1a} . We found, however, that intestinal epithelia express very low levels of caveolin-1. Furthermore, caveolin-1 does not colocalize with G_{M1} on the apical membrane of normal intestinal epithelia or T84 cells in monolayer culture, and two structurally distinct populations of DIGs are isolated from cultured T84 cells. One DIG

population contains vesicles enriched in the apical membrane CT-G_{M1} complex but depleted in caveolin-1, and the other contains vesicles enriched in caveolin-1 but depleted in apical membrane CT-G_{M1}. Thus, when applied to the physiologically relevant apical membrane of T84 cells, CT binds G_{M1} associated with apical membrane DIGs that lack caveolin-1. These data and those from human jejunum imply that the mechanism(s) for recognition of the CT-G_{M1} structural motif in T84 and native intestinal epithelial cells may not require caveolin-1 directly. Our results indicate heterogeneity in structure and likely function of DIGs in intestinal epithelia and are consistent with the idea that detergent-insoluble membranes mediate CT-induced disease even in the absence (or near absence) of caveolae in this cell type.

METHODS

Materials

CT was purchased from Calbiochem (San Diego, CA), and fluorescein-labeled CTB (CTB-FITC) was purchased from List Biological Laboratories (Campbell, CA). Mouse monoclonal anti-caveolin-1 clones 2297 and 2234 were from Transduction Laboratories (Lexington, KY), and affinity-purified rabbit polyclonal anti-caveolin-1 IgG (N20) was from Santa Cruz Biotechnology (Santa Cruz, CA). Antibodies against ZO-1 protein were from Zymed (South San Francisco, CA). Polyclonal rabbit antisera against CT and LTIIB were previously described (16,24). Recombinant LTIIB was prepared and purified as previously described (8,37). All secondary antibodies and chemical reagents were from Sigma Chemical (St. Louis, MO) unless otherwise specified.

Cell culture

T84 cells were purchased from the American Type Culture Collection (Rockville, MD) and grown to confluency on collagen-coated Transwell inserts (Costar, Cambridge, MA) as previously described (20). Cell passages 70–100 were used for these experiments. A431 cells (gift of Dr. David Fitzgerald, National Cancer Institute, Bethesda, MD) were grown to confluency in 75-cm² tissue culture flasks in DMEM with 5% FCS and standard antibiotics.

Normal human intestinal epithelial cells

Normal human small intestinal epithelial cells were isolated from segments of proximal jejunum resected from patients undergoing gastric bypass surgery for weight control. Tissue was obtained with patients' informed consent under a protocol approved by the Human Studies Committee of the Brigham and Women's Hospital. Resected tissue was immediately placed in ice-cold complete RPMI culture medium, and the mucosa was dissected away from the submucosa and the muscularis propria within ~30–45 min of tissue resection. Intestinal epithelial cells were nonenzymatically isolated as described (2,4).

Sucrose equilibrium density centrifugation

Confluent monolayers of T84 cells in 45-cm² inserts (representing ~7–10 mg of total protein) were used for isolation of DIGs. All steps were performed at 4°C. Cells were equilibrated with Hanks balanced salt solution without sodium bicarbonate or phenol red, buffered with 10 mM HEPES pH 7.5 (HBSS), and scraped into 1 ml of ice-cold 1% Triton X-100 solution in 10 mM Tris-150 mM NaCl, pH 7.6 (TBS). All buffers contained 5 mM 4-(2-aminoethyl)-benzenesulfonyl fluoride (Pefabloc SC, Boehringer Mannheim, Indianapolis, IN) and complete protease inhibitor cocktails at 1× final concentration according to manufacturer's instructions (Boehringer). Cells were homogenized slowly in the detergent solution on ice with 15 strokes in a tight-fitting Dounce homogenizer (Kontes, Vineland, NJ). The homogenate was adjusted to 40% sucrose by addition of an equal volume of 80% sucrose in TBS. Typically, 4 ml of this 40% sucrose mixture was layered under a step gradient consisting of 4 ml of 30% sucrose

followed by 4 ml of 5% sucrose in TBS (some experiments were scaled to 5 ml total volume including 2 ml of 40% sucrose, 2 ml of 30% sucrose, and 1 ml of 5% sucrose). The gradient was centrifuged for 2 h to overnight either at 39,000 rpm in a SW-41 (12-ml tubes) or at 48,000 rpm in a MLS-50 (5-ml tubes) swinging bucket rotor (both from Beckman Instruments, Palo Alto, CA). Sequential 1-ml fractions from the top of the gradient or representative fractions of the floating membranes and soluble fractions were collected as needed. Isolated intestinal epithelial cells (IECs) were treated in a similar way. To maintain the same detergent-to-total protein ratio as in T84 monolayers, 1 ml of ice-cold 1% Triton X-100 solution in TBS was used per 0.25 ml of IEC pellet representing 7–10 mg of total protein. Isolation of DIGs from A431 cells was carried out in an essentially identical fashion. The only difference was that A431 cells, which are a nonpolarized epithelial cell line, were grown to confluency, incubated with CT, washed, and treated with detergent extraction buffer in tissue culture flasks instead of Transwell inserts.

Immunohistology and confocal microscopy

Normal human small intestine and polarized T84 cell monolayers (0.33-cm² inserts) were washed in PBS and embedded in optimum cutting temperature compound (Tissue-Tek, Torrance, CA) for frozen sectioning on a Leica CM3050 cryomicrotome (Leica, Nussloch, Germany). Five-micrometer frozen sections were air dried at room temperature, fixed in 4% paraformaldehyde in PBS, washed in PBS, and blocked in 10% normal goat serum (Zymed). Sections were stained with primary antibodies diluted to 2–2.5 µg/ml in the blocking solution and detected either with a fluorophore-conjugated secondary antibody for fluorescent microscopy or prepared for bright-field microscopy using a diaminobenzidine (DAB) chromogen kit according to the manufacturer's instructions (Histostain-Plus; Zymed). For some experiments, inserts were directly fixed in 4% paraformaldehyde, washed in PBS, stained as above, and mounted en face on glass slides for epifluorescence and/or confocal microscopy. T84 cells and tissue stained with CTB-FITC were similarly prepared but were blocked with 3% BSA in PBS and directly stained with 2 nM CTB-FITC. All sections prepared for fluorescent microscopy were mounted in ProLong anti-fade reagent according to the manufacturer's instructions (Molecular Probes, Eugene, OR) and examined either using a Bio-Rad MRC-1024 confocal microscope (Bio-Rad Laboratories, Hercules, CA) or an Axiophot microscope (Zeiss) equipped with a Spot digital camera (Diagnostic Instruments, Sterling Height, CA). Electronic images were captured, cropped, and edited using Adobe Photoshop v. 5 (Adobe Systems, San Jose, CA).

