Caveolin is present in intestinal cells: role in cholesterol trafficking?

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Abstract **It was postulated that specialized microdomains of the plasma membrane, consistent with caveolae, might play a role in cholesterol trafficking in intestinal cells. The existence, therefore, of caveolin and the role of detergent-resistant microdomains of the plasma membrane in cholesterol trafficking were investigated in human small intestine and CaCo-2 cells. Caveolin mRNA was detected by RT-PCR in small intestinal brushings and biopsies and in CaCo-2 cells. Northern hybridization of caveolin mRNA detected 3 kb and 0.8 kb transcripts in CaCo-2 cells. From brushings of distal duodenum and in CaCo-2 cells, Western analysis for detection of caveolin protein demonstrated a 21 kDa-sized protein and a 600 kDa homooligomer. In CaCo-2 cells, caveolin was demonstrated by immunoflourescence in apical membranes as well as within cells. Using sucrose-density gradients, caveolin was localized to detergent-resistant microdomains of the plasma membrane. As determined by cholesterol oxidase-accessible cholesterol, 3–5% of plasma membrane cholesterol in CaCo-2 cells was estimated to be in these detergent-resistant microdomains. After the absorption of cholesterol from bile-salt micelles, more plasma membrane cholesterol moved to these specialized microdomains within the plasma membrane and was esterified. In CaCo-2 cells, filipin, N-ethyl maleimide, and cholesterol depletion, treatments that disrupt caveolar function, interfered with the transport of plasma membrane cholesterol to the endoplasmic reticulum, whereas okadaic acid, sphingomyelinase, and cholesterol oxidase did not. Changes in cholesterol flux at the apical membrane of the cell did not alter mRNA levels or mass of caveolin. The results suggest that caveolin is present in intestinal and CaCo-2 cells and is associated with detergent-resistant microdomains of cellular membranes. With the influx of micellar cholesterol from the lumen, plasma membrane cholesterol moves or "clusters" to these microdomains and is transported to the endoplasmic reticulum for esterification and eventual transport. Caveolin/caveolae may play a role in cholesterol trafficking in intestinal cells.**—Field, F. J., E. Born, S. Murthy, and S. N. Mathur. **Caveolin is present in intestinal cells: role in cholesterol trafficking? *J. Lipid Res.* 1998.** 39: **1938–1950.**

Supplementary key words CaCo-2 cells • caveolae • intestine • cholesterol transport

With the influx of fatty acids, the small intestinal absorptive cell must recruit cholesterol for the normal assembly and secretion of triacylglycerol-rich lipoprotein particles. The absorptive cell is unique from other cells in that it can utilize dietary or biliary cholesterol absorbed from the apical membrane to meet these increased cholesterol requirements. This cholesterol, derived from the lumen, is in addition to what is available from newly synthesized cholesterol or cholesterol delivered to the cell within a lipoprotein particle. Although the intestinal cell has lipoprotein receptors, it is unlikely that this source of cholesterol is utilized for the assembly of a chylomicron particle following the ingestion of a fatty meal (1). Similarly, we have shown in CaCo-2 cells, that the contribution of newly synthesized cholesterol to the cholesterol secreted in triacylglycerol-rich lipoprotein particles is minimal (2). We did find, however, that interfering with the normal trafficking of cholesterol from the plasma membrane to the endoplasmic reticulum resulted in a decrease in the transport of lipids and the secretion of apolipoprotein B (3). When the origins of triacylglycerol-rich lipoprotein cholesterol were investigated, it was observed that cholesterol derived from the plasma membrane was the predominant source of cholesterol utilized for assembly and secretion of lipoprotein particles. During the influx of oleic acid and/or cholesterol, the amount of cholesterol transported from the plasma membrane to the endoplasmic reticulum was increased and furthermore, this cholesterol was found as part of the secreted lipoprotein particle (2). To maintain cholesterol homeostasis during the influx of fatty acid, more newly synthesized cholesterol was transported to the plasma membrane to replenish the cholesterol that was being utilized for lipoprotein assembly.

While performing these studies, we found that the absorption of cholesterol from a micellar solution at the apical membrane caused plasma membrane cholesterol to move to the endoplasmic reticulum where it was esterified

Abbreviations: RT-PCR, reverse transcription-polymerase chain reaction; ACAT, acyl-CoA:cholesterol acyltransferase; PBS, phosphate-buffered saline; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; LDL, low density lipoprotein; GPI, glycosyl-phosphatidylinositol; NEM, N-ethyl-maleimide.

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by acyl-CoA:cholesterol acyltransferase (ACAT) (2). This suggested to us that when excess cholesterol transiently accumulates in the apical membrane of the absorptive cell, structural cholesterol of the plasma membrane is displaced to a particular microdomain within the membrane. It is this cholesterol within this domain, which is destined for transport to the endoplasmic reticulum. We postulated, therefore, that there are specialized areas within the plasma membrane of an intestinal cell which functioned to "cluster" excess cholesterol molecules, directing them, likely by vesicles derived from the plasma membrane, to the endoplasmic reticulum (2).

The apical membrane of an intestinal cell is enriched in sphingomyelin, glycosphingolipids, and cholesterol (4). Domains of the plasma membrane that are enriched in these particular lipids are resistant to solubilization by nonionic detergents (5). Several studies have suggested that these microdomains that are resistant to detergents represent caveolae, flask-shaped invaginations of the plasma membrane which are proposed to be involved in potocytosis, signal transduction, and recently, cholesterol efflux (6–12). Caveolin, a 21 kDa cholesterol-binding protein whose function is unknown, is found in these detergent-insoluble domains of the plasma membrane (13, 14). Caveolin has been shown to traffick from these microdomains within the plasma membrane to the endoplasmic reticulum and Golgi apparatus (15, 16). We postulated, therefore, that in intestinal cells these microdomains of the plasma membrane may be involved in cholesterol trafficking from the plasma membrane to the endoplasmic reticulum and that perhaps caveolin, as a cholesterol-binding protein, may play a role in directing plasma membrane cholesterol to ACAT. Although other types of epithelial cells have been shown to contain caveolin, there remains debate as to whether intestinal cells even contain this protein (17–21). The present study was performed, therefore, to determine whether caveolin is present in intestinal cells and whether detergent-resistant microdomains have a role in cholesterol influx from the plasma membrane to the endoplasmic reticulum.

MATERIALS AND METHODS

[1,2-^{3}H(N)]cholesterol was purchased from New England Nuclear (Boston, MA). Cholesterol, cholesterol oxidase, cholestenone, Trireagent, filipin, N-ethyl maleimide, sphingomyelinase, and sodium taurocholate were from Sigma Chemical Co. (St. Louis, MO). Anti-human caveolin polyclonal antibody (250 μg/mL) raised in rabbit was purchased from Transduction Laboratories (Lexington, KY). Another polyclonal antibody raised in rabbit directed against the N-terminus of human caveolin (sc-894, 200 μg/mL) was purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Anti-human e-cadherin monoclonal antibody (114 μg/mL) was a gift from Dr. Warren Bishop, University of Iowa, Iowa City, IA. Anti-rabbit (4 mg/mL) and anti-mouse IgG (1.1 mg/ml) conjugated to fluorescein isothiocyanate (FITC) were purchased from Sigma Chemical Co. (St. Louis, MO). Anti-rabbit IgG cross-linked to horseradish peroxidase was from Amersham (Arlington Heights, IL). A pcDNA3 plasmid containing an 838 bp sequence of human caveolin was a generous gift from Drs. Chris and Phoebe Fielding, University of California, San Francisco, CA.

Cell culture and human intestinal biopsies/brushings

CaCo-2 cells were grown in T-75 flasks as described previously (22). They were subcultured on either 6-well plastic clusters or semipermeable micropore membranes (0.4 μm pore size) inserted in Transwells (Costar, Cambridge, MA). Cells were used 14 days after plating and medium was changed every 2 days. Biopsies and brushings of the distal duodenum were procured from individuals undergoing diagnostic upper endoscopy. The biopsies and cells obtained by brushings were kept on ice in normal saline. RNA was extracted from the samples on the same day. The biopsies were histologically normal.

