Caveolin-2 Localizes to the Golgi Complex but Redistributes to Plasma Membrane, Caveolae, and Rafts when Co-expressed with Caveolin-1*

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We have characterized comparatively the subcellular distributions of caveolins-1 and -2, their interactions and their roles in caveolar formation in polarized epithelial cells. In Fischer rat thyroid (FRT) cells, which constitutively express both caveolin-1 and -2, caveolin-2 localizes exclusively to the Golgi complex but is partially redistributed to the plasma membrane upon co-expression of caveolin-1 by transfection or by adenovirus-mediated transduction. In Madin-Darby canine kidney (MDCK) cells, which constitutively express both caveolin-1 and -2, caveolin-2 localized to both the Golgi complex and to the plasma membrane, where it co-distributed with caveolin-1 in flat patches and in caveolae. In FRT cells, endogenous or overexpressed caveolin-2 did not associate with low density Triton insoluble membranes that floated in sucrose density gradients but was recruited to these membranes when co-expressed together with caveolin-1. In MDCK cells, both caveolin-1 and caveolin-2 associated with low density Triton-insoluble membranes. In FRT cells, transfection of caveolin-1 promoted the assembly of plasma membrane caveolae that localized preferentially (over 99%) to the basolateral surface, like constitutive caveolae of MDCK cells. In contrast, as expected from its intracellular distribution, endogenous or overexpressed caveolin-2 did not promote the assembly of caveolae; rather, it appeared to promote the assembly of intracellular vesicles in the peri-Golgi area. The data reported here demonstrate that caveolin-1 and -2 have different and complementary subcellular localizations and functional properties in polarized epithelial cells and suggest that the two proteins co-operate to carry out specific as yet unknown tasks between the Golgi complex and the cell surface.

Plasmalemmal caveolae are specialized plasma membrane microdomains, first discovered on the surface of endothelial cells (1) where they function in transport processes (2, 3) and then described in a variety of cell types (4). By transmission electron microscopy, caveolae can be distinguished by their characteristic morphology: flash-shaped 60–90-nm vesicles located at or near the plasmalemma (1, 5). By rapid freeze, deep etch replica methods, caveolae are seen to be coated cytoplasmically by a distinct striated filamentous coat, that contains a 21-kDa protein, caveolin (4). Caveolin was also identified as a component of post-Golgi vesicles, hence its other name VIP21 (vesicle integral protein of 21 kDa) (6).

Recently, a multigenic family of caveolin-related proteins has been identified (7). The original caveolin/VIP21 was renamed caveolin-1. Caveolin-2, with 38% sequence identity and 58% similarity to caveolin-1, has an overlapping tissue distribution with caveolin-1; both proteins are found as long (α) and short (β) isoforms lacking a few N-terminal amino acids (8–10). Caveolin-3 is muscle-specific (11, 12). The presence of caveolin-1 in the caveolar coat suggested that it might be involved in the formation of caveolae. Indeed, recent experiments in which caveolin-1 was transiently (13, 14) or permanently (15, 16) transfected into caveolin-deficient cell lines have shown that caveolin-1 promotes de novo formation of caveolae. In vivo experiments have shown that the long (α) isoforms of the two proteins can form homo- and hetero-oligomers during biosynthesis (8–10, 17, 18) and that these oligomers are differentially transported to the cell surface; whereas the smaller caveolin-1 and -2 hetero-oligomers are transported to the basolateral surface, the larger caveolin-1 homo-oligomers are transported to the apical surface of MDCK cells (8). The primary determinants of oligomerization are in the protein moieties; however, the cholesterol binding ability of caveolin-1 (19) and the attachment of palmitoyl chains to the C-terminal region (20) does promote additional clustering. Oligomers can be isolated from tissues taking advantage of their insolubility in detergents, such as Triton X-100 and CHAPS at 4 °C (6, 21). To date, the individual ability of caveolins-1 and -2 to form detergent insoluble oligomers in vivo has not been characterized because of their parallel tissue distribution.

Here we utilize an epithelial cell line (FRT) that expresses low levels of caveolin-2 and, as shown before (15, 22), no caveo-
lin-1, as a tool to study the subcellular distribution of these two proteins, their ability to interact with each other, and their individual contribution to the biogenesis of caveolae. We show that caveolin-2 has a strikingly different subcellular distribution than caveolin-1; the former protein localizes to the Golgi complex, whereas the latter one is preferentially found at the cell surface. In addition, we show that, despite their different distributions, the two proteins interact with each other and that furthermore caveolin-1 promotes redistribution of a fraction of caveolin-2 to the cell surface, to detergent-insoluble complexes, and to caveolae. Finally, we demonstrate that caveolin-2 does not share the capability of caveolin-1 to promote the formation of plasmalemmal caveolae but may contribute to the formation of post Golgi vesicles. Our data confirm and extend previous observations that indicate that caveolae are assembled with a striking selectivity for the basolateral surface of epithelial cells (16, 23).