Immunoblotting

T84 cells and IECs were extracted in boiling 5% SDS in water. Protein concentrations were determined by the bicinchoninic acid method (Pierce, Rockford, IL) with BSA standards. Samples were resolved on 10–20% denaturing Tris-HCl polyacrylamide gels (Bio-Rad) and transferred onto nitrocellulose membranes (Bio-Rad) by electroblotting. Nitrocellulose membranes were blocked with 1% BSA-1% nonfat milk in TBS containing 0.1% Tween-20, and probed with the primary antibody. Bound primary antibody was labeled with horseradish peroxidase-conjugated goat secondary antibody and detected with an enhanced chemiluminescence reagent (Pierce).

RT-PCR

Total RNA was purified with TRIzol reagent (GIBCO BRL, Gaithersburg, MD) from polarized T84 and Caco-2E cells grown to confluency on 1-cm² inserts, human endothelial cells (HECs, generously provided by the Vascular Research Division, Brigham and Women's Hospital, Boston, MA), and isolated human small intestinal cells from four different individuals. RNA (~5–10 µg) was reverse transcribed to cDNA with an oligo(dT) primer (Promega, Madison,

WI) and avian myeloblastosis virus reverse transcriptase (Promega). One-third of the cDNA preparation was used in a PCR reaction with primers specific for human caveolin-1 (forward primer 5'-AGCGAGAAGCAAGTGTAC-3'; reverse primer 5'-GATGCGGACATTGCTGAA-3') and human β -actin.

Immunoisolation of DIGs

T84 cell DIGs were isolated from 45-cm² inserts that had been incubated apically with 10 ml of 2–4 nM CT in HBSS for 1 h at 4°C, and A431 cell DIGs were isolated from 75-cm² flasks that had been incubated with 20 ml of 4 nM CT in HBSS for 1 h at 4°C. Floating membranes were collected and pelleted for 30 min at 48,000 rpm in an MLS-50 rotor (Beckman). The pellet was resuspended in TBS containing 1% Triton X-100 and finely dispersed by gently pipetting in a sonicator bath for 20 s. Resuspended DIGs prepared in this fashion typically consisted of single vesicles with occasional small clusters of vesicles when examined by electron microscopy. For immunoisolation, DIGs were aliquoted in equal portions, each containing ~50 μ g of total protein and precleared with 25 μ l of protein G Sepharose beads (Pharmacia, Piscataway, NJ) for 2 h at 4°C with gentle mixing. The beads were then removed by centrifugation at 200 g for 2 min at 4°C, and DIGs were incubated overnight at 4°C with polyclonal antibodies against CTB (1 ng/ml final concentration), polyclonal (N20) or monoclonal (clone 2234) antibodies against caveolin-1 (5 ng/ml), anti-ovalbumin antiserum (5 ng/ml), or normal mouse IgG (5 ng/ml). Twenty-five microliters of protein-G Sepharose beads were then added to each sample and incubated for an additional 2 h at 4°C. Beads were removed by centrifugation at 200 g for 2 min at 4°C, washed three times in TBS, and resuspended in 50 μ l of Laemmli sample buffer for immunoblotting.

Electron microscopy

Samples were fixed in 2% glutaraldehyde-2.5% paraformaldehyde in 0.2M cacodylate buffer at 4°C and absorbed onto formvar-carbon-coated gold grids (Electron Microscopy Sciences, Fort Washington, PA). Un-stained or positively stained grids with adsorbed DIGs or Sepharose-bound DIGs were examined and photographed using a Phillips 300 electron microscope.

RESULTS

CT-G_{M1} but not LTIIb-G_{D1a} complexes fractionate with DIGs in normal human intestinal epithelium

We have recently hypothesized that signal transduction by CT in polarized T84 cells requires specific association with DIGs via toxin binding to its cell surface receptor ganglioside G_{M1} (23,51). To demonstrate the ability of native intestinal epithelial DIGs to do the same, we utilized the heat-labile enterotoxin of *E. coli*, LTIIb. LTIIb is a serotype II toxin whose enzymatically active A subunit has significant homology with the A subunits of serotype I enterotoxins such as CT but lacks significant homology with CT in its B subunits (37). Because of the differences in the receptor-binding B subunits, CT preferentially binds G_{M1}, whereas LTIIb prefers binding G_{D1a} and does not bind G_{M1} at all (14). We recently showed that, in T84 cells, toxin binding and/or clustering G_{M1} is associated with biochemical fractionation in DIGs and induction of a chloride secretory response, whereas binding and/or clustering G_{D1a} excludes the toxin from DIGs and is not associated with induction of a secretory response (51). These data provided evidence that T84 cells distinguish between G_{M1} and G_{D1a} (or pentameric clusters of these) by virtue of their interaction with DIGs and that this separation is critical to toxin action in vitro.