Reverse transcription-polymerase chain reaction (RT-PCR)

Total RNA from CaCo-2 cells was prepared using Trireagent. Reverse-transcribed cDNA was synthesized by reverse transcription of 0.5 μg total RNA with MMLV reverse transcriptase and random hexamers by standard procedure. PCR primers Hcaveolin-142 PCR: 5′-GAGCGAGAAGCAAGTGTACGA-3′ and Hcaveolin-502: 5′-ACAGACGGTGTGGACGTAGAT-3′ were designed from human caveolin sequence, accession #Z18951 S49856 (23). PCR amplification of cDNA for human caveolin was performed for 30 cycles by denaturation at 94°C for 30 sec, annealing at 55°C for 45 sec, extension at 72°C for 120 sec, and final extension at 72°C for 10 min. The PCR product of 361 bp was analyzed on an 8% polyacrylamide gel and visualized by silver staining (24). Briefly, the gel was incubated for 3 min with fixing solution containing 10% ethanol + 0.5% acetic acid. It was stained for 5 min with 0.2% silver nitrate dissolved in the fixing solution. After washing once for 20 sec and once for 2 min with water, the gel was exposed for 5 min to developing solution containing 3% sodium hydroxide and 0.3% formaldehyde. After visualization of the DNA bands the gel was rinsed with water and stored in fixing solution.

Size determination of caveolin mRNA by Northern blotting

Total RNA (25 μg) was resolved on 1% agarose, and transferred to a positively charged NYTRAN PLUS membrane (Schleicher and Schuell, Keene, NH). The RNA was immobilized by UV irradiation. Human caveolin-pcDNA3 plasmid was used as a template to synthesize a ^{32}P-labeled probe using oligolabeling kit from Pharmacia LKB, Uppsala, Sweden. The prehybridization and hybridization solution contained 0.25 m Na_2HPO_4, pH 7.2, 1 mm EDTA, 10% sodium dodecyl sulfate, and 0.5% blocking reagent from Boehringer Mannheim, Indianapolis, IN. After prehybridization for 1 h at 68°C, the blot was incubated for 18 h at 68°C in hybridization solution containing 7 million counts in the probe. This was followed by 3 washings of 20 min each at 58°C. To increase the specific activity of the probe, the unlabeled caveolin-pcDNA3 plasmid was separated from the labeled probe on a 1% agarose gel (25). The blot was exposed to a Kodak BIOMAX film and a BIOMAX intensifying screen. The bands on the X-ray film were quantitated by scanning on a UMAX ASTRA 1200S scanner and analysis by SIGMA GEL software (Jandel Scientific, San Rafael, CA).

Western blot

CaCo-2 cell homogenates were used directly with or without boiling as indicated in the figure legend. In experiments in which membranes were first isolated by sucrose density gradients, proteins were concentrated by precipitation with 10% trichloroacetic acid (TCA) followed by a wash with acetone to re-

move TCA. The proteins in the pellet obtained by centrifugation at 13000 *g* for 10 min was dissolved in 1.25 × Laemmli buffer. These samples were not boiled as in these preparations the caveolin protein was present predominantly as a 21 kDa band. The proteins were resolved on an 8% porous gel (26) and transferred for 18 h at 10 volts to a PVDF membrane (Immobilon-P, Millipore, Bedford, MA). The membranes were blocked for 30 min at 37°C with 5% milk in TBST buffer (10 mm Tris, 0.15 m NaCl, pH 7.6, 0.1% Triton X-100). An anti-human caveolin polyclonal antibody raised in rabbit was used as primary antibody followed by a secondary anti-rabbit IgG antibody raised in donkey and linked to horseradish peroxidase (Amersham, Arlington Heights, IL). Each antibody was diluted 4000 times in TBST and 0.1% milk and incubated with the blot for 1 h at room temperature on a shaker. After incubation with each antibody, the blots were washed 3 times for 10 min each with TBST. After the final wash with TBS, the horseradish peroxidase signal was detected with ECL+plus chemiluminescent reagent (Amersham, Arlington Heights, IL). The bands on the X-ray film were quantitated by scanning on a UMAX ASTRA 1200S scanner and analysis by SIGMA GEL software (Jandel Scientific, San Rafael, CA).

Immunofluorescence microscopy

CaCo-2 cells cultured on semipermeable filters were rinsed in ice-cold phosphate-buffered saline (PBS), pH 7.4, and fixed for 30 min at 4°C with 4% paraformaldehyde diluted with PBS. After quenching the paraformaldehyde with 25 mm glycine, some cells were permeabilized with 0.1% Triton X-100 for 5 min at room temperature. Cells were then blocked for 30 min with PBS containing 5% bovine serum albumin and incubated for 1 h at room temperature in PBS containing 1% bovine serum albumin and a 100-fold dilution of either anti-cadherin monoclonal antibody or anti-caveolin antibody. This was followed by three washes with PBS. The cells were then incubated at room temperature for 1 h with the appropriate fluoroscein isothiocyanate-conjugated secondary antibody diluted 50-fold in PBS containing 1% bovine serum albumin as stated in the figure legend. Finally, the cells were again washed with PBS and filters were mounted on glass slides in 90% glycerol. Cells were observed and photographed using a confocal laser scanning microscope (Bio-Rad MRC 1024). Cells incubated with no primary antibody but with the appropriate secondary antibody were used as negative controls.

Oxidation of plasma membrane cholesterol

Cholesterol oxidase-accessible cholesterol on the surface of live cells was estimated by treating cells with the agents described in the text, followed by incubation with cholesterol oxidase (10 IU/ml) for 1 h at 37°C in the absence of the agent. The lipids were extracted with hexanes–isopropanol–water, 3:2:0.1 (v/v). Unlabeled cholestenone was added as a carrier. Cholesterol, cholestenone, and cholesteryl esters were separated by thin-layer chromatography. The plates were eluted twice in heptane–diethyl ether–acetic acid, 85:15:1 (v/v). Bands corresponding to cholesterol, cholestenone, and cholesteryl esters were scraped from the plate and counted. Using this procedure, recoveries for radiolabeled cholestenone were between 92 and 95%. The amount of cholesterol on the cell surface was calculated by dividing the radioactivity in the cholestenone fraction by the total cholesterol.

The cholesterol oxidase methodology used to estimate the amount of cholesterol found on the cell surface of CaCo-2 cells after fixation has been previously described (2). Briefly, after the incubation as described for each experiment, the cells were washed twice with ice-cold phosphate-buffered saline. After washing two more times with 10 mm sodium phosphate buffer (pH 7.4), the cells were incubated for 10 min at 4°C with water. The water was removed and the cells were fixed by incubating them for 10 min at 4°C with 1% glutaraldehyde. After removing the glutaraldehyde, the cells were washed twice with 10 mm sodium phosphate buffer (pH 7.4) containing 310 mm sucrose that had been warmed to 37°C. They were then incubated in this buffer for 15 min at 37°C. Cholesterol oxidase, 10 IU/ml, was added and the cells were incubated for 45 min at 37°C. The cells were again washed twice with cold phosphate-buffered saline. The lipids from these cells were extracted and analyzed as described above.

Subcellular fractionation of CaCo-2 cells

CaCo-2 cells grown on plastic clusters were incubated for 18 h at 37°C with 1 μCi/ml of [1,2-^{3}H(N)]cholesterol in DMEM containing 10% FCS. Labeled cells from 12 wells were combined for the sucrose gradient. The cells were suspended in 2 ml of hypotonic buffer, pH 7.4, containing 1 mm Tris, 75 mm sucrose, 0.5 mm dithiothreitol, 42 μm leupeptin, and 0.5 mg/ml soybean trypsin inhibitor. The cell suspensions were homogenized in a Potter-Elvehjem homogenizer with 50 downward strokes. Total membranes were sedimented by ultracentrifugation at 100,000 *g* for 1 h. The total membrane pellet was resuspended in 2.2 ml of 60% sucrose/1 mm Tris-HCl solution and layered in a 13.2-ml tube on a cushion of 1.2 ml 66% sucrose solution followed by 1.2 ml of 55–20% sucrose solutions at a 5% interval. The gradients were centrifuged for 18 h in a Beckman SW 41 Ti rotor at 118,610 *g*. The fractions were collected from the top in 0.5-ml aliquots, kept on ice, and diluted with cold 1 mm Tris-HCl to a total volume of 1.5 ml. Aliquots were taken for determining protein, cholesterol mass, RNA, labeled cholesterol, and alkaline phosphatase activity. Alkaline phosphatase activity was estimated by measuring absorbance of p-nitrophenol at 405 nm generated by the hydrolysis of 6 mm disodium *p*-nitrophenyl phosphate in a buffer of pH 10.4 containing 0.1 m glycine, 1 mm magnesium chloride, and 1 mm zinc chloride. Protein was estimated using Eosin B dye to reduce interference by sucrose as described by Waheed and Gupta (27). For RNA estimation, 1 ml of ice-cold 6% perchloric acid was added to 500 μL of the diluted fraction and kept on ice for at least 3 h. The precipitate was collected by centrifugation at 16,000 *g* for 7 min and washed once with 3% perchloric acid. The sediment was dissolved in 200 μl 1 N NaOH by incubation at 37°C for 1 h. The protein and DNA were precipitated by adding 800 μl ice-cold 3% perchloric acid and centrifugation at 16,000 *g* for 10 min. The RNA in the supernatant was estimated at 260 nm (28). Cholesterol mass was determined using a total cholesterol kit (Catalog #352-100) from Sigma, St. Louis, MO. Golgi marker galactosyltransferase was assayed as described by Beaufay et al. (29).