**EXPERIMENTAL PROCEDURES**

**Materials—**Cell culture reagents were purchased from Life Technol-
ogies, Inc., and chemicals were from Sigma unless otherwise specified. The pCMV-myc vector, and the myc-tagged caveolin-2 cDNAs were purchased from G. R. W. Andersen (University of Texas, Southwest of Dallas, TX). Caveolin-2 cDNAs were provided by Michael Lisanti (Albert Einstein College of Medicine, Bronx, NY). Monoclonal (mAb 2297) and polyclonal antibodies to caveolin-1, a monoclonal antibody to caveolin-2 (mAb 65) (10) and a monoclonal antibody to γ-adaptin were purchased from Transduction Laboratories (Lexington, KY). The monoclonal 9E10 and polyclonal A-14 antibodies to c-myc epitope were from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). A polyclonal antibody to manno-
sidase II was provided by Kelley Moremen (University of Georgia, Athens, GA). A rabbit polyclonal antibody against the Golgi marker GOS-28 was provided by Tomas Sollner (Memorial Sloan-Kettering Cancer Center). A rabbit polyclonal antibody to TGN-38 was provided by G. Banting (University of Bristol, Bristol, UK). The pAdlox plasmid, CRES cells and 65 virus were provided by Steve Hardy (Somatix Ther-
apy Corp., Alameda, CA). The ECL Western blot detection kit was purchased from Amersham Pharmacia Biotech.

**Cell Culture—**Cells were grown for 3–5 days at high density either in Dulbecco’s modified Eagle’s medium (MDCK cells) or in Ham’s F-12/Coon’s (Sigma) modified media (FRT cells) supplemented with 5% fetal bovine serum. Cells were plated either on tissue culture dishes, glass coverslips or polycarbonate Transwell R chambers (Corning Costar Corporation, Cambridge, MA).

**Transfection with Caveolin cDNAs—**Full-length human caveolin-1 cDNAs (untagged or myc-tagged), were subcloned into either pcDNA3 (Invitrogen, San Diego, CA) or pC87 vectors (15), which contained a neomycin- or a hygromycin-selectable marker, respectively. Full-length as well as truncated versions of human caveolin-2 cDNAs were purchased from American Type Culture Collection. For dual immunofluorescence studies, the cDNAs were subcloned into the pC87 vector (9). Liposome-mediated transfection of MDCK or FRT cells with LipofectAMINE was carried out according to manufacturer’s instructions. Selections were performed in the presence of 500 μg/ml geneticin or 200 μg/ml hygromycin. Individual clones were obtained by limiting dilution.

**Western Blot Analysis—**Cells were frozen for 60 min on ice with Tris-buffered saline (25 mM Tris, pH 7.5, 0.15 M NaCl) containing 60 mM n-octyl β-D-glucopyranoside (24). After centrifugation at 13,000 rpm for 10 min in a tabletop microcentrifuge, the protein content of the extracts was determined using a Bio-Rad DC protein assay kit with bovine serum albumin as a standard. After SDS-polyacrylamide (15%) gel electrophoresis and transfer to nitrocellulose (Schleicher & Schuell), blots were probed with anti-caveolin-1 IgG (pAb 1/10,000 or mAb 2297, 1/1000), anti-caveolin-2 IgG (mAb 65, 1/250), or polyclonal anti-myc IgG (1/5000). 20–50 μg of cell extract were routinely loaded per lane. The bound horseradish peroxidase-conjugated reporter IgG was detected on the ECL system. Band intensities were quantified by imaging the appropriate fluoroscein isothiocyanate- or Texas Red-conjugated (Jackson Immunoresearch Laboratories, Inc., West Grove, PA) secondary antibodies at a dilution of 5 μl IgG μl with primary and secondary antibodies were for 3 and 1 h, respectively.

Cells were washed five times with PBSB during incubations. Control experiments for the specificity of the antibodies consisted in: (i) immunofluorescence staining for caveolin-1 of wild type FRT cells that express caveolin-2 but no caveolin-1; (ii) immunostaining of wild type MDCK cells, wild type FRT cells, and FRT cells transfected with caveo-
lin-1 with anti-myc epitope antibodies; and (iii) immunostaining of cells in the absence of primary antibodies. Coverslips and polycarbonate Transwell filters were mounted onto slides with Vectashield (Vector Laboratories Inc., Burlingame, CA) and observed under Molecular Dynamics or Bio-Rad laser scanning confocal fluorescence microscopes. Alternately, fluorescence microscopy was performed on a Nikon E-600 microscope using the following filter cubes (Chroma Technologies, Barbelltovo, VT): rhodamine (G-2/E/C DM 565), Cy5 (HYQ, DM 66), fluorescein (B-2/E/C DM 505); a UV filter cube (EF-4ex430/em460 DC 450), a green filter cube (EF-5ex517/em530 DC 50), and a blue filter cube (EF-6ex630/em650 DC 50). Images were acquired using a cooled charged coupled device camera (CCD 1000 PB, Princeton Instru-
ments) and were transferred to a computer workstation running the metaMorph imaging software (Universal Imaging, West Chester, PA).

**Immunoelectron Microscopy on Ultrathin-frozen Sections—**Cells grown on 6-well plastic Petri dishes were fixed for 20 min in 4% paraformaldehyde/0.1% glutaraldehyde and were washed and stored in PBS containing 0.02% azide. They were cryoprotected in 2.3 M sucrose in PBS, scraped from the plates, and placed on a cryostat support for freezing. Cells were sectioned at −110 °C on a Reichert FC4D ultracryo- microscopy (Leica Instruments, Deerfield, IL), and sections were collected on nickel grids. For immunostaining, the grids were blocked on a drop of PBS containing 1% bovine serum albumin/1% normal goat serum for 30 min. They were incubated with anti-caveolin-2 monoclonal antibody 65 diluted 1:300 (in commercial antibody diluent; DAKO Corp.) overnight at 4 °C. After rinsing three times for 5 min each time in PBS, they were incubated with goat anti-mouse IgG coupled to 10 nm of colloidal gold, diluted 1:25 in antibody diluent, for 1 h at room temperature. After further rinsing three times for 5 min in PBS, the sections were fixed in 1% glutaraldehyde for 10 min, rinsed in distilled water, and stained with 2% methylcellulose containing 0.2% uranyl acetate for 10 min, on ice. Sections were examined and photographed using a Philips CM10 electron microscope.