To obtain evidence that such specificity of ganglioside function extends to normal human intestinal epithelia, we studied intestinal epithelial cells isolated from proximal small intestine

of three different individuals. The macroscopic appearance of the mucosa was normal in all three cases, and microscopic examination of random areas revealed no abnormalities. Nonenzymatic isolation of the epithelial cells typically yielded a preparation composed of a mixture of single epithelial cells and small clusters of epithelial cells as well as intraepithelial lymphocytes that constituted ~5% of the cell population based on morphological appearance on hematoxylin and eosin-stained preparations. No other cell types or vessels were identified in any of these preparations. The purely epithelial nature of these preparations was confirmed by microscopic examination of the tissue left after isolation of the epithelial cells, which revealed an intact basement membrane with nearly complete loss of villous epithelium and loss of the majority of the crypt epithelium (data not shown).

To test the ability of native intestinal DIGs to distinguish between G_{M1} and G_{D1a} , cell surface receptors for CT or LTIIB were bound to steady state by incubating the IECs (between 7 and 10 mg of total protein) with 20 ml of 2 nM CT or LTIIB for 30 min at 4°C. Cells were then washed to remove unbound toxin, homogenized in 1% Triton X-100 in TBS, and subjected to sucrose equilibrium density centrifugation. After centrifugation, a turbid floating fraction was always present at the 30%-to-5% sucrose interface. The floating fraction of DIGs consisted primarily of vesicles of lipid bilayers ranging from 40 to 200 μ m in diameter as assessed by electron microscopy (data not shown). DIGs typically contained <1% of total cellular protein but were highly enriched in caveolin-1 and CT- G_{M1} complexes compared with the soluble fractions at the bottom of the gradient (Fig. 1). On the other hand, the relative concentration of LTIIB, assessed by signal intensity on the Western blot, was roughly similar between DIGs and the soluble fractions, which contained nearly two orders of magnitude more total protein (Fig. 1). Thus the bulk of the LTIIB- G_{D1a} complex fractionated separately from DIGs and was found at the bottom of the gradient, together with other Triton X-100-soluble proteins. These data indicate that human IECs, like T84 cells, exhibit the ability to distinguish between CT- G_{M1} and LTIIB- G_{D1a} complexes at the cell surface on the basis of the pattern of their association with DIGs.

Expression of caveolin-1 in normal human intestinal epithelial cells

The presence of caveolin-1 in intestinal epithelia (human IECs, T84 cells, and Caco-2E cells) was demonstrated biochemically by immunoblotting and by RT-PCR (Figs. 1 and 2A and additional data not shown). Specific mRNA for caveolin-2 was also detected in both native IECs, T84, and Caco-2E preparations (data not shown). Neither T84 cells nor IECs, however, were rich sources for caveolin-1, as evidenced by comparison to cultured HECs (Fig. 2A). Endothelial cells, like fibroblasts, exhibit abundant caveolae and caveolins, and both cell types have been used to study structure and function of caveolae, DIGs, and related membrane structures (1,36,39,45,48).

Fig. 2A shows that with 1 μ g of protein per lane, caveolin-1 was not detectable in Western blots of whole cell lysates and only modestly detectable in DIGs isolated from T84 cells and native human IECs. In contrast, expression of caveolin-1 in whole cell lysates of HECs was readily apparent and the immunoblot signal for caveolin-1 in isolated DIG fractions from HECs saturated the detection system. As assessed by serial dilutions and Western blotting, human endothelial cell DIGs contained between 10- and 100-fold more caveolin-1 than epithelial cells isolated from human jejunum or from cultured T84 cells (data not shown).

That the immunoreactive band for caveolin-1 originated from epithelial cells isolated from human jejunum was confirmed by immunocytochemistry (Fig. 2, B and C). When freshly isolated IECs were fixed and permeabilized, a diffuse specific immunoreactive signal against caveolin-1 was detected in columnar epithelial cells and occasional goblet cells. Similar results were obtained using polyclonal goat anti-caveolin-1 antibodies (N20, Santa Cruz Biotechnology) followed by peroxidase-conjugated secondary antibody detected and amplified

either with the DAB chromogen (Zymed; Fig. 2*B*) or tyramide-fluorescein (NEN Life Sciences, Boston, MA; data not shown). Caveolin-1 was not convincingly detectable by indirect immunofluorescence without amplification. The DAB and tyramide-fluorescein signals were specific for caveolin-1 because no signal was apparent in control studies using nonspecific primary antibody at a similar concentration or secondary antibody alone (Fig. 2*C*).

Steady-state distribution of caveolin-1 and G_{M1} in intestinal epithelial cells

To determine the extent of physical colocalization between caveolin-1 and the cell surface receptors for CT, the steady-state distribution of each was determined morphologically. Cell surface receptors for CT were highlighted by ligand overlay at low ligand concentrations (comparable to those used in the biochemical studies). Caveolin-1 was stained by standard immunocytochemistry using goat polyclonal (N20) or mouse monoclonal (clone 2297) primary antibodies followed by detection with either fluorescent secondary antibodies or peroxidase-conjugated secondary antibodies for detection and amplification using the DAB chromogen.

Sections of formalin-fixed paraffin-embedded tissue or paraformaldehyde-fixed frozen sections of human small intestine incubated with 2 nM CTB-FITC showed distinct villous and crypt staining patterns. In the villous epithelium, CTB at this concentration uniformly highlighted binding sites on the apical plasma membrane (Fig. 3*A*). In the crypt epithelium, CTB-FITC was also primarily apical, but its distribution was coarse and somewhat irregular and included the apical membrane and the apical cytoplasm (Fig. 3*B*). With the use of 2 nM CTB, the only prominent basolateral staining was that of the Paneth cells at the base of the crypts (not shown). Except for mucin granules within goblet cells, receptor binding by CTB-FITC was blocked in the presence of competing excess CTB (2 μ M), indicating specificity for the CT receptor, which is primarily ganglioside G_{M1} (data not shown). The patterns of fluorescence described above were not detectable in cells or sections treated in parallel with 2 nM CTB instead of CTB-FITC.

