Caveolin Forms a Hetero-Oligomeric Protein Complex That Interacts with an Apical GPI-linked Protein: Implications for the Biogenesis of Caveolae

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Abstract. Glycosyl-phosphatidylinositol (GPI)-linked proteins are transported to the apical surface of epithelial cells where they undergo cholesterol-dependent clustering in membrane micro-invaginations, termed caveolae or plasmalemmal vesicles. However, the sorting machinery responsible for this caveolar-clustering mechanism remains unknown. Using transfected MDCK cells as a model system, we have identified a complex of cell surface molecules (80, 50, 40, 22–24, and 14 kD) that interact in a pH- and cholesterol-dependent fashion with an apical recombinant GPI-linked protein. A major component of this hetero-oligomeric protein complex is caveolin, a type II transmembrane protein. As this hetero-oligomeric caveolin complex is detectable almost immediately after caveolin synthesis, our results suggest that caveolae may assemble intracellularly during transport to the cell surface. As such, our studies have implications for understanding both the intracellular biogenesis of caveolae and their subsequent interactions with GPI-linked proteins in epithelia and other cell types.

GLYCOSYL-phosphatidylinositol (GPI)[1] is attached to the COOH terminus of a diverse group of proteins, where it acts to anchor the protein's ectodomain to cellular membranes (Cross, 1990; Ferguson, 1991; Low, 1989). After transport to the cell surface, GPI-anchored proteins are excluded from clathrin-coated pits, but undergo cholesterol-dependent clustering in another membrane microinvagination, termed plasmalemmal vesicles or caveolae (Bretscher et al., 1980; Anderson et al., 1992; Rothberg et al., 1990*b*). To date, only three non-GPI-linked protein components of caveolae have been identified. These include the cell surface 230-kD IP_3 receptor, the plasma membrane Ca^{2+} ATPase, and caveolin-a type II transmembrane protein (Fujimoto et al., 1993; Fujimoto, 1993; Rothberg et al., 1992).

Caveolin is an important structural component of caveolae and forms part of their cytoplasmic coat, helping to maintain their characteristic striped bipolar structure (Rothberg et al., 1992). Multiple species of caveolin (22–29 kD) have been identified and all are identical in protein sequence, but differ in some unknown modification which leads to a decrease in electrophoretic mobility (Glenney and Soppet, 1992; Kurzchalia et al., 1992). This modification may represent phosphorylation as caveolin was originally identified as a major v-Src substrate (Glenney, 1989; Glenney and Zokas, 1989). Recent immunolocalization studies reveal that although caveolin is present predominantly in cell surface micro-invaginations (plasmalemmal caveolae) it is also localized in endosome-like structures and in tubulovesicular structures within the TGN (Kurzchalia et al., 1992; Rothberg et al., 1992). In accordance with these morphological observations, biochemical studies indicate that caveolin is present in roughly equal quantities in apically or basolaterally destined TGN transport vesicles isolated from MDCK cells (Kurzchalia et al., 1992). These results suggest that caveolae form intracellularly at the level of the Golgi and may represent a subclass of exocytic–endocytic vesicular carriers that shuttle between the cell surface and the *trans*-Golgi. This hypothesis is consistent with the proposed transcytotic function of caveolae in endothelial cells, where they are thought to form patent *trans*-endothelial channels (Severs, 1988).

In epithelial cells, GPI-anchored proteins are selectively transported to the luminal or apical plasma membrane and are excluded from the basolateral membrane (Lisanti et al., 1988; Lisanti et al., 1990*a*,*b*). Using the model renal epithelial cell line, MDCK, several independent reports demonstrate that GPI-anchorage is sufficient to target the attached protein to the apical surface, acting as a dominant apical trafficking signal (Brown et al., 1989; Lisanti et al., 1989, 1991*a*; Simons and Wandinger-Ness, 1990; Rodriguez-Boulan and Powell, 1992).

During transport to the apical surface, GPI-anchored pro-

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1. *Abbreviations used in this paper*: ConA^r, ConA-resistant; DTSSP, 3,3′-Dithio*bis*(sulfo-succinimidylpropionate); GPI, glycosyl-phosphatidylinositol.

teins become resistant to solubilization by non-ionic detergents, i.e., Triton X-100 (Hooper and Turner, 1988), at the level of the early Golgi (Brown and Rose, 1992). Insolubility also occurs in non-epithelial cells, suggesting that this association is not epithelial cell specific (Hoessli and Rungger-Brandle, 1985). Similarly, biophysical studies demonstrate that the newly arrived population of an apical GPI-linked protein is immobile, while the steady-state population is freely diffusible (Hannan et al., 1993; Lisanti and Rodriguez-Boulan, 1992). Such immobility appears to depend on acidic pH as (*a*) immobilization of the newly arrived population can be inhibited by a weak base (chloroquine); and (*b*) immobilization of the steady-state population can be induced by slightly acidic extracellular pH (6.5). In addition, immobilization does not occur in a mutant MDCK cell line (Hannan et al., 1993), that selectively missorts GPI-anchored proteins (Lisanti et al. 1990*b*). As GPI-linked proteins cluster in cell surface caveolae, the above insolubility and immobility could reflect an association between GPI-linked proteins and caveolar transmembrane components.