**Preembedding Immunocytochemistry—**Cells grown on 6-well clusters were fixed in 3% paraformaldehyde (10 min, 37 °C), from the wells, and exposed to 0.1% Triton X-100 for 5 min at room temperature (25). They were then washed for 30 min in PBS containing 0.01% glutaraldehyde. For dual immunofluorescent labeling, the cells were incubated overnight with mAb 65 to caveolin-2 (1:100) and anti-caveolin-1 pAb (1:100) followed by 5 nm of gold goat anti-rabbit IgG conjugate (1:50) and 10 nm gold-goat anti-mouse IgG conjugate, (1:50) for 3 h. After washing, the cells were fixed for 30 min in 1% glutaraldehyde in 0.1 M cacodylate buffer, pH 7.4. The cells were then fixed and postfixed in 1% OsO4, stained with 0.5% tannic acid (10 min), followed by staining in block for 20 min in 1% uranyl acetate, dehydrated, and embedded in epon.

**Post-embedding Immunostaining—**Cells grown on filters were fixed in 4% paraformaldehyde for 30 min, dehydrated in cold solutions of increasing ethanol concentrations, and then embedded in Unicryl according to the protocol described by British BioCell International (Cardiff, UK). Thin sections were collected on nickel grids, rehydrated in PBS containing 1% bovine serum albumin and 10% normal goat serum and then incubated with the mAb 65 to caveolin-2 followed by 5 or 10 nm of gold goat anti-mouse IgG conjugate. The grids were then counterstained with uranyl acetate and lead citrate.

**Thin Section Electron Microscopy—**Cells grown on polycarbonate Transwell® filters were processed for thin section electron microscopy by standard procedures as described previously (26). They were then cut parallel to the plane of the culture with a Reichert F 4500 microtome and stained with uranyl acetate and lead citrate. Specimens were examined and photographed in a JEOL 100 CXII electron microscope.

For quantitation of caveolae, the specimens were treated with 1% tannic acid in 0.05 M cacodylate buffer for 30 min to increase membrane density with the NIH Image 1.60 package.
and lysed for 30 min at 4 °C in 2 ml of 1% Triton X-100 in TNE buffer.

Shuttle plasmids were co-transfected along with purified full-length myc-caveolin-2 cDNAs were subcloned into pAdlox shuttle constructed by using CRE/Lox assisted recombination according to previously described methods (27). In brief, full-length caveolin-1 and previously described methods (27). In brief, full-length caveolin-1 and caveolin-2 cDNAs were subcloned into pAdlox shuttle plasmids and co-transfected along with purified viral genomic DNA into CRE8 cells (293 cells stably transfected with CRE recombinase). After 1 week of transfection the crude viral lysate was subjected to immunoblot analysis. Molecular mass standards (Sigma) were as follows: carbonic anhydrase (29 kDa), bovine serum albumin (66 kDa), alcohol dehydrogenase (150 kDa), b–amylase (200 kDa), apoferritin (443 kDa), and thyroglobulin (670 kDa).

**Construction of Adenovirus Vectors**—E1 deleted replication-defective adenovirus vectors carrying caveolin-1 or myc-caveolin-2 cDNAs were constructed by using CRE/Lox assisted recombination according to previously described methods (27). In brief, full-length caveolin-1 and full-length myc-caveolin-2 cDNAs were subcloned into pAdlox shuttle plasmids and co-transfected along with purified viral genomic DNA into CRE8 cells (293 cells stably transfected with CRE recombinase). After 1 week of transfection the crude viral lysate was subjected to immunoblot analysis. Molecular mass standards (Sigma) were as follows: carbonic anhydrase (29 kDa), bovine serum albumin (66 kDa), alcohol dehydrogenase (150 kDa), b–amylase (200 kDa), apoferritin (443 kDa), and thyroglobulin (670 kDa).

**Velocity Gradient Centrifugation**—To estimate the oligomeric state of caveolins in FRT and MDCK cells, we used a previously described protocol (10) with minor modifications. Briefly, 500 µl of samples prepared in 2.5 ml Mes, pH 6.5, 150 mM NaCl buffer plus 60 µM n-octyl β-D-glucopyranoside were loaded atop a 5–50% linear sucrose gradient (4.3 ml) and centrifuged at 40,000 rpm for 18 h in a SW-50.1 rotor. Twelve gradient fractions were harvested from the top, and aliquots were subjected to immunoblot analysis. Molecular mass standards (Sigma) were as follows: carbonic anhydrase (29 kDa), bovine serum albumin (66 kDa), alcohol dehydrogenase (150 kDa), b–amylase (200 kDa), apoferritin (443 kDa), and thyroglobulin (670 kDa).

**Results**

**Expression of Caveolin-1 and Caveolin-2 in FRT and MDCK Cells**—To characterize the localization of caveolin-1 and caveolin-2 in FRT and MDCK cells and the effect of caveolin-1 expression on caveolin-2 localization, we prepared stable clones expressing either one or both of these proteins simultaneously (Fig. 1). In the FRT clones selected for study the levels of expression of transfected caveolin-2 were three to five times higher than the levels of endogenous caveolin-2. To distinguish it from endogenous caveolin-2, transfected caveolin-2 had a myc epitope; as shown below, possession of this myc epitope did not affect the localization of the protein or its ability to interact with caveolin-1. The levels of transfected caveolin-1 expressed in FRT cells ranged from half to twice the levels of endogenous caveolin-1 in MDCK cells.