Identical fresh frozen or formalin-fixed paraffin sections of small intestine incubated with anti-caveolin-1 antibodies showed strong staining of endothelial cells and myofibroblasts in the lamina propria (Fig. 3, *C* and *D*). Positive immunoreactivity was also noted at the interface between epithelial cells and the lamina propria in both crypts and villi (Fig. 3, *C* and *D*). This signal may represent basal membrane localization of caveolin-1 or, more likely, expression of caveolin-1 in the tightly adherent pericrypt myofibroblasts that occupy the immediate subepithelial space in this tissue (Fig. 3, *C* and *D*). A faint diffuse epithelial cell staining pattern similar to that of IECs (Fig. 2*B*) was only detectable with signal amplification (data not shown). Fresh frozen sections fixed in -20°C acetone, -20°C methanol, or 4°C ethanol (70% aqueous) gave similar results to those described above for 4% paraformaldehyde at 4°C (data not shown).

Polarized T84 cells grown on filter supports were also examined for CT receptor and caveolin-1 as described for native human intestine. With 2 nM CTB-FITC overlay, T84 cells showed patchy staining on the apical plasma membrane as well as a coarse granular staining just below the apical membrane (data not shown). The signal intensity was variable from cell to cell, and occasionally the basolateral membrane was faintly highlighted, consistent with our previous studies showing expression of G_{M1} on basolateral membranes (22). When polarized monolayers were incubated with apical CTB-FITC before fixation and sectioning, CTB-FITC was restricted to the apical membrane (Fig. 4*A*). Identical results were obtained when nonfixed T84 monolayers, paraformaldehyde-fixed frozen sections of T84 cells, or formalin-fixed paraffin-embedded sections of T84 cell monolayers were studied (data not shown). CTB-FITC signal could be blocked in the presence of excess nonfluorescent CTB (2 μ M), and no signal was detected in cells or sections treated in parallel with 2 nM CTB instead of CTB-FITC (data not shown).

In contrast to IECs, we were able to detect caveolin-1 by immunocytochemistry in cultured T84 cells. When examined for caveolin-1 using rabbit polyclonal (N20) or mouse monoclonal (clone 2297) anti-caveolin-1 antibodies, T84 cells showed a sparse punctuate staining pattern typically below the level of the tight junction protein ZO-1 (not shown) and often superimposed on the basolateral plasma membrane (Fig. 4, *B* and *C*). Although a faint immunofluorescent signal was occasionally present in the apical portions of the cells, no specific staining of the apical plasma membrane was appreciable. Control studies using nonspecific antibody controls or secondary antibody alone were negative (data not shown).

Heterogeneity of DIG membranes in polarized epithelial T84 cells

In nature, CT must enter polarized cells from the apical (luminal) membrane and, as discussed in the introduction, this likely depends on toxin association with plasma membrane DIGs via binding or clustering G_{M1} . T84 cells and intestinal epithelial cells from human jejunum, however, exhibit few if any structures consistent with caveolae on the apical membrane (data not shown) and relatively small amounts of total cellular caveolin-1 (Figs. 2 and 3). Furthermore, in T84 cells caveolin-1 primarily localizes to the basolateral membranes (Fig. 4). These data suggested to us that the apical membrane DIGs functioning in CT-induced signal transduction may not contain caveolin-1 as a structural component.

To test this idea, we immunoprecipitated intact DIGs using antibodies against CT or caveolin-1. For these studies, CT was bound selectively to the apical membrane DIGs by applying CT to the apical reservoirs of polarized T84 monolayers (45 cm^2) at 4°C for 45 min (as seen in Fig. 4A for CTB-FITC binding under similar conditions). Under these conditions, CT binds only apical membrane receptors (22). After washing to remove unbound CT, freshly isolated DIGs were dispersed by brief sonication and incubated with antibodies against CTB, caveolin-1 (N20), or a matched nonspecific antibody. DIGs coated with antibody were pulled down with protein G Sepharose beads. When analyzed by SDS-PAGE and Western blotting, DIGs immunoisolated with anti-CTB antibodies were enriched in CT but not caveolin-1 (Fig. 5A) and DIGs immunoisolated with anti-caveolin-1 antibodies were enriched in caveolin-1 but not CT (Fig. 5A). No signal for either antigen was detectable in DIGs immunoisolated with matched nonspecific antibodies (Fig. 5A).

To demonstrate that precipitation of DIGs by this method is specific to preincubation with relevant primary antibodies, in selected experiments immediately before the addition of electrophoresis sample buffer, samples of beads were fixed and examined by electron microscopy (Fig. 5B). Vesicles consistent with DIGs were only identifiable on Sepharose beads in the presence of antibodies to CT or caveolin-1 (Fig. 5B). In contrast, no DIGs were identified on beads with antibodies to chicken ovalbumin (Fig. 5B).

To show that DIG membranes and not soluble antigens were precipitated, a set of experiments was performed in which just before the addition of protein A beads, membranes were separated from the soluble phase by centrifugation at $100,000 \text{ g}$ for 30 min at 4°C . Beads were then added to the clarified supernatant. In these experiments, although CT and caveolin-1 could be precipitated from the suspension containing DIGs, no detectable antigen could be precipitated from the soluble phase (Fig. 5C). These data indicate that solubilized antigen represents little (if any) of the total antigen precipitated by the procedure.

Finally, to confirm that this methodology has the sensitivity to detect colocalization between CT and caveolin-1 in DIGs, a cell line in which CT and caveolin-1 are known to colocalize was used. CT has been used by others to localize G_{M1} to caveolae in the epidermoid carcinoma cell line A431 (35). A431 cells were grown to confluency, incubated with CT, and subjected to immunoprecipitation in a fashion identical to that described for T84 cells. Unlike polarized T84 monolayers containing CT bound to the apical membrane, immunoprecipitation of DIGs

in A431 cells using antibodies to CT or caveolin-1 showed colocalization between CT and caveolin-1 (Fig. 5D). These data indicate that this method would have had the sensitivity to detect colocalization between caveolin-1 and apical CT in T84 cells had this association existed to any significant degree. Collectively, these data suggest that DIGs isolated from polarized T84 cells exhibit heterogeneity in structure (and presumably function) and that caveolin-1 is not likely a structural component of apical membrane DIGs that is enriched for CT-G_{M1}.