Triton X-100-insoluble complexes in CaCo-2 cells

CaCo-2 cells were incubated for 18 h with 1 μCi/ml of [1,2-^{3}H(N)]cholesterol in DMEM containing 10% FCS with or without treatments. The cell monolayers were washed 4 times with M199. To each well, 0.5 ml 25 mm MES (2-[N-morpholino] ethanesulfonic acid), 0.15 m NaCl, pH 6.5 (MES buffer) containing 1 mm PMSF and 20 μm leupeptin was added and cells were frozen at −80°C for 1 h (20, 30, 31). The cells were thawed on ice and homogenized by passing 5 times through a 25-gauge needle. The homogenate was centrifuged at 800 *g* to obtain cell "ghosts." The cell "ghost" pellet or freshly prepared cell homogenates were homogenized in 1% Triton X-100 in MES buffer by passing 40 times through a 25-gauge needle. This preparation was made to 40% in sucrose by addition of 66% sucrose in MES buffer. All sucrose solutions were made in MES buffer. This mixture (3 ml) was overlaid in a 13.2-ml tube on a 0.9-ml cushion of 66% sucrose. A sucrose gradient was formed by addition of 0.9 ml of solutions containing 36–4% solutions at 4% intervals. The gradients were centrifuged for 18 h in a Beckman SW 41 Ti rotor at

118,610 *g*. The fractions were collected and analyzed for protein, cholesterol mass, labeled cholesterol, and alkaline phosphatase activity as described above for subcellular fractionation of CaCo-2 cells.

Esterification of plasma membrane cholesterol

Plasma membrane cholesterol was labeled by incubating cells for 90 min at 4°C with 3 μCi [1,2-^{3}H(N)]cholesterol in 1 ml M199 (medium-199/Earle's, Gibco, Grand Island, NY) containing 1% delipidated fetal calf serum. To remove unincorporated labeled cholesterol, cells were washed twice with cold M199. They were then incubated with 1 ml M199 containing 5 mm sodium taurocholate and 50 μm oleate with or without the treatment at 37°C for 5 h. Control cells were handled in the same fashion except that the medium contained only the vehicle for the agent. The lipids were extracted with hexanes–isopropanol–water, 3:2:0.1 (v/v). The lipids were separated by thin-layer chromatography and cholesterol and cholesteryl ester bands were localized by authentic standards, scraped from the plate, and counted.

Statistical analysis

The data were analyzed by Dunnett's method to compare treatment groups with the control group at alpha value of 0.05 using SIGMASTAT software from Jandel Scientific (San Rafael, CA).

RESULTS

Caveolin expression in CaCo-2 cells

Caveolin mRNA. There is conflicting information regarding the existence of caveolin in CaCo-2 cells (19–21). Anticipating difficulty in detecting a suspected low-abundance message, caveolin mRNA was first amplified by RT-PCR. The results are shown in **Fig. 1A**. In CaCo-2 cells, the expected PCR product from caveolin mRNA was detected (lane 1). To verify that this amplified product represented caveolin mRNA, the product was sequenced and found to match that of human caveolin mRNA. To address whether caveolin mRNA was present in human intestine, RNA extracted from small intestinal biopsies and brushings were also amplified by RT-PCR (lanes 2 and 3, respectively). Again, the expected amplified product was present in human small intestine as well.

The results with RT-PCR strongly suggested that intestinal cells contain mRNA for caveolin. Using a human caveolin cDNA probe, we next performed Northern blot hybridization of RNA extracted from CaCo-2 cells (Fig. 1B). The major transcript detected was approximately 3 kb in size. A minor transcript of approximately 0.8 kb was also detected. The two observed transcripts of caveolin mRNA are in agreement with the results of others in other cell types (23). It can be concluded, therefore, that CaCo-2 cells and human small intestine express the gene for caveolin.

Caveolin protein. It is recognized that caveolin, a 21 kDa protein, can exist as 200-, 400-, and 600-kDa homooligomers within cells (32–34). To address whether caveolin protein was expressed in human small intestine, enterocytes were collected by brushings of the distal duodenum. After separation of the proteins by SDS-PAGE and transfer to a membrane, the membrane was probed with an anti-caveolin polyclonal antibody. As a control, proteins isolated from endothelial cells were probed on the same membrane. The results are shown in **Fig. 2**. In human small intestinal cells, the antibody detected a 21 kDa-sized protein as well as a 600 kDa protein suggesting that within intestinal cells caveolin may exist as an homooligomer (lane 2). Similarly, in CaCo-2 cells, caveolin protein was detected predominately as an homooligomer as well as a 21 kDa-sized protein (lanes 3, 4, and 5). If samples from CaCo-2 cells were boiled prior to loading on the gel, all the protein detected by the antibody existed as the smaller molecular weight 21 kDa protein (lanes 6, 7, and 8).

Immunofluorescence. As further proof for the existence of caveolin in CaCo-2 cells, caveolin was detected by immunofluorescent microscopy (**Fig. 3**). E-cadherin, an epithelial cell adhesion molecule that is excluded from apical membranes of intestinal and CaCo-2 cells (35, 36), was used to verify the technique and to compare its location to that of caveolin within the cell. Panels A and B show the immunofluorescence pattern for e-cadherin in cross-sec-

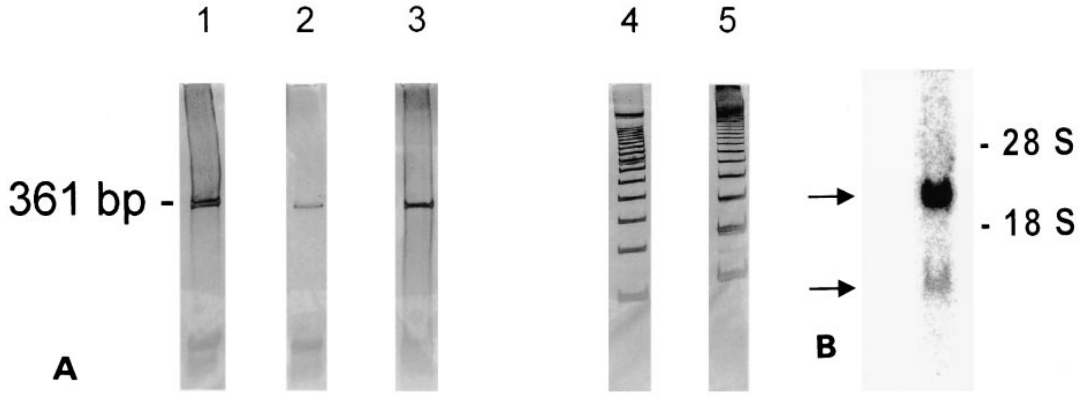

Fig. 1. Caveolin mRNA in CaCo-2 cells and human intestine. A: RNA isolated from CaCo-2 cells or human intestine was analyzed for caveolin mRNA by RT-PCR as described in Methods. Lane 1: CaCo-2 cells; lane 2: human intestinal cells collected by biopsy; lane 3: human intestinal cells collected by brushing; lanes 4 and 5: 100 and 123 bp DNA ladder, respectively. B: RNA from CaCo-2 cells was isolated and Northern blot for caveolin mRNA was performed as described in Methods. The bands corresponding to caveolin mRNA are indicated by the arrows. The positions of 28 S and 18 S RNA relative to the caveolin transcripts are also shown.