**Localization of Caveolin-1 and Caveolin-2 in MDCK and FRT Cells**—The subcellular localization of caveolin-1 and -2 in FRT and MDCK cell lines was studied by indirect immunofluorescence on a confocal microscope or on a Nikon Eclipse fluorescence microscope linked to an image collection system (see “Experimental Procedures”). Endogenous (rat) caveolin-2 detected in FRT cells by immunostaining with an antibody against human caveolin-2 (mAb 65) exhibited an almost exclusively juxtanuclear distribution (Fig. 2A, W7). FRT cell lines overexpressing full-length myc-caveolin-2 displayed the same juxtanuclear localization as detected by a polyclonal antibody against the myc epitope (Fig. 2B, ML-5), indicating that the epitope did not interfere with the localization of the protein. The monocular antibody 65 against human caveolin-2 did not recognize the endogenous canine caveolin-2 in MDCK cells either by immunofluorescence (not shown) or by Western blot (Fig. 1A, WT), but transfection of human caveo-
Lin-2 into MDCK cells, either the wild type α isoform (Fig. 2, pool) or the myc-tagged α isoform (Fig. 2H, R10), resulted in a distinct juxtanuclear signal by immunofluorescence and in a strong Western blot signal (Fig. 1). The localization pattern of the truncated β isoform of caveolin-2 in stable transfectants (B). A similar juxtanuclear distribution of caveolin-2 was detected in MDCK cells after transfection with wild type full-length caveolin-2 cDNA (G) or of myc-tagged caveolin-2 (H). In contrast, transfected wild type and myc-tagged caveolin-1 displays a punctate and main surface localization in FRT cells (C–F) identical to that displayed by endogenous caveolin-1 in MDCK cells (I and J). In xz confocal sections, caveolin-1 is seen equally distributed on the apical and basolateral membranes in FRT cells (E and F) and in MDCK cells (J). Arrow in C and J point at small intracellular vesicular compartments positively stained for caveolin-1. Bars, 5 μm.

Caveolin-2 Co-localizes with Golgi Cisternae and TGN Markers in MDCK and FRT Cells—Double-labeling immunofluorescence experiments with antibodies against Golgi markers (mannosidase II and GOS-28) or TGN markers (TGN-38) demonstrated an overlapping distribution of caveolin-2 with these markers in FRT cells (Fig. 3, A–F). Caveolin-2 partially co-localized with γ-adaptin in MDCK (Fig. 3, G and H) cells. Immunogold localization on ultracytosections revealed caveolin-2 on tubulovesicular structures in the Golgi area (Fig. 4).}

**FIG. 2.** Differential localization of caveolin-1 and caveolin-2 in MDCK and FRT cells. Confluent monolayers of MDCK or FRT cell lines grown on polycarbonate filters expressing various combinations of endogenous and exogenous caveolin-2 and caveolin-1 were analyzed by immunofluorescence and laser scanning confocal microscopy. Note the predominantly juxtanuclear localization of the endogenous caveolin-2 in wild type FRT cells (A) as well as of the myc-tagged overexpressed caveolin-2 in FRT stable transfectants (B). A similar juxtanuclear distribution of caveolin-2 was detected in MDCK cells after transfection with wild type full-length caveolin-2 cDNA (G) or of myc-tagged caveolin-2 (H). In contrast, transfected wild type and myc-tagged caveolin-1 displays a punctate and main surface localization in FRT cells (C–F) identical to that displayed by endogenous caveolin-1 in MDCK cells (I and J). In xz confocal sections, caveolin-1 is seen equally distributed on the apical and basolateral membranes in FRT cells (E and F) and in MDCK cells (J). Arrow in C and J point at small intracellular vesicular compartments positively stained for caveolin-1. Bars, 5 μm.

**FIG. 3.** Double-immunofluorescence of caveolin-2 with Golgi or TGN markers in FRT and MDCK cells. In wild type FRT cells (A–F) there is a partial overlapping staining of caveolin-2 (A, C, and E) with mannosidase II (A and B), with GOS-28 (C and D), and with TGN 38 (E and F). In MDCK cells (G and H) a partial overlapping distribution of caveolin-2 with γ-adaptin is observed. Bars, 5 μm.

**FIG. 4.** Localization of caveolin-2 in the Golgi region of FRT cells. Ultrathin cryosections of FRT cells overexpressing caveolin-2 (clone ML-12) were stained by immunogold. The gold labeling is restricted to numerous vesicles and tubules in the Golgi region of the cell. Little or no label can be detected over cellular compartments, demonstrating the specificity of the labeling. G, Golgi complex; M, mitochondria. Bar, 0.1 μm.
FIG. 5. Caveolin-2 is recruited to the plasma membrane in the presence of caveolin-1. Caveolin-2 is confined to the Golgi area of confluent monolayers of FRT cells overexpressing caveolin-2, α isoform (A and C) or β isoform (E) but redistributes partially to the plasma membrane in FRT cell lines co-expressing transfected caveolin-1 (B, D, and F). Caveolin-2 expression was detected with pAb A-14 that recognizes the myc epitope. Control experiments confirmed the specificity of the antibody for the myc epitope. The levels of expression of caveolin-1 and caveolin-2 in the cell lines shown in this figure can be found in the Western blots of Fig. 1. Bars, 5 μm.

nous caveolin-2 either in wild type FRT cells or in FRT cells overexpressing caveolin-2 tagged with myc epitope (three to four times higher levels than the endogenous caveolin-2) (Fig. 2, A and B, and Fig. 5, A, C, and E). These results suggested that the simultaneous expression of both caveolin-1 and caveolin-2 might promote the recruitment of caveolin-2 at the plasma membrane. To test this hypothesis, we generated double transfectant FRT cell lines expressing caveolin-1 and caveolin-2. In these clones the immunostaining of caveolin-2 resembled that seen in MDCK cells: mainly Golgi complex but with a definite labeling of the plasma membrane (Fig. 5, B, D, and F). Interestingly, the same observation was true for both the α isoform (Fig. 5, BM1 and BM2) and for the shorter β isoform (Fig. 5, D2).