DISCUSSION

The results of these studies show that native human intestinal epithelial cells contain detergent-insoluble membranes that are enriched in caveolin-1 and distinguish between CT-G_{M1} and LTIIB-G_{D1a} complexes. DIGs isolated from normal human intestinal epithelia, like those isolated from cultured T84 cells (51), exclude LTIIB-G_{D1a} and enrich for CT-G_{M1}. Toxigenic strains of *V. cholerae* or *E. coli* that secrete type I enterotoxins such as CT or LTI cause severe diarrhea in humans (17,47), but human diarrheal disease associated with type II toxins such as LTIIB has rarely been described (44). It is therefore likely that DIGs isolated from native human intestine represent the functional moiety critical to the pathogenesis of cholera and the secretory diarrheas induced by LTI. DIGs that contain the functional CT-G_{M1} complex, however, do not appear to be structurally dependent on caveolin-1. Relatively little caveolin-1 is found in native small intestinal epithelia or cultured T84 cells, and although caveolin-1 is enriched in DIGs, detergent-insoluble membranes isolated from T84 cells that carry the apical membrane receptors for CT are depleted in caveolin-1.

Caveolins have been implicated in a variety of normal and malignant cellular processes (1,7,10,31,45). Although caveolin-1 has been demonstrated in the epithelia of different organs (6,19,30), its presence in intestinal epithelial cell lines has been controversial (5,11,27,28,49). Furthermore, native intestinal epithelial cells have been examined in only one study (11), the results of which may be indefinite due to the potential presence of mesenchymal elements in endoscopically obtained tissue samples. The presence of caveolins in intestinal epithelia is also perhaps somewhat overlooked, because these cells rarely exhibit plasmalemmal caveolae. Although occasional uncoated vesicles may be encountered in the apical or basolateral membranes of native enterocytes, classical Ωshaped caveolae are rarely identifiable [isolated “caveolated” cells are present in mammalian intestinal epithelia (29), but the tubulovesicular structures in caveolated cells are distinctly different from classical caveolae].

The data reported here provide direct evidence that epithelial cells of the normal human small intestine contain detergent-insoluble membranes enriched in caveolin-1. Expression of caveolin-1 in intestinal epithelia, however, is not prominent, consistent with the paucity of caveolae in these cells. Caveolin-1 is a major component of the caveolar coat protein (36,39), and in this role it would not be as abundant in intestinal epithelia as in endothelial cells or fibroblasts that are typically decorated with numerous plasmalemmal caveolae.

Because of the paucity of the antigen, the morphological demonstration of caveolin-1 in normal intestinal epithelia was only possible by signal amplification. By light microscopy in intact tissue sections, we cannot distinguish between caveolin-1 staining at the basal surface of native epithelial cells and staining of the tightly adherent subepithelial myofibroblasts. Our view is that the basal staining apparent in intact tissue most likely represents caveolin-1 expression in the pericrypt myofibroblasts. We could not demonstrate specific apical staining for caveolin-1 with or without signal amplification using a variety of different fixatives.

The idea that caveolin-1 may not represent a structural component of apical membrane DIGs in polarized intestinal epithelial cells was supported by experiments designed to immunoprecipitate isolated DIGs. The results indicate that, in polarized T84 cells, DIGs

enriched for apical membrane CT-G_{M1} complexes do not contain biochemically detectable caveolin-1 and that DIGs enriched for caveolin-1 do not contain detectable amounts of the apical CT-G_{M1} complex. Collectively, these observations lead us to believe that the functionally active CT-G_{M1} complex at the apical membrane of intestinal epithelia is associated with noncaveolar DIGs and that caveolin-1 is not a structural component of these DIGs. However, these data do not exclude the possibility that caveolin-1 may affect or control the biological function of CT-G_{M1} indirectly or downstream of receptor binding. Caveolin-1 has recently been shown to indirectly affect the biological function of DIGs, possibly by controlling the trafficking of cholesterol in and out of these cholesterol-rich membranes (40,41).

Finally, we conclude that in polarized intestinal epithelial cells DIGs represent biochemically and morphologically heterogeneous membranes. Absence of caveolin-1 in the apical membrane seen morphologically and exclusion of caveolin-1 from DIGs containing the apical CT shown biochemically provide evidence in support of the presence of at least two distinct populations of DIGs in T84 cells. Structural and functional heterogeneity in detergent-insoluble membranes has become increasingly apparent in the past several years. Biochemically isolated DIGs typically consist of a mixture of classical caveolae and noncaveolar membranes, and several recent studies have shown that the caveolar fraction is morphologically and biochemically distinct from the noncaveolar fraction (18,43,48,50). Although it is possible that the structural and functional heterogeneity of DIGs extends beyond this simple binary division, direct evidence in support of this hypothesis is not currently available. Thus it is possible that our data may represent a manifestation of the separation between caveolae and noncaveolar DIGs. On the other hand, given the rarity of caveolae in intestinal epithelia, we hypothesize that the two distinct populations of DIGs isolated from T84 cells represent structural (and possibly functional) heterogeneity in noncaveolar DIGs or between DIGs originating from apical and basolateral membranes.

In summary, the results of these studies show that native human intestinal epithelial cells contain detergent-insoluble membranes that distinguish between CT-G_{M1} and LTIIb-G_{D1a} complexes, thus providing a possible mechanism for enterotoxicity associated with CT and LTI but not LTIIb. We find direct evidence for the presence of caveolin-1 in detergent-insoluble membranes from human intestinal epithelial cells but find that these membranes are heterogeneous and that caveolin-1 is not a structural component of apical membranes that contain the CT-G_{M1} complexes.