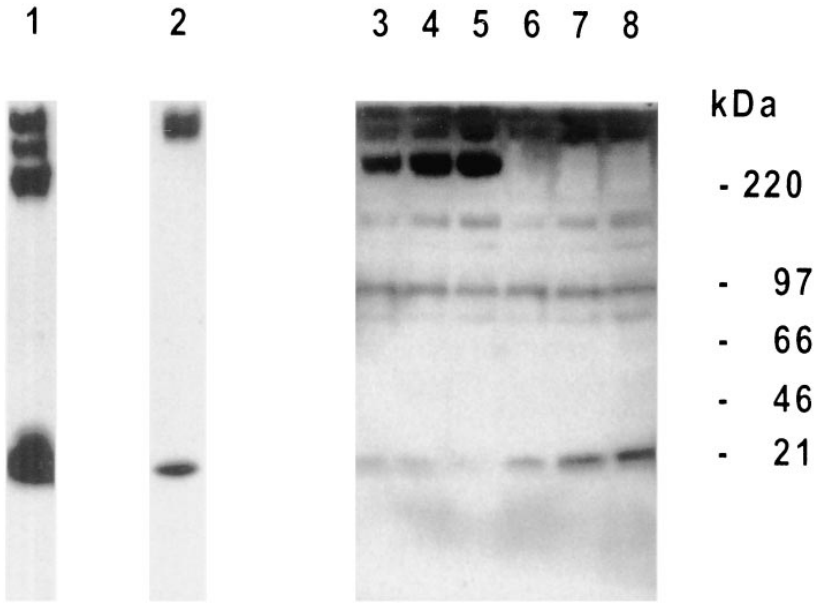

Fig. 2. Caveolin protein in human intestine and CaCo-2 cells. Caveolin protein in human intestine and CaCo-2 cells was analyzed by Western blot as described in Methods. The samples were dissolved in 30 μl Laemmli buffer and treated as follows before SDS-PAGE. Lane 1: human endothelial cells, room temperature; lane 2: human intestinal cells collected by brushing, heated at 100°C, 5 min; lanes 3–5: CaCo-2 cell homogenates containing 5, 10, or 30 μg protein, room temperature; lanes 6–8: CaCo-2 cell homogenates containing 5, 10, or 30 μg protein, heated at 100°C, 5 min.

tion and vertical section, respectively, of CaCo-2 cells that had been permeabilized. The fluorescence is localized to the lateral and basolateral membranes of the cells. Apical membranes are essentially free of fluorescence. In contrast, the fluorescent pattern for caveolin in permeabilized cells (panels D and E) stain not only the apical surface but also more diffusely within the cell. In cells that have not been permeabilized, the fluorescence pattern for caveolin is mostly confined to the apical surfaces (panels G and H). In panel E, caveolin was detected by the polyclonal antibody (sc-894) used by Vogel, Sandvig, and Vandeurs (21). Similar results were obtained with the other primary polyclonal antibody. Controls for each experiment, shown in panels C, F, and I, demonstrate no appreciable fluorescence.

Subcellular localization. To further investigate the location of caveolin within CaCo-2 cells, sucrose-density gradients were used to separate intracellular organelles. Prior to subcellular fractionation, the cells were equilibrated with labeled cholesterol. The fractions recovered from the sucrose gradient were analyzed for alkaline phosphatase activity, labeled cholesterol, and cholesterol mass to identify plasma membrane fractions, and RNA and galactosyltransferase activity to identify endoplasmic reticulum and Golgi membranes, respectively. To determine the location of caveolin, immunoblot analyses were performed on total protein combined from two fractions after SDS-PAGE and transfer to membranes. The results are shown in **Fig. 4**. Each point represents a single 0.5-ml fraction taken from the sucrose gradient (except for caveolin, in which two fractions were combined). Markers for plasma membrane and endoplasmic reticulum were recovered in fractions 1–12 (12–42% sucrose) and 15–23 (48–64% sucrose), respectively. Based on results from a separate experiment, Golgi membranes were recovered in fractions 10–16 (38–50% sucrose). Caveolin was found only in fractions that were enriched in markers for the plasma membrane. This experiment was also performed in cells grown on semipermeable supports and the results were similar (data not shown).

Detergent-insoluble complexes in CaCo-2 cells

Detergent-insoluble complexes are defined as cellular membranes that, due to their high content of glycosphingolipids, resist solubilization at low temperatures by nonionic detergents (5, 37). Although detergent-insoluble microdomains are known to exist in cells without caveolin or caveolae, in most cell types studied, caveolin when present is recovered in these detergent-insoluble complexes (14, 33, 38–42). To examine whether caveolin is confined to these microdomains in CaCo-2 cells and to determine whether these microdomains could be separated from the remaining bulk of the plasma membrane, cells were first equilibrated with labeled cholesterol. The cells were then homogenized in Triton X-100 and loaded on a sucrose-density gradient. Detergent-insoluble complexes, because of their low buoyant density, can be separated from the soluble cellular proteins (5). Fractions collected from the gradient (represented by a single point) were analyzed for the glycosyl-phosphatidylinositol (GPI)-linked enzyme, alkaline phosphatase, cholesterol mass, labeled cholesterol, and protein. The results are shown in **Fig. 5**. Fractions 5–9 (9–18% sucrose) contained the Triton-insoluble complexes as evidenced by the enrichment of the above markers in these fractions. Caveolin was found only in these fractions suggesting that caveolin in CaCo-2 cells is confined to these domains. Although we could clearly demonstrate an enrichment of caveolin in membranes resistant to Triton X-100, it was clear that in CaCo-2 cells, the majority of total cellular cholesterol and alkaline phosphatase activity, 65% and 68%, respectively, were recovered in detergent-insoluble complexes. Thus, with this technique, it was not possible to isolate these detergent-insoluble complexes containing caveolin from the remainder of the bulk lip-