To confirm that caveolin-1 was required for the recruitment of caveolin-2 to the plasma membrane and to completely eliminate the possibility that this observation might be due to clonal variation or to the saturation of the intracellular machinery responsible for the Golgi retention of caveolin-2, we carried out additional experiments using an adenovirus-mediated gene transfer approach. For these experiments, FRT cell lines expressing both transfected and endogenous caveolin-2 were transduced with adenoviruses carrying the α subunit of caveolin-1 (Adcav-1) and, in control experiments, with adenoviruses carrying caveolin-2 (Adcav-2). Fig. 6 shows that transduction of FRT cells with Adcav-1 or Adcav-2 resulted in the expression of high levels of caveolin-1 or caveolin-2, respectively, as detected by Western blot. Control immunofluorescence experiments demonstrated that treatment of ML-5, ML-12 and 18/FRT clones (Fig. 7, A, D, and G) with Adcav-2 did not affect the juxtanuclear localization of caveolin-2 (Fig. 7, B, E, and H), indicating that the higher levels of expression of the protein did not modify its stringent localization to the Golgi complex. In contrast, transduction of caveolin-1 into ML-5, ML-12/FRT sublines, resulted in a redistribution of a fraction of caveolin-2 to the cell surface (Fig. 7, C and F). A similar observation was made for an FRT cell line overexpressing the short (β) subunit of caveolin-2: transduction of caveolin-1 caused a considerable redistribution of this protein to the cell surface (Fig. 7, I).

Analysis of double immunofluorescence experiments indicated a clear co-localization of caveolin-1 and caveolin-2 in plasma membrane patches (Fig. 8, D and E, arrows). Immunogold electron microscopy demonstrated the co-distribution of both caveolins in plasma membrane patches and on plasmalemmal caveolae (Fig. 8).

Expression of Caveolin-1 in FRT Cells Promotes Recruitment of Caveolin-2 to the Low Density Triton-insoluble Membrane Fraction—The partial change in distribution of caveolin-2 in
the presence of caveolin-1 and the codistribution of caveolin-1 and caveolin-2 in the plasma membrane suggested that the two proteins interact in vivo. Previous work has shown that a fraction of both proteins co-immunoprecipitates in MDCK cells and gets incorporated in detergent insoluble complexes (8). However, it is not clear whether both proteins share the ability to incorporate into detergent-insoluble clusters (rafts) or only one of them has this ability and the other one follows. To study this important point, we analyzed the floatation characteristics of caveolin-2 in the presence or in the absence of caveolin-1. We

**FIG. 8.** Caveolin-2 is recruited to caveolae in the presence of caveolin-1. A–C, post-embedding immunogold staining of caveolin-2. In FRT cells expressing exogenous caveolin-1 (clone BM1), immunogold detects caveolin-2 (arrows) both on the apical (A) and basal membrane (B). Note that the concentration of caveolin-2 is higher on the basolateral membrane (B) where is present in flat patches (B) or on caveolae (C, arrows). Bars, 0.05 μm. D and E, double immunofluorescence analysis of FRT cells transfected with caveolin-1 (clone T2) using a caveolin-2 mAb (D) and a caveolin-1 pAb (E) show that the two caveolins co-patch at the plasma membrane (arrows) and in perinuclear regions (arrowheads). Bars, 5 μm.Control experiments confirmed the specificity of these antibody probes; no cross-reaction was observed. F–H, pre-embedding immunogold staining for caveolin-1 and caveolin-2. The two caveolins (10 nm of gold for caveolin-2; 5 nm of gold for caveolin-1) colocalize on caveolae (F) and on cytoplasmic vesicles apparently not connected to plasmalemma (G) and are also seen in the same plasmalemmal noncaveolar domains (H)(arrowheads, caveolin-2; arrows, caveolin-1). Bars, 0.05 μm.