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REFERENCES

1. Anderson RG. The caveolae membrane system. *Annu Rev Biochem* 1998;67:199–225. [PubMed: 9759488]
2. Balk SP, Burke S, Polischuk JE, Frantz ME, Yang L, Porcelli S, Colgan SP, Blumberg RS. Beta 2-microglobulin-independent MHC class Ib molecule expressed by human intestinal epithelium. *Science* 1994;265:259–262. [PubMed: 7517575]
3. Bastiaens PI, Majoul IV, Verveer PJ, Soling HD, Jovin TM. Imaging the intracellular trafficking and state of the AB5 quaternary structure of cholera toxin. *EMBO J* 1996;15:4246–4253. [PubMed: 8861953]

4. Blumberg RS, Terhorst C, Bleicher P, McDermott FV, Allan CH, Landau SB, Trier JS, Balk SP. Expression of a nonpolymorphic MHC class I-like molecule, CD1D, by human intestinal epithelial cells. *J Immunol* 1991;147:2518–2524. [PubMed: 1717564]
5. Bradbury NA, Clark JA, Watkins SC, Widnell CC, Smith HI, Bridges RJ. Characterization of the internalization pathways for the cystic fibrosis transmembrane conductance regulator. *Am J Physiol Lung Cell Mol Physiol* 1999;276:L659–L668.
6. Breton S, Lisanti MP, Tyszkowski R, McLaughlin M, Brown D. Basolateral distribution of caveolin-1 in the kidney. Absence from H⁺-ATPase-coated endocytic vesicles in intercalated cells. *J Histochem Cytochem* 1998;46:205–214. [PubMed: 9446827]
7. Brown DA, London E. Functions of lipid rafts in biological membranes. *Ann Rev Cell Dev Biol* 1998;14:111–136. [PubMed: 9891780]
8. Connell TD, Holmes RK. Characterization of hybrid toxins produced in *Escherichia coli* by assembly of A and B polypeptides from type I and type II heat-labile enterotoxins. *Infect Immun* 1992;60:1653–1661. [PubMed: 1548088]
9. Engelman JA, Wykoff CC, Yasuhara S, Song KS, Okamoto T, Lisanti MP. Recombinant expression of caveolin-1 in oncogenically transformed cells abrogates anchorage-independent growth. *J Biol Chem* 1997;272:16374–16381. [PubMed: 9195944]
10. Engelman JA, Zhang X, Galbati F, Volonte D, Sotgia F, Pestell RG, Minetti C, Scherer PE, Okamoto T, Lisanti MP. Molecular genetics of the caveolin gene family: implications for human cancers, diabetes, Alzheimer disease, and muscular dystrophy. *Am J Hum Genet* 1998;63:1578–1587. [PubMed: 9837809]
11. Field FJ, Born E, Murthy S, Mathur SN. Caveolin is present in intestinal cells: role in cholesterol trafficking? *J Lipid Res* 1998;39:1938–1950. [PubMed: 9788240]
12. Fra AM, Masserini M, Palestini P, Sonnino S, Simons K. A photo-reactive derivative of ganglioside GM1 specifically cross-links VIP21-caveolin on the cell surface. *FEBS Lett* 1995;375:11–14. [PubMed: 7498456]
13. Fra AM, Williamson E, Simons K, Parton RG. De novo formation of caveolae in lymphocytes by expression of VIP21-caveolin. *Proc Natl Acad Sci USA* 1995;92:8655–8659. [PubMed: 7567992]
14. Fukuta S, Magnani JL, Twiddy EM, Holmes RK, Ginsburg V. Comparison of the carbohydrate-binding specificities of cholera toxin and *Escherichia coli* heat-labile enterotoxins LTh-I, LT-IIa, and LT-IIb. *Infect Immun* 1988;56:1748–1753. [PubMed: 3290106]
15. Holmes, RK.; Jobling, MG.; Connell, TD. Cholera toxin and related enterotoxins of gram-negative bacteria. In: Moss, J.; Iglewski, B.; Vaughan, M.; Tu, AT., editors. *Bacterial Toxins and Virulence Factors in Disease*. Dekker; New York: 1995. p. 225-255.
16. Holmes RK, Twiddy EM. Characterization of monoclonal antibodies that react with unique and cross-reacting determinants of cholera enterotoxin and its subunits. *Infect Immun* 1983;42:914–923. [PubMed: 6196297]
17. Holmes, RK.; Twiddy, EM.; Pickett, CL.; Marcus, H.; Jobling, MG.; Petitjean, FMJ. The *Escherichia coli/Vibrio cholera* family of enterotoxins. In: Pohland, AE.; Dowell, VR.; Richard, JL., editors. *Symposium on Molecular Mode of Action of Selected Microbial Toxins in Foods and Feeds*. Plenum; New York: 1990. p. 91-102.
18. Iwabuchi K, Handa K, Hakomori S. Separation of “glycosphingolipid signaling domain” from caveolin-containing membrane fraction in mouse melanoma B16 cells and its role in cell adhesion coupled with signaling. *J Biol Chem* 1998;273:33766–33773. [PubMed: 9837965]
19. Lee SW, Reimer CL, Oh P, Campbell DB, Schnitzer JE. Tumor cell growth inhibition by caveolin re-expression in human breast cancer cells. *Oncogene* 1998;16:1391–1397. [PubMed: 9525738]
20. Lencer WI, Constable C, Moe S, Jobling MG, Webb HM, Ruston S, Madara JL, Hirst TR, Holmes RK. Targeting of cholera toxin and *Escherichia coli* heat labile toxin in polarized epithelia: role of COOH-terminal KDEL. *J Cell Biol* 1995;131:951–962. [PubMed: 7490296]
21. Lencer WI, de Almeida JB, Moe S, Stow JL, Ausiello DA, Madara JL. Entry of cholera toxin into polarized human intestinal epithelial cells. Identification of an early brefeldin A sensitive event required for A1-peptide generation. *J Clin Invest* 1993;92:2941–2951. [PubMed: 8254049]