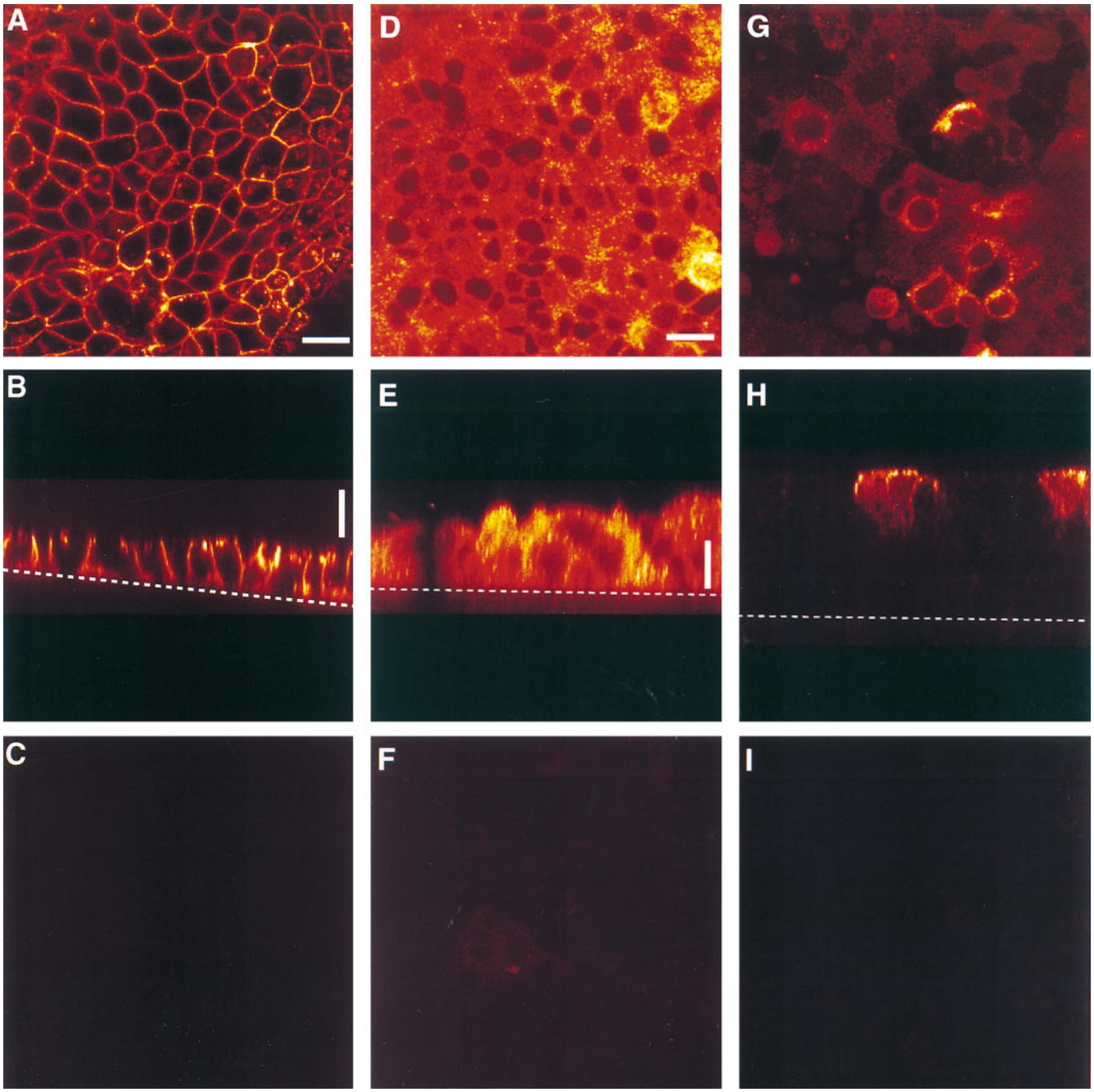

Fig. 3. Immunofluorescence microscopy. CaCo-2 cells cultured on semipermeable supports were fixed and treated as described in Methods. A: e-cadherin in cross-section permeabilized cells; B: e-cadherin in vertical section permeabilized cells; C: negative control for cadherin; D: caveolin in cross-section permeabilized cells; E: caveolin (detected with sc-894 antibody) in vertical section permeabilized cells; F: negative control for caveolin in permeablized cells; G: caveolin in cross-section nonpermeabilized cells; H: caveolin in vertical section nonpermeabilized cells; I: negative control for caveolin in nonpermeabilized cells. Caveolin was detected with an anti-rabbit polyclonal antibody purchased from Transduction Labs (Lexington, KY) except in panel E. Bar, 20 microns.

ids of the plasma membrane. In contrast, the amount of protein found in these microdomains represented only 4% of the total cell membrane protein.

Cholesterol influx from detergent-resistant microdomains

We had speculated that in intestinal cells, cholesterol transported from the plasma membrane to the endoplasmic reticulum likely originated from "specialized" domains within the plasma membrane. Because caveolin is a cholesterol-binding protein, we postulated a role for this protein in cholesterol trafficking from the plasma membrane to the endoplasmic reticulum. In studies with other cell types, the membrane-impermeable enzyme, cholesterol oxidase, has been used to selectively oxidize cholesterol contained within detergent-resistant domains of the plasma membrane (11, 15). When CaCo-2 cells were equilibrated

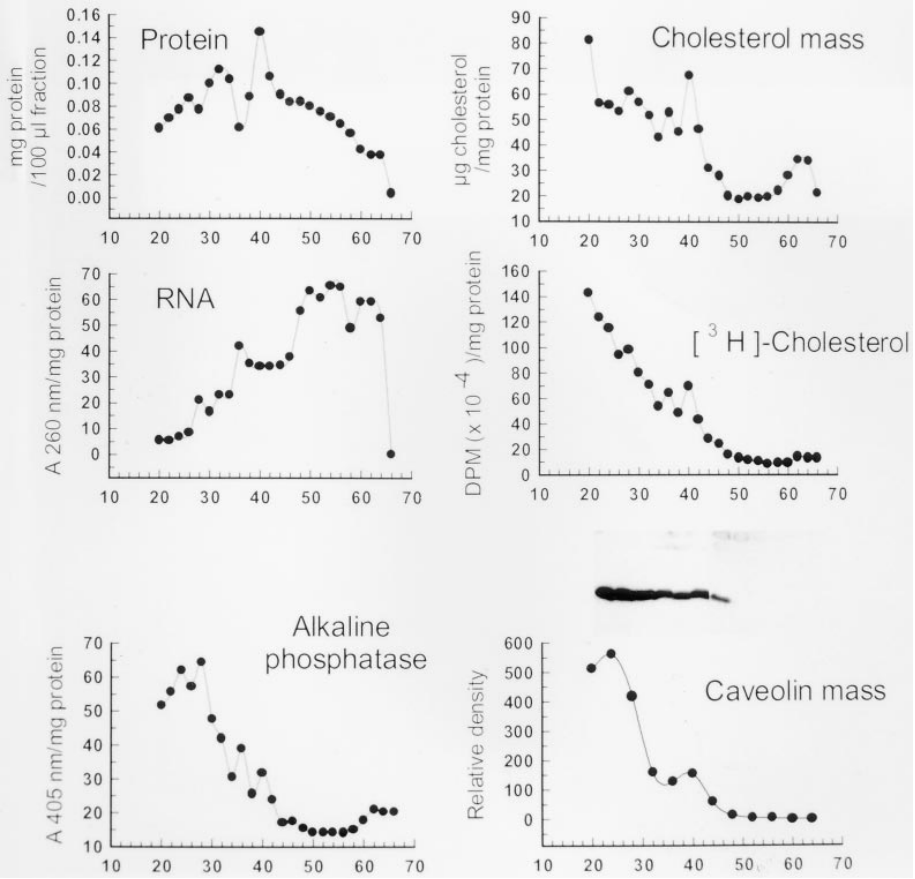

Fig. 4. Subcellular localization of caveolin in CaCo-2 cells. CaCo-2 cells labeled for 18 h at 37°C with 1 µCi/ml of [1,2-^{3}H(N)]cholesterol in DMEM containing 10% FCS from 12 wells were combined for subcellular fractionation by sucrose density gradients. The total cell membrane fraction was resolved on the gradient and the 24 fractions (0.5 ml each) were analyzed for protein, cholesterol mass, RNA, labeled cholesterol, and alkaline phosphatase activity as described in Methods. To localize caveolin protein, two fractions were pooled and concentrated by precipitation with TCA. The TCA precipitate was dissolved in 0.1 ml Laemmli buffer and 0.03 ml was applied on the gel for SDS-PAGE and Western analysis. The insert shows the relative density units of caveolin mass in the fractions.

with labeled cholesterol and then exposed to cholesterol oxidase, approximately 3–5% of the labeled cholesterol was recovered as cholest-4-en-3-one (cholestenone). In contrast, when cells were fixed prior to the addition of cholesterol oxidase, 75% of the label was recovered as cholestenone (data not shown). To investigate whether an influx of cholesterol at the apical membrane would cause "clustering" of plasma membrane cholesterol to these regions of the membrane accessible to cholesterol oxidase, cells were equilibrated with labeled cholesterol and incubated for 4 h with taurocholate micelles containing oleic acid and increasing concentrations of cholesterol. To estimate cholesterol influx from the plasma membrane to the endoplasmic reticulum, cholesteryl esters synthesized from plasma membrane cholesterol were determined (**Fig. 6**). As shown on the left side of Fig. 6, the amount of cholesteryl esters synthesized from cholesterol derived from the plasma membrane increased with increasing concentrations of cholesterol in the micelle. This suggests that labeled plasma membrane cholesterol was being displaced by the influx of luminal cholesterol driving plasma membrane cholesterol to the endoplasmic reticulum for esterification. With the influx of labeled plasma membrane cholesterol into the cell and the accumulation of excess unlabeled micellar cholesterol into the plasma membrane, it might be expected that less labeled plasma membrane cholesterol would be accessible to the action of cholesterol oxidase. This turned out not to be the case, however. In a concentration-dependent manner, the percent of labeled cholesterol recovered as cholestenone increased with increasing concentrations of micellar cholesterol (Fig. 6, right panel). This suggests that the incoming micellar cholesterol caused labeled plasma membrane cholesterol to move to a domain within the membrane that was now accessible to cholesterol oxidase. The effect of micellar cholesterol on the displacement of plasma membrane cholesterol was detected as early as 30 min after adding micellar cholesterol and increased progressively over the 4 h of the experiment (**Fig. 7**).

Influx of plasma membrane cholesterol: caveolae involved?