**FIG. 9.** Expression of caveolin-1 in FRT cells causes recruitment of caveolin-2 to the Triton insoluble low density membrane fraction. A, floatation of caveolin-2 in the absence or presence of caveolin-1. Confluent monolayers of FRT and MDCK cell lines were extracted with Triton X-100 at 4 °C (see “Experimental Procedures”). The cell extract was made 40% in sucrose concentration and loaded under a 0–35% sucrose density gradient. After centrifugation for approximately 20 h, fractions were collected and analyzed by immunoblot (mAb 2297 for caveolin-1 and mAb 65 for caveolin-2). In wild type FRT cells (A, panel a) most of caveolin-2 was detected in the bottom fractions (9–12) corresponding to the Triton-soluble material. In contrast, in FRT cells expressing transfected caveolin-1 (A, panel b) as well as in MDCK cells (A, panel c) a large fraction of caveolin-2 was also found in the top fractions (3–5), corresponding to the low density regions of the gradient. In B, the floatation pattern of caveolin-1 in FRT and MDCK cells is shown for comparison. B, velocity gradient analysis of the oligomeric state of caveolin-2 in the absence or presence of caveolin-1. FRT and MDCK cells were extracted (see “Experimental Procedures”), loaded atop a 5–50% sucrose density gradient and centrifuged for 18 h. The arrows mark the positions of molecular mass standards. The distribution of caveolin-2 and caveolin-1 in the 12 gradient fractions collected was detected by immunoblot analysis using the mAb 65 for caveolin-2 and the mAb 2297 for caveolin-1. Note that in WT/FRT cells (C, panel a) that lack caveolin-1 expression, a significant fraction of caveolin-2 was present in fractions 4–8, corresponding to approximate apparent molecular masses of 66–443. The distribution of transfected overexpressed myc-caveolin-2 in ML-12/FRT cells was virtually identical to that of endogenous caveolin-2. Note that in ML-12/FRT cells 2 bands are visible. The lower band corresponds to the endogenous caveolin-2, and the upper one corresponds to the full-length exogeneous myc-tagged caveolin-2. Overexpression of caveolin-2 as well as the myc epitope did not affect the size of caveolin-2 oligomers. In FRT cells (C, panel b) that co-express caveolin-1 (T2/FRT cells), caveolin-2 sediments with a higher apparent molecular mass (fractions 6–10, corresponding to 200–669 kDa), which is also the apparent molecular mass of caveolin-1 (D) under the same conditions.
FIG. 10. Caveolin-1, but not caveolin-2, promotes the formation of basolateral caveolae in FRT cells. Confluent monolayers of MDCK or FRT cells were processed for transmission electron microscopy as described under “Experimental Procedures.” For all MDCK panels (A–F) and FRT panels (G–L), the abbreviations are as follows: Ap, apical plasmalemma; Bl, basolateral plasmalemma; v and arrowheads, coated vesicles; c and arrows, plasmalemmal caveolae. Specimens shown in A–E and in G and H and in K–L were additionally treated with 1% tannic acid. A–F, basolateral caveolae in MDCK cells. In MDCK cells, which express both caveolin-1 and caveolin-2, caveolar structures (arrow) are rare on the apical plasma membrane, whereas coated vesicles (arrowheads) are frequent (A). In contrast, on the basolateral membrane (B) plasmalemmal caveolae (arrows) are very abundant. Note the absence of tannic acid staining for the caveolar structure (arrow) shown in C. D and E show higher magnification of the apical vesicles. The caveolar structure in D is apparently fused to the apical plasmalemma. The two apical coated vesicles in E (V1 and V2) have different sizes and appear to be decorated by distinct coats. F illustrates basolateral plasmalemmal caveolae with different morphological appearances: vesicles with long-neck (C1), with open stoma (C2), and with fused straight diaphragm (C3). Transfection of caveolin-1 into FRT cells promotes the formation of basolateral caveolae (G–J). On the apical (Ap) plasmalemma mainly coated pits and vesicles (arrowheads) are seen (G). The plasmalemmal caveolae (arrows) are predominantly found on the basolateral (Bl) membrane (H). Structural variation of plasmalemmal caveolae induced by expression of caveolin-1 in FRT cells (I and J). The apical caveolae had the following distinctive morphology: vesicles with a straight fusion membrane (I, 1), caveolae displaying a well-defined single-layered stomatal diaphragm (I, 2), and flask-shaped caveolae with elongated and narrow neck and sharply bent rims (I, 3), apparently in apposition with an intracellular vesicle (v) or caveolae fused in a tubular structure (I, 4–6). The typical caveolar structures found on the basolateral plasmalemma were: vesicles with five-layered (J, 1) or...
initially compared the floatation of caveolin-2 in FRT and MDCK cells solubilized with Triton X-100 at low temperature (24). To separate LDTIM from the Triton X-100 soluble material, we placed the Triton extract, made 40% in sucrose concentration, under 0–35% sucrose density gradients. After overnight centrifugation, aliquots from the different fractions were subjected to immunoblot analysis using caveolin-1 or caveolin-2 specific antibodies. Fig. 9 shows that, in FRT cells, endogenous (Fig. 9A, WT) and overexpressed (Fig. 9A, myc-cav2) caveolin-2 is found at the bottom of the sucrose gradient (fractions 9–12), indicating that this protein lacks intrinsic capability to associate with LDTIM. In contrast, the transfection of caveolin-1 into FRT cells (Fig. 9A, panel b) or the presence of endogenous caveolin-1 in MDCK cells (Fig. 9A, panel c) results in the association of a substantial fraction of caveolin-2 with LDTIM (fractions 3–5 in sucrose density gradient). A large fraction of caveolin-1 was found in association with LDTIM in MDCK and in FRT cells (Fig. 9B). These results indicate that the simultaneous expression of caveolin-1 and caveolin-2 is required for the incorporation of caveolin-2 into rafts. On the other hand, because we do not have a cell line in which caveolin-1 is expressed in the absence of caveolin-2, our data do not allow us to conclude that caveolin-1 can associate with rafts independently of caveolin-2.