To evaluate this possibility, we have pursued a chemical cross-linking strategy. Using MDCK cells recombinantly expressing of an apical GPI-linked protein, we identify here a hetero-oligomeric complex of cell surface protein molecules that contains caveolin and associates with a model GPI-linked protein in a pH- and cholesterol-dependent manner. We suggest that these caveolin hetero-oligomers may represent the assembly units of caveolar membrane domains. As such, our results provide the first demonstration of a direct interaction between a GPI-linked molecule and a caveolar protein complex at the apical surface of epithelial cells.

Materials and Methods

Materials

Sulfo-NHS biotin and DTSSP (3,3'-Dithio*bis*[sulfo-succinimidylpropionate]) were from Pierce (Rockford, IL). Polyclonal and monoclonal antibodies directed against the gD-1-ectodomain were described (Lisanti et al., 1989). Anti-caveolin IgG (mAb clone 2234) was as described and generously provided by John Glenney (University of Kentucky College of Medicine, Lexington, KY) (Glenney, 1989; Glenney and Zokas, 1989). Recombinant PI-PLC (from *Bacillus thuringiensis*) was the generous gift of Martin Low (Columbia University, New York). Nystatin was tissue culture grade obtained from Sigma (St. Louis, MO). Fetal bovine lipoprotein deficient serum was from Organon Tecknika (Biotechnology Research Institute, Bethesda MD) and was used at the same concentration as normal serum.

Cell Culture

Wild-type and mutant (Con A-resistant; ConA^r) MDCK cells expressing the gD-1-DAF fusion protein were derived by transfection and characterized previously (Hannan et al., 1993; Lisanti et al., 1989). Growth media consisted of DME supplemented with antibiotics (penicillin and streptomycin only) and 10% FBS. MDCK cells were grown to confluence for 2–3 d in 6-well dishes or for 4 d on 24.5-mm tissue culture inserts (Transwells; Costar Corp., Cambridge, MA) before overnight induction of the GPI-anchored fusion protein with 10 mM sodium butyrate (Gorman and Howard, 1983; Gottlieb et al., 1986). To upregulate endogenous cholesterol synthesis, MDCK cells expressing gD-1-DAF were grown in normal medium supplemented with 10% lipoprotein-deficient FBS and passaged three to five times at a dilution of 1:7 before use in experiments.

Metabolic Labeling

For metabolic labeling, cells were starved in media lacking methionine and cysteine for 30 min, and pulsed with 1 mCi/ml Expre^{35}S^{35}S label in the same media for 15 or 30 min at 37°C as described previously (Lisanti et al., 1989). Metabolically labeled proteins were detected after SDS-PAGE (10% acrylamide gels) by fluorography using sodium salicylate (Chamberlain, 1979).

Surface Biotinylation

MDCK cells were surface labeled with the biotinylating reagent, sulfo-NHS-biotin (Lisanti et al., 1988; Sargiacomo et al., 1989). Briefly, sulfo-NHS-biotin (0.5 mg/ml; 1 ml) was added to the surface to be labeled in ice-cold PBS containing 0.1 mM $CaCl_2$ and 1 mM $MgCl_2$ (PBS$^+$). After 30 min on ice, the solution was removed and remaining unreacted biotin quenched by incubation with ice cold serum-free DME. After quenching, cells were washed twice with PBS$^+$ and subjected to cell extraction.

Detection of Endogenous GPI-linked Proteins

Endogenous MDCK GPI-anchored protein were isolated through release with PI-specific phospholipase C after cell surface biotinylation, as described (Lisanti et al., 1988, 1990*b*); cell lysates were prepared by extraction for 20 min at 37°C with buffers containing 1% Triton X-114 and multiple protease inhibitors (Lisanti et al., 1988, 1990*b*), a condition that effectively solubilizes GPI-linked proteins (Brown and Rose, 1992).

Transport Assay

To study the newly arrived population of gD-1-DAF, we used an assay we previously designed in which the steady-state population of gD-1-DAF is masked by repeated cell surface exposure (6×) to sulfo-SHPP (lacking a biotin moiety) (Lisanti et al., 1990*a*). After quenching cell surface amino groups with sulfo-SHPP, only newly arrived amino groups are detected after warming to 37°C, followed by cell surface biotinylation. After various times of chase in normal growth medium, cells were washed with PBS$^+$, and biotinylated on ice for 30 min as described above. Of the newly arrived population of gD-1-DAF, 95% is due to biosynthetic delivery while the remaining 5% may represent a recycling pool (