There have been a number of agents that have been used to disrupt caveolar function. To investigate whether

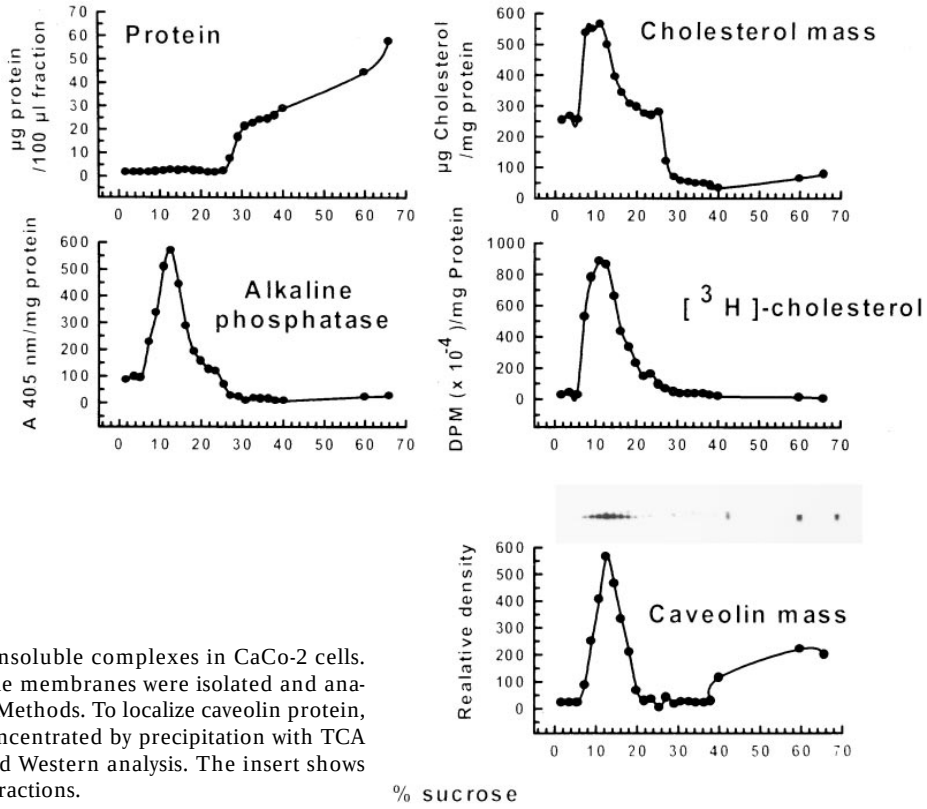

Fig. 5. Detergent-insoluble complexes in CaCo-2 cells. Triton X-100-insoluble membranes were isolated and analyzed as described in Methods. To localize caveolin protein, the fractions were concentrated by precipitation with TCA before SDS-PAGE and Western analysis. The insert shows caveolin mass in the fractions.

the influx of plasma membrane cholesterol to the endoplasmic reticulum required normal caveolar function, we examined the effect of filipin, N-ethyl maleimide (NEM), cholesterol depletion, okadaic acid, sphingomyelinase, and cholesterol oxidase on the esterification of cholesterol derived from the plasma membrane. The results are shown in **Table 1**. Exposure of other cell types to filipin, a cholesterol-binding agent, has been shown to cause disassembly of caveolae and unclustering of folate receptors found there (43, 44). As shown in Table 1, filipin was very potent in inhibiting the esterification of plasma membrane cholesterol. Caveolae contain N-ethyl-maleimide sensitive factor and the agent NEM has been used to inhibit caveolar function (11, 45). NEM also caused a pro-

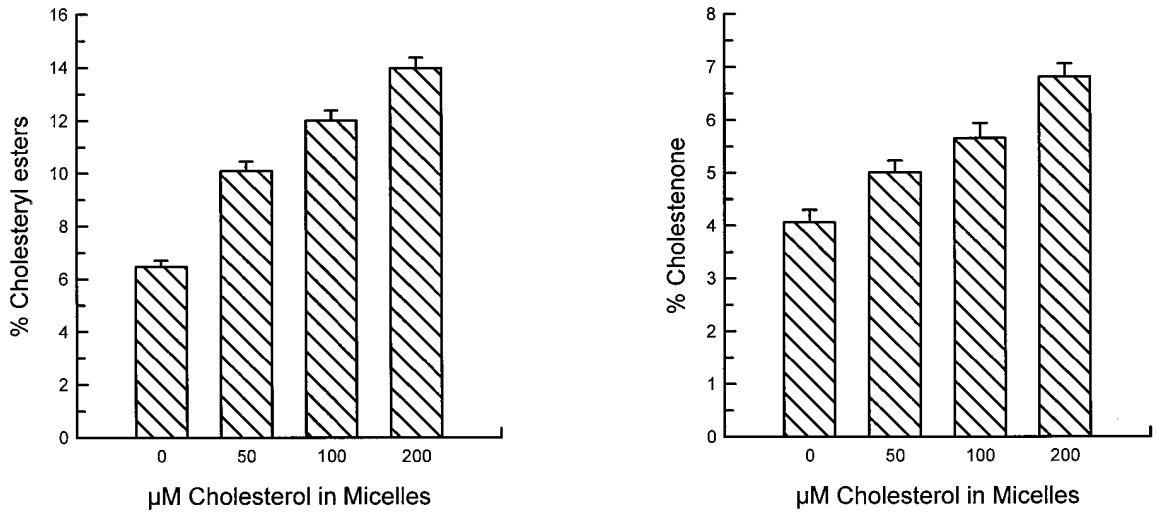

Fig. 6. Effect of micellar cholesterol concentration on the transport of plasma membrane cholesterol to the endoplasmic reticulum and cholesterol oxidase accessibility. CaCo-2 cells were prelabeled with cholesterol overnight as described in Methods. They were then incubated at 37°C for 4 h in medium containing 5 mm sodium taurocholate, 50 µm oleate, and 0, 50, 100, or 200 µm cholesterol. At the end of this incubation, cells were washed extensively and incubated for an additional hour at 37°C with 10 IU / ml cholesterol oxidase. The percent of cholesteryl esters and cholestenone in cells was determined as described in Methods. Each value is the mean ± SEM of 6 wells.

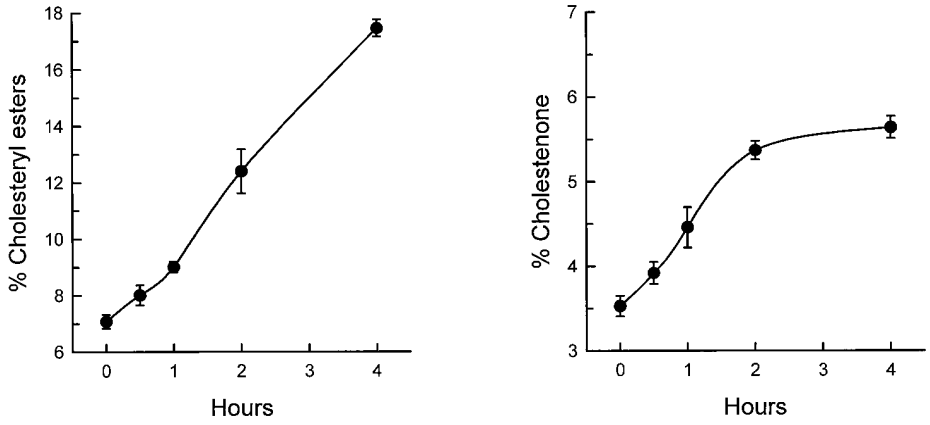

Fig. 7. Effect of micellar cholesterol on the transport of plasma membrane cholesterol to the endoplasmic reticulum and cholesterol oxidase accessibility over time. CaCo-2 cells were prelabeled with cholesterol overnight as described in Methods. They were then incubated at 37°C for 0, 0.5, 1, 2, or 4 h with medium containing 5 mm sodium taurocholate, 50 μm oleate, and 200 μm cholesterol. At the end of this incubation cells were washed extensively and incubated for an additional hour at 37°C with 10 IU/ml cholesterol oxidase. The percent of cholesteryl esters and cholestenone in cells was determined as described in Methods. Each value is the mean ± SEM of 6 wells.

found decrease in the esterification of plasma membrane-derived cholesterol. Cholesterol depletion in fibroblasts disrupts caveolar structure, causing unclustering of folate receptors in caveolae and suppressing the transport of folate (46). In CaCo-2 cells depleted of cholesterol by growing them in delipidated serum and in the presence of an HMG-CoA reductase inhibitor, the esterification of cholesterol originating from the plasma membrane was significantly diminished. In contrast, okadaic acid, an agent which is said to decrease the activity and number of caveolae found on the cell surface (47), did not inhibit cholesterol trafficking. As expected, the addition of sphingomyelinase increased the esterification of plasma membrane cholesterol (48). However, when cells were first depleted in plasma membrane sphingomyelin by incubating them for 18 h with sphingomyelinase, the addition of sphingomyelinase had little effect on plasma membrane cholesterol esterification. Incubation of cells with cholesterol oxidase, which should oxidize caveolar cholesterol to cholestenone, had no effect on cholesterol transport.