Finally, velocity gradients were performed to study the formation of caveolin-1 and caveolin-2 oligomers (Fig. 9C). In these gradients, caveolin-2 from FRT cells not expressing caveolin-1 was present in fractions 4–8, corresponding to an estimated molecular mass of 66–443 kDa. In contrast, simultaneous expression of caveolin-1 resulted in an increased sedimentability of caveolin-2 to fractions 6–10 in the velocity gradient, corresponding to an estimated molecular mass of 200–669 kDa. Importantly, in MDCK cells, caveolin-2 also had a high apparent molecular mass and was detected in fractions 6–10. Parallel experiments demonstrated that in both MDCK and FRT cells, caveolin-1 sedimented into fractions 6–10 (Fig. 9D). These experiments demonstrate that expression of caveolin-1 promoted the formation of caveolin-2 oligomers larger than those formed in the absence of caveolin-1.

Formation of Basolateral Plasma Membrane Caveolae Is Induced by Caveolin-1 but Not by Caveolin-2—Ultrastuctural examination revealed an abundance and polarized distribution of plasmalemmal caveolae in MDCK cells (Fig. 10). On the apical membrane mainly coated vesicles were observed; caveolae were very rarely seen (Fig. 10A). Some of the coated vesicles had the same size as the infrequent apical caveolae (Fig. 10D) and exhibited fine cytoplasmic striations (Fig. 10E, v1) morphologically distinct from the “classical” clathrin coat (Fig. 10, v2). In contrast, on the basolateral plasma membrane, caveolar profiles, highlighted by tannic acid (see “Experimental Procedures”), were more abundant than coated vesicle profiles (Fig. 10B). Some basolateral caveolae were seen near the cell surface apparently not connected to the plasma membrane (Fig. 10C), others had open stomata or displayed a characteristic straight trilaminar fusion membrane at the contact with the plasmalemma (Fig. 10F).

Unlike MDCK cells, FRT cells do not have plasmalemmal caveolae (15) and lack both caveolin-1 protein and mRNA (21, 22). Fig. 10 (G and H) shows that expression of caveolin-1 in FRT cells induces the morphogenesis of plasmalemmal caveolae with the same predominantly basolateral distribution as described for MDCK cells. Coated pits/vesicles are practically the only vesicle type observed on the apical plasmalemma (Fig. 10G), whereas both caveolae and coated pits/vesicles are found on the basolateral plasma membrane (Fig. 10H). The surface distributions of caveolae and coated pits/vesicles were quantified and are presented in Table I. Caveolae were vastly more frequent on basolateral membranes than on apical membranes, by a factor of over 99.3:1–99.5:1 in FRT cells and 99.7:1 in MDCK cells. In contrast, coated vesicles were almost evenly distributed on apical and basolateral surfaces in both cell lines.

Fig. 10 (I and J) shows the structure of plasmalemmal caveolae induced by the expression of caveolin-1 in FRT cells. Most caveolae had either open stoma or an elongated neck with a sharply angled contact with the plasmalemma. Some caveolae exhibited distinctive neck diaphragms, which may represent intermediate stages in the fusion to or fission from the plasma membrane.

As demonstrated by the quantitative data in Table I, wild type FRT cells that express low levels of caveolin-2 but not caveolin-1 displayed practically no plasmalemmal caveolae. Overexpression of caveolin-2 in FRT cells after transfection of full-length myc-caveolin-2 resulted in an approximately 4-fold increase in the total level (endogenous plus exogenous) of this protein (Fig. 1). Nonetheless, the electron microscopy experiments indicated that overexpression of caveolin-2 (full-length or β isoform) in FRT cells did not induce the assembly of plasmalemmal caveolae (Fig. 10, K and L).

DISCUSSION

The functional significance of the existence of various caveolin types is not understood. Because caveolin-1 and caveolin-2 are three-layered (J, 2) diaphragms or caveolae with open stomata (J, 3) or an elongated neck (J, 4). Note the sharp angular edges at the bent rims of caveolae (J, 1–4). Overexpression of full-length caveolin-2 (K and L) in FRT cells does not promote the morphogenesis of plasmalemmal caveolae.

Note the absence of caveolar profiles both on apical (Ap) and basolateral (Bl) plasmalemmal membrane domains. The appearance of the membrane is exactly the same as in wild type FRT cells. Bars indicate 0.5 μm (A), 0.1 μm (B–F), 0.25 μm (G, H, K, and L), or 0.05 μm (I and J).
are expressed in similar tissues and the expression of both is increased simultaneously upon differentiation of adipocytes (9), it is likely that they may have either redundant or complementary functions. In this report, we took advantage of the availability of the FRT cell line, which expresses no caveolin-1 and low levels of caveolin-2, to study the effects of caveolin-1 expression on the subcellular localization and biochemical properties (oligomerization, association with rafts) of caveolin-2.

We show that, in confluent FRT cells, caveolin-2, either endogenous or overexpressed by transfection or adenovirus transduction of its cDNA, has a predominant localization at a juxtanuclear compartment that is co-labeled with markers of the Golgi cisternae (Man II, GOS-28) and the TGN (TGN-38). A similar localization of caveolin-2 was observed in polarized MDCK cells; for these experiments we used myc-tagged caveolin-2, because our caveolin-2 antibody recognized human and rat caveolin-2 but not canine caveolin-2. Control experiments showed that myc-tagged caveolin-2 had an identical localization as endogenous and overexpressed wild type caveolin-2 in FRT cells. In contrast to caveolin-2, caveolin-1 had a predominant plasma membrane localization in both polarized FRT and MDCK cells. Importantly, a substantial fraction of caveolin-2 changed its distribution from the Golgi complex to the plasma membrane in the presence of caveolin-1, in agreement with previous data that demonstrate an interaction of the two caveolins (8, 10, 29). This differential localization of caveolins -1 and -2 was striking because it was recently shown that in a nonpolarized fibroblastic cell line (3T3-L1 cells), epitope-tagged caveolin-2 and endogenous caveolin-2 are strictly co-localized with endogenous caveolin-1, both intracellularly and at the level of the plasma membrane (9, 10). This suggests that the differential localization of caveolins-1 and -2 may be at least in part the result of the polarized epithelial cell phenotype. Indeed, experiments with subconfluent MDCK and FRT cells detected a large component of intracellular caveolin-1 in a juxtanuclear localization (data not shown). Because it is now known that caveolin-1 and caveolae are dynamic structures that can be internalized under certain conditions (30, 31), the different localization of caveolins in polarized cells may reflect different recycling patterns of the proteins or their preferential utilization for different purposes, e.g. new membrane synthesis and post-Golgi transport in growing subconfluent cells and plasma membrane signaling processes in quiescent confluent cells.