22. Lencer WI, Delp C, Neutra MR, Madara JL. Mechanism of cholera toxin action on a polarized human intestinal epithelial cell line: role of vesicular traffic. *J Cell Biol* 1992;117:1197–1209. [PubMed: 1318883]
23. Lencer WI, Hirst TR, Holmes RK. Membrane traffic and the cellular uptake of cholera toxin. *Biochim Biophys Acta* 1999;1450:177–190. [PubMed: 10395933]
24. Lencer WI, Moe S, Rufo PA, Madara JL. Transcytosis of cholera toxin subunits across model human intestinal epithelia. *Proc Natl Acad Sci USA* 1995;92:10094–10098. [PubMed: 7479732]
25. Li S, Galbiati F, Volonte D, Sargiacomo M, Engelman JA, Das K, Scherer PE, Lisanti MP. Mutational analysis of caveolin-induced vesicle formation. Expression of caveolin-1 recruits caveolin-2 to caveolae membranes. *FEBS Lett* 1998;434:127–134. [PubMed: 9738464]
26. Majoul IV, Bastiaens PI, Soling HD. Transport of an external Lys-Asp-Glu-Leu (KDEL) protein from the plasma membrane to the endoplasmic reticulum: studies with cholera toxin in Vero cells. *J Cell Biol* 1996;133:777–789. [PubMed: 8666663]
27. Mayor S, Rothberg KG, Maxfield FR. Sequestration of GPI-anchored proteins in caveolae triggered by cross-linking. *Science* 1994;264:1948–1951. [PubMed: 7516582]
28. Mirre C, Monlauzeur L, Garcia M, Delgrossi MH, Le Bivic A. Detergent-resistant membrane microdomains from Caco-2 cells do not contain caveolin. *Am J Physiol Cell Physiol* 1996;271:C887–C894.
29. Nabeyama A, Leblond CP. “Caveolated cells” characterized by deep surface invaginations and abundant filaments in mouse gastro-intestinal epithelia. *Am J Anat* 1974;140:147–165. [PubMed: 4363601]
30. Newman GR, Campbell L, von Ruhland C, Jasani B, Gumbleton M. Caveolin and its cellular and subcellular immunolocalisation in lung alveolar epithelium: implications for alveolar epithelial type I cell function. *Cell Tissue Res* 1999;295:111–120. [PubMed: 9931357]
31. Okamoto T, Schlegel A, Scherer PE, Lisanti MP. Caveolins, a family of scaffolding proteins for organizing “preassembled signaling complexes” at the plasma membrane. *J Biol Chem* 1998;273:5419–5422. [PubMed: 9488658]
32. Orlandi PA. Protein-disulfide isomerase-mediated reduction of the A subunit of cholera toxin in a human intestinal cell line. *J Biol Chem* 1997;272:4591–4599. [PubMed: 9020187]
33. Orlandi PA, Fishman PH. Filipin-dependent inhibition of cholera toxin: evidence for toxin internalization and activation through caveolae-like domains. *J Cell Biol* 1998;141:905–915. [PubMed: 9585410]
34. Orlandi PA, Fishman PH. Orientation of cholera toxin bound to target cells. *J Biol Chem* 1993;268:17038–17044. [PubMed: 8349592]
35. Parton RG. Ultrastructural localization of gangliosides; GM1 is concentrated in caveolae. *J Histochem Cytochem* 1994;42:155–166. [PubMed: 8288861]
36. Peters KR, Carley WW, Palade GE. Endothelial plasmalemmal vesicles have a characteristic striped bipolar surface structure. *J Cell Biol* 1985;101:2233–2238. [PubMed: 4066756]
37. Pickett CL, Twiddy EM, Coker C, Holmes RK. Cloning, nucleotide sequence, and hybridization studies of the type IIb heat-labile enterotoxin gene of *Escherichia coli*. *J Bacteriol* 1989;171:4945–4952. [PubMed: 2670900]
38. Rietveld A, Simons K. The differential miscibility of lipids as the basis for the formation of functional membrane rafts. *Biochim Biophys Acta* 1998;1376:467–479. [PubMed: 9805010]
39. Rothberg KG, Heuser JE, Donzell WC, Ying YS, Glenney JR, Anderson RG. Caveolin, a protein component of caveolae membrane coats. *Cell* 1992;68:673–682. [PubMed: 1739974]
40. Roy S, Luetterforst R, Harding A, Apolloni A, Etheridge M, Stang E, Rolls B, Hancock JF, Parton RG. Dominant-negative caveolin inhibits H-ras function by disrupting cholesterol-rich plasma membrane domains. *Nat Cell Biol* 1999;1:98–105. [PubMed: 10559881]
41. Scheel J, Srinivasan J, Honnert U, Henske A, Kurzchalia TV. Involvement of caveolin-1 in meiotic cell-cycle progression in *C. elegans*. *Nat Cell Biol* 1999;1:127–129. [PubMed: 10559886]
42. Schlegel A, Volonte D, Engelman JA, Galbiati F, Mehta P, Zhang XL, Scherer PE, Lisanti MP. Crowded little caves: structure and function of caveolae. *Cell Signal* 1998;10:457–463. [PubMed: 9754713]