TABLE 1. Effect of "caveolae" modification on the influx of plasma membrane cholesterol to the endoplasmic reticulum

Treatment	Cholesteryl Esters Formed Relative to Control
	%
Control[a]	100
Filipin (5 μm)	28 ± 3[e]
N-ethyl maleimide (1 mm)	27 ± 6[e]
Cholesterol depletion[b]	29 ± 2[e]
Okadaic acid (1 μm)	165 ± 8[e]
Sphingomyelinase, 5 h	435 ± 21[e]
Sphingomyelinase, 18 h and 5 h[c]	122 ± 4[e, f]
Cholesterol oxidase[d]	93 ± 5

Plasma membrane cholesterol was labeled by incubating cells for 90 min at 4°C with 3 μCi [1,2-^{3}H(N)]cholesterol in 1 ml M199 (medium-199/Earle's, Gibco, Grand Island, NY) containing 1% delipidated fetal calf serum. Unless noted in the footnotes below, the medium was replaced with 1 ml M199 containing 5 mm sodium taurocholate and 250 μm oleate with or without the treatment at 37°C for 5 h. The cholesteryl esters formed were measured as described in Methods. The values are expressed relative to corresponding controls and represent mean ± SEM of 6 individual dishes.

[a] In control cells 2.1 ± 0.09% of the cellular cholesterol was found as cholesteryl esters and corresponds to 100% in the table.

[b] Cholesterol was depleted in cells by culturing them for 8 days with 1 μg/ml lovastatin in 10% delipidated fetal calf serum/DMEM containing mevalonate to rescue.

[c] Sphingomyelin content was modified by incubating cells for 18 h, at 37°C with 100 mU/ml sphingomyelinase. The cells were then labeled as described and incubated an additional 5 h with sphingomyelinase.

[d] After labeling the cells, they were preincubated for 0.5 h at 37°C with 10 IU/ml cholesterol oxidase before incubation with 250 μm oleic acid and 83 μm bovine serum albumin in M 199. Cholesterol oxidase was present throughout the entire 5-h incubation.

[e] $P < 0.05$ compared to control.

[f] $P < 0.05$ compared to 100 mU/ml sphingomyelinase, 5 h.

Effect of cholesterol flux on caveolin mass and mRNA levels

To investigate whether changes in cholesterol flux at the apical membrane would regulate the expression of caveolin in CaCo-2 cells, cells were incubated with taurocholate micelles alone, micelles containing oleic acid, or micelles containing oleic acid and cholesterol. After the incubation, caveolin mass and mRNA levels were estimated by Western analysis and Northern hybridization, respectively. **Fig. 8** and **Fig. 9** show these results. An influx of micellar cholesterol, which causes displacement of plasma membrane cholesterol to domains that are more accessible to cholesterol oxidase and causes the transport of cholesterol from the plasma membrane to the endoplasmic reticulum, failed to significantly alter either caveolin mRNA levels or the amount of caveolin protein. Similarly, in data not shown, compared to cells incubated in control media, neither caveolin mRNA nor caveolin protein was altered in cells incubated with either taurocholate micelles alone or with 10% delipidated fetal calf serum containing 25 μm lovastatin for 3 days. Cells incubated with

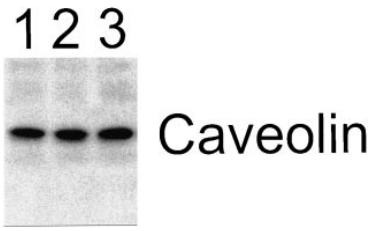

Fig. 8. Effect of cholesterol influx on caveolin mass in CaCo-2 cells. CaCo-2 cells were incubated for 18 h at 37°C with 5 mm taurocholate (lane 1); 5 mm taurocholate and 50 μm oleate (lane 2); or 5 mm taurocholate, 50 μm oleate, and 200 μm cholesterol (lane 3). Western analysis for caveolin mass was performed as described in Methods. Three separate experiments with 3 wells for each treatment per experiment were performed. A representative blot is shown.

taurocholate micelles efflux cellular cholesterol into the apical media (49). Compared to control cells cultured in 10% fetal calf serum, cells cultured for 3 days in delipidated fetal calf serum and lovastatin contained 13% less total cholesterol. It is clear, therefore, that in CaCo-2 cells, caveolin mass and mRNA levels are not altered by changes in cholesterol flux.

DISCUSSION

The results of this study clearly demonstrate the presence of caveolin in the small intestine and CaCo-2 cells. Although the majority of cells recovered in small intestinal biopsies are epithelial cells, muscle and endothelial cells are invariably present. As these cell types contain significant amounts of caveolin, one cannot attribute the amplified caveolin RT-PCR product solely to intestinal epithelial cells. Brushings taken from the upper intestinal tract, however, contain cells from the superficial epithelial layer without contamination from cells of the submucosa. The observed caveolin RT-PCR product and the identification of caveolin protein in these preparations, together with the identification of caveolin expression in CaCo-2 cells by Northern, immunoblotting, and immunofluorescent microscopy analyses, are convincing evidence that intestinal cells contain caveolin. Caveolin in CaCo-2 cells exists in detergent-resistant complexes that are associated with the plasma membrane. Immunofluorescent data shown in Fig. 3E, however, suggest that caveolin might also exist in other intracellular compartments. Electron microscopy will be required to provide more definitive information as to the location of the intracellular fluorescence. Although the presence of caveolin suggests the possible existence of caveolae in intestinal cells, the results provided here do not directly address that point. Although it has been stated that caveolae are not observed in the densely packed microvilli of the enterocyte brush border membrane, this issue has not been rigorously studied (20, 50). Clearly, CaCo-2 cells do contain detergent-resistant domains (20, 50). Other cell types, however, have also been shown to contain detergent-resistant domains but lack caveolin (40, 42). Thus, detergent-insoluble membranes are not synonymous with caveolae. Moreover, in Madin-Darby canine kidney cells, caveolae are exclusively localized to the basolateral membrane, and yet, caveolin in these cells is found in the apical membrane (51). Thus, the role of caveolin in the apical membrane, in the absence of caveolae, is open to question. We agree with the postulate offered by Scheiffele et al. (51) that perhaps caveolin is being used to organize lipid-raft microdomains of the apical membrane. We would further postulate that, in CaCo-2 cells, micellar cholesterol taken up into the apical membrane causes cholesterol to cluster in these lipid-raft domains prior to being transported to the endoplasmic reticulum for esterification.

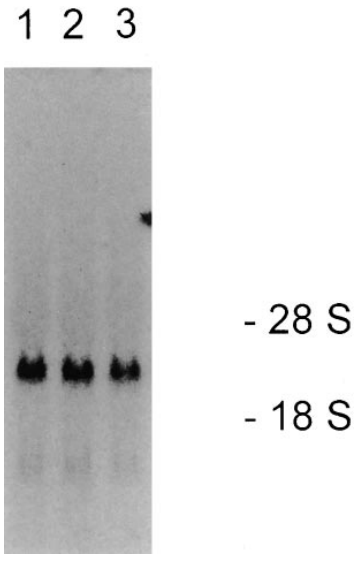

Fig. 9. Effect of cholesterol influx on caveolin mRNA in CaCo-2 cells. CaCo-2 cells were incubated for 18 h at 37°C with 5 mm taurocholate (lane 1); 5 mm taurocholate and 50 μm oleate (lane 2); or 5 mm taurocholate, 50 μm oleate, and 200 μm cholesterol (lane 3). RNA was isolated and analyzed by Northern blot as described in Methods. Two separate experiments (n = 4) were performed for each treatment. A representative blot is shown.

Our results are in agreement with Mayor, Rothberg, and Maxfield (19) who demonstrated caveolin in CaCo-2 cells using immunofluorescence techniques. Not only did these investigators demonstrate its presence, but they showed that caveolin colocalized with clusters of a caveolar receptor, the folate receptor, suggesting that caveolae likely exist in these cells. In contrast to our findings and those of Mayor et al. (19), Mirre et al. (20) and Vogel et al. (21) failed to find expression for caveolin-1 in CaCo-2 cells at either the mRNA or protein level. There is not a good explanation for this discrepancy except to implicate possible differences in the two CaCo-2 cell lines studied. We and others have observed variability among CaCo-2 cell lines and heterogeneity of protein expression due to transient mosaics within the same CaCo-2 cell clone (52, 53).