Because in both MDCK and FRT cells, the distribution of both caveolins appears to overlap at the level of the intracellular compartment, we speculate that they may cooperate specifically to carry out an intracellular function, perhaps protein sorting in the TGN. Perhaps a specific function of the caveolin-2 is to help in the formation of large oligomers, which may be essential for a sorting function. Oligomerization of caveolin is best understood for caveolin-1 and occurs in two steps; in the first step the protein forms oligomers of about 200 kDa in the endoplasmic reticulum, and in the second step, which occurs later, probably in the Golgi apparatus, it forms very large oligomers (> 600 kDa) that are detergent insoluble (18, 20). The ability to form smaller oligomers depends on sequences in the N-terminal domain, whereas the ability to form very large oligomers depends on the palmitoylation of cysteine residues in the C-terminal domain. Our data show that, when expressed singly, caveolin-2 sediments in a velocity gradient with an apparent molecular mass of 66–450 kDa, indicating that it can form homo-oligomers of up to ~24 molecules. However, in the presence of caveolin-1, caveolin-2 sediments with an apparent molecular mass of 200–660 kDa (oligomers up to ~36 molecules), indicating that the interaction of the two proteins promotes both more efficient oligomerization and the formation of larger oligomers. In part, this may be due to a differential ability to bind to cholesterol. In contrast to caveolin-1, which binds cholesterol and associates with LDTIM or rafts (see Introduction), caveolin-2 does not associate with LDTIM unless caveolin-1 is also expressed (Fig. 9). These experiments suggest that caveolin-2 lacks the intrinsic ability to bind cholesterol or LDTIM and that its association with rafts may depend on its interaction with caveolin-1. The regulation of the association with caveolin-1 may have important functional consequences, because Scheiffele et al. (8) have presented data suggesting that large homo-oligomers of caveolin-1 are involved in apical sorting whereas hetero-oligomers of caveolin-1 and caveolin-2 might be involved in basolateral sorting.

Another important difference between caveolin-1 and caveolin-2 highlighted by the experiments in this report appears to lie in their ability to promote the formation of caveolae. FRT cells lacking caveolin-1 have no caveolae (15, 22). Previous work has shown that transfection of caveolin-1 into FRT cells promotes the assembly of typical plasma-membrane caveolae (15), in agreement with results in other cell lines from other laboratories (see the Introduction). The results presented here demonstrate that overexpression of caveolin-2 in FRT cells does not have the same effect; no plasma-membrane caveolae are formed (Fig. 10). Quantitation of caveolae in both MDCK cells and FRT cells indicates that the numbers of caveolae promoted by caveolin-1 in FRT cells are roughly similar to the number of caveolae found constitutively in MDCK cells. Intriguingly, these results are different from those recently reported by Vogel et al. (16) for Caco-2 cells, which also lack caveolae and caveolin-1. In intestinal Caco-2 cells, transfection of caveolin-1 promotes assembly of caveolae, but the levels observed are vastly different from those in MDCK cells. Because Caco-2 cells lack caveolin-2, these data, taken together with the data in this report, suggest that an association between caveolin-1 and caveolin-2 may promote a more efficient assembly of caveolae. Alternatively, Caco-2 cells may lack an additional component that facilitates assembly of caveolae. Intriguingly, although it appears to localize at both apical and basolateral surfaces, caveolin-1 promotes the formation of typical caveolae almost exclusively at the basolateral plasma membrane of FRT cells. This is the same localization observed in MDCK cells (Ref. 16 and this work) and in transfected Caco-2 cells. One possible explanation of this observation may be that the critical concentration of caveolin-1 needed for formation of caveolae is only reached at the basolateral surface. Alternatively, if caveolin-1/2 hetero-oligomers are indeed preferentially targeted basolaterally (8), this may provide additional support for a requirement for both caveolins in the formation of caveolae. Polarized caveolar formation may also reflect the different lipid composition of apical and basolateral membranes (32). The very high concentration of glycosphingolipids and cholesterol at the apical surface may result in a very rigid membrane that cannot be easily folded into invaginated caveolae. On the other hand, basolateral membranes may allow patches of GSL/cholesterol to be folded into caveolae with the more flexible phospholipids acting as hinges. Additionally, differences in the submembrane cytoskeleton present in apical and basolateral membranes (33) may restrict differentially the formation of apical caveolae. Finally, apical membranes may lack a caveolar component essential for the formation of caveolae. The known functional properties of caveolins and their fascinating variations in subcellular distribution guarantee that further exploration will result in novel roles in intracellular trafficking and polarized protein and lipid sorting.

Acknowledgments—We gratefully acknowledge Lee Cohen-Gould and Peg McLaughlin for the expert assistance with electron microscopy.
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Golgi Localization of Caveolin-2