43. Schnitzer JE, McIntosh DP, Dvorak AM, Liu J, Oh P. Separation of caveolae from associated microdomains of GPI-anchored proteins. *Science* 1995;269:1435–1439. [PubMed: 7660128]
44. Seriwatana J, Echeverria P, Taylor DN, Rasrinaul L, Brown JE, Peiris JS, Clayton CL. Type II heat-labile enterotoxin-producing *Escherichia coli* isolated from animals and humans. *Infect Immun* 1988;56:1158–1161. [PubMed: 2833443]
45. Shaul PW, Anderson RG. Role of plasmalemmal caveolae in signal transduction. *Am J Physiol Lung Cell Mol Physiol* 1998;275:L843–L851.
46. Simons K, Ikonen E. Functional rafts in cell membranes. *Nature* 1997;387:569–572. [PubMed: 9177342]
47. Spangler BD. Structure and function of cholera toxin and the related *Escherichia coli* heat-labile enterotoxin. *Microbiol Rev* 1992;56:622–647. [PubMed: 1480112]
48. Stan RV, Roberts WG, Predescu D, Ihida K, Saucan L, Ghitescu L, Palade GE. Immunoisolation and partial characterization of endothelial plasmalemmal vesicles (caveolae). *Mol Biol Cell* 1997;8:595–605. [PubMed: 9247641]
49. Vogel U, Sandvig K, van Deurs B. Expression of caveolin-1 and polarized formation of invaginated caveolae in Caco-2 and MDCK II cells. *J Cell Sci* 1998;111:825–832. [PubMed: 9472010]
50. Waugh MG, Lawson D, Tan SK, Hsuan JJ. Phosphatidylinositol 4-phosphate synthesis in immunoisolated caveolae-like vesicles and low buoyant density non-caveolar membranes. *J Biol Chem* 1998;273:17115–17121. [PubMed: 9642278]
51. Wolf AA, Jobling MG, Wimer-Mackin S, Ferguson-Maltzman M, Madara JL, Holmes RK, Lencer WI. Ganglioside structure dictates signal transduction by cholera toxin and association with caveolae-like membrane domains in polarized epithelia. *J Cell Biol* 1998;141:917–927. [PubMed: 9585411]

Fig. 1.

Differential fractionation of cholera toxin (CT), *Escherichia coli* heat-labile toxin type IIb (LTIIb), and caveolin-1 in native human small intestinal epithelial cells. Isolated intestinal epithelial cells were incubated with 2 nM CT or LTIIb, and cells were extracted in 1% Triton X-100, homogenized, and separated into soluble fractions and detergent-insoluble membranes by sucrose equilibrium density centrifugation. Approximately 4 μ g of protein from each fraction was analyzed by Western blot. CT and caveolin-1 fractionated predominantly in detergent-insoluble membranes, whereas the relative concentration of LTIIb was essentially the same, indicating that the bulk of this protein-ganglioside complex resides in the soluble fractions that contain 99% of total cellular protein.

Fig. 2.

Relative density of caveolin-1 in human endothelial and epithelial cells. *A*: comparison of human endothelial cells (HECs) with T84 cells and isolated human intestinal epithelial cells (IECs) indicates that intestinal epithelia contain significantly less caveolin-1 (whole cells). When normalized with respect to the detergent-insoluble glycosphingolipid-rich membrane (DIG) protein content, the relative concentration of caveolin-1 in intestinal epithelial DIGs is significantly less than that in endothelial cell DIGs (1 μ g of protein loaded in each lane). *B*: immunocytochemical staining for caveolin-1 in isolated human intestinal epithelial cells. Caveolin-1 can be detected in the columnar epithelial cells after signal amplification. *C*: negative control shows specificity of staining for caveolin-1.

Fig. 3.

Localization of CTB-G_{M1} and caveolin-1 in human small intestine. Ligand overlay with B subunit of CT (CTB)-FITC in 5- μ m sections of normal human small intestine shows distinct villous and crypt staining patterns for G_{M1} (A and B, respectively). In the villous epithelium, CTB-FITC was uniformly restricted to the apical plasma membrane (A, arrowheads). Nonspecific staining was only seen in the goblet cells' mucin (A, asterisks). In the crypt epithelium, CTB-FITC was primarily apical but more widespread, with irregular staining of the apical membrane and coarse granular staining of the apical cytoplasm as shown in a representative cross-section (B). Identical sections of small intestine immunostained for caveolin-1 show strong staining of endothelial cells in the lamina propria (C and D, asterisks). Staining was also noted at the interface between the epithelial cells and the lamina propria in both crypt and villous epithelium (C and D, arrowheads). This pattern of staining was not distinguishable from subepithelial myofibroblasts by light microscopy. Faint diffuse epithelial cell staining was only detectable with signal amplification (not shown). Intense staining of the microvascular endothelium (asterisks) saturated the image. Bars = $\sim 10 \mu$ m.

Fig. 4.

Localization of CTB-G_{M1} and caveolin-1 in T84 cells. *A*: apical membrane of polarized T84 monolayers was incubated with 2 nM CTB-FITC at 4°C before fixation and sectioning. Under these conditions, CTB was restricted to the apical surface (arrowheads). *B*: immunofluorescent staining for caveolin-1 in 5-μm frozen sections of polarized T84 monolayers shows a sparse punctate staining pattern (arrows), which in occasional cells highlighted the lateral membranes. No specific staining of the apical membrane was apparent. *C*: immunofluorescent staining of intact T84 monolayers for caveolin-1 reveals a vague "chicken-wire" pattern also suggestive of lateral membrane staining. Bars = ~10 μm.

Fig. 5.

Immunoprecipitation of DIGs from T84 cells. T84 cells were incubated apically with 2–4 nM CT at 4°C, and DIGs were isolated as described. *A*: freshly isolated DIGs were immunoprecipitated using antibodies against CTB (*left*), caveolin-1 (*right*), or chicken ovalbumin (*middle*) and analyzed by Western blotting for CT and caveolin-1. Caveolin-1 was only detectable in samples immunoprecipitated using anti-caveolin-1 antibodies, and CT was only detectable in samples immunoprecipitated using anti-CTB antibodies. Neither CT nor caveolin-1 was detected in samples immunoprecipitated using anti-chicken ovalbumin antiserum. *B*: electron microscopy of immunoprecipitated DIGs shows a string of vesicles (some indicated by arrowheads) at the periphery of the agarose bead from samples immunoprecipitated using anti-CTB (*top*) or anti-caveolin-1 (*bottom*) antibodies. In contrast, no vesicles were present on agarose beads isolated from samples containing anti-ovalbumin antibody (*middle*). Bars = ~200 nm. *C*: there was no “leakage” of ligand from DIGs during the process, because CT could only be immunoprecipitated from the suspension of DIGs and not from aqueous phase of the suspension alone. *D*: in contrast to T84 cells, DIGs isolated from A431 cells show coprecipitation of membrane-bound CT with antibodies against caveolin-1 (*right*) and coprecipitation of caveolin-1 with antibodies against CT (*left*).