It is well recognized that mammalian cells tightly control the amount of unesterified cholesterol they contain. It would seem reasonable to assume, therefore, that after the uptake of additional cholesterol from the gut lumen,

the intestinal cell would have an efficient and orderly mechanism for transporting excess cholesterol from the plasma membrane to the endoplasmic reticulum. This would then ready the sterol for secretion (as a part of a lipoprotein particle) or regulate expression of sterol responsive genes, such as HMG-CoA reductase and the low density lipoprotein receptor (49, 54). In previous studies performed in CaCo-2 cells, we have postulated that this process involves transport vesicles likely originating from the plasma membrane (2, 3). It would seem unreasonable to assume that cholesterol molecules within the plasma membrane, which are destined for transport to the endoplasmic reticulum, would be distributed randomly throughout the membrane. This would imply that the "participants" involved in the transport of cholesterol would exist over the entire plane of the plasma membrane. This does not make good sense, particularly in an intestinal absorptive cell whose plasma membrane is expanded several-fold to allow more surface area for absorption to occur. It is more likely that special microdomains exist within the plasma membrane which function to direct and "cluster" the excess cholesterol so that the sterol can be rapidly shuttled to intracellular membranes. It is also likely that these microdomains contain the putative "sterol sensor" which has been postulated to sense the amount of cholesterol within the plasma membrane, which in turn, leads to the regulation of sterol responsive genes (55).

Lipids contained within biological membranes are not homogeneous. Cholesterol, too, is not distributed evenly throughout the membrane but instead is localized into cholesterol-poor and cholesterol-rich domains (56). Because of the recognized attraction between cholesterol and sphingomyelin, it is likely that these cholesterol-rich domains are synonymous with detergent-insoluble complexes. Caveolin, a protein confined to these domains of the plasma membrane, whose function is unknown, is a cholesterol-binding protein (14). Moreover, caveolin is thought to travel from the plasma membrane to the endoplasmic reticulum and then to the trans-Golgi (15, 16). Similar to what we and others have observed for cholesterol influx from the plasma membrane, the movement of caveolin is inhibited at 18°C and is independent of new protein synthesis (15, 57). Thus, it appeared reasonable to postulate that this protein, or even caveolae per se, may play a role in cholesterol trafficking (58). In support of this, the Fieldings (11) have recently demonstrated that caveolin/caveolae are involved in cellular cholesterol efflux, suggesting that these plasma membrane microdomains regulate the amount of intracellular cholesterol by providing a mechanism by which the cell can release or deplete itself of excess sterol. More recently, in Madin-Darby canine kidney cells, caveolin/caveolae expression were shown to decrease in response to cellular cholesterol depletion (59).

The results of the present study are suggestive that detergent-resistant complexes in CaCo-2 cells play a role in cholesterol transport from the plasma membrane to the endoplasmic reticulum. Plasma membrane cholesterol that is accessible to the action of cholesterol oxidase is thought to be confined within caveolae (11, 15). The data generated in fibroblasts, on which this statement is based, are convincing (15). However, in cells that lack caveolae, detergent-resistent complexes exist and a percentage of plasma membrane cholesterol remains susceptible to cholesterol oxidase (40, 42). Moreover, in CaCo-2 cells, cholesterol confined to detergent-resistant complexes make up approximately 65% of the total cellular cholesterol, an observation also confirmed by Stevens and Tang (31). Thus, in intestinal cells that have very high contents of sphingolipids and cholesterol in their apical microvillar membranes, it is not possible to separate these microdomains from the bulk of the remaining membrane lipids by fractionation techniques used in other cell types. Similar to other cells studied, however, approximately 3–5% of CaCo-2 cellular cholesterol is accessible to cholesterol oxidase. With an influx of excess cholesterol coming from micelles, more plasma membrane cholesterol becomes susceptible to the action of cholesterol oxidase. Under these conditions, more plasma membrane cholesterol moves to the endoplasmic reticulum; more plasma membrane cholesterol is secreted in lipoproteins; gene expression for HMG-CoA reductase and the LDL receptor is inhibited; and cholesterol synthesis and LDL uptake are suppressed (2, 49, 54). This implies that cholesterol of the plasma membrane shifts or is displaced by the absorbed cholesterol and is now in a domain of the membrane that becomes exposed to cholesterol oxidase. We would postulate that this domain represents "clustering" of cholesterol to these detergent-resistant complexes that contain caveolin, and it is from these domains that cholesterol is directed to intracellular membranes.

The present data, however, do not conclusively implicate caveolae as a mechanism for transporting plasma membrane cholesterol inward in CaCo-2 cells. Filipin, NEM, and cholesterol depletion did interfere with the transport of cholesterol from the plasma membrane to the endoplasmic reticulum. Filipin and NEM are agents that have been shown to disrupt caveolar function in other cells and, likewise, cholesterol depletion has had similar effects (11, 43, 44, 46). Filipin, in particular, has been shown to cause flattening of caveolae invaginations, disorganization of the caveolin, and unclustering of GPI-linked proteins (43, 44). In contrast to the findings of Fielding and Fielding (11), however, okadaic acid, an inhibitor of protein phosphatases 1 and 2A which is said to reduce the number and activity of caveolae at the cell surface, did not reduce the trafficking of cholesterol in CaCo-2 cells. We have previously used this protein phosphatase inhibitor in CaCo-2 cells and are convinced of its "hyperphosphorylation" effects (60). In the original observation demonstrating the effect of okadaic acid on caveolar function in A431 cells, Parton, Joggerst, and Simons (47) found that it was necessary to first cross-link (cluster) the GPI-linked alkaline phosphatase with an anti-alkaline phosphatase antibody in order for okadaic acid to inhibit caveolar function (internalization of alkaline phosphatase). Without cross-linking, okadaic acid was without effect. It is possible that in intestinal cells with a microvillar mem-

brane so enriched in glycosphingolipids, cholesterol, and the GPI-linked alkaline phosphatase, okadaic acid alone might not affect caveolar function. In the work of Fielding and Fielding (11), however, okadaic acid was effective in reducing cholera toxin uptake into fibroblasts (a measure of caveolar function) without first cross-linking the receptor. Why their results using okadaic acid to disrupt caveolar function should differ from those of Parton et al. (47) is unclear. The observation that cholesterol oxidase did not affect cholesterol transport was not unexpected. In a previous study performed in another cell type, the number and morphology of caveolae were found to be unaffected by cholesterol oxidation suggesting that cholestenone likely does not interfere with caveolar function (15). In fact, if cholesterol oxidation causes caveolin to move to the endoplasmic reticulum as suggested, one might have expected more efficient transport of cholesterol to the endoplasmic reticulum after the addition of cholesterol oxidase. This did not occur. Thus, although the results are convincing that microdomains exist within the plasma membrane of intestinal cells that "cluster" excess cholesterol, we do not have experimental proof to suggest that caveolae per se are involved in this process.

In fibroblasts, Fielding, Bist, and Fielding (61) found that gene expression for caveolin was increased, as was the amount of free cholesterol mass within caveolae, after the delivery of cholesterol to the cell via LDL. They proposed that caveolin mRNA increased in response to the influx of unesterified cholesterol and that the stimulation of cholesterol efflux that subsequently followed was due to an increase in caveolin mRNA levels. These investigators, however, did not report the effect of cholesterol on caveolin mass. In the present study, we could find no evidence for the regulation of caveolin mass or mRNA levels by cholesterol influx or efflux at the apical membrane. The half-life for caveolin has been estimated to be 10.5 h (16). It would seem unlikely, therefore, that the cell would acutely regulate caveolin expression by altering its rate of synthesis. Moreover, stimulation of caveolin internalization to the endoplasmic reticulum and trans-Golgi occurs in the absence of new protein synthesis, suggesting that synthesis of new caveolin is not required for caveolin transport (15). Likewise, new protein synthesis is not required for the transport of plasma membrane cholesterol to the endoplasmic reticulum (57). In agreement with our results, Hanada et al. (62), in CHO cells, failed to observe changes in caveolin mass in response to cholesterol enrichment or depletion. Interestingly, the expression of the two major participants in lipoprotein assembly and secretion, apolipoprotein B and microsomal triglyceride transfer protein, are also not acutely regulated by changes in protein synthesis or gene expression. This suggests that the intestinal cell synthesizes sufficient amounts of these proteins to accommodate large influxes of dietary fatty acids (63, 64). If caveolin is involved in cholesterol transport in intestinal cells, thereby linking it to lipoprotein assembly and secretion, it would not necessarily be expected that caveolin mass or mRNA levels would change in response to fatty acid or cholesterol influx.

Addendum: During the review of this manuscript, Orlandi and Fishman (65) confirmed the presence of caveolin in CaCo-2 cells.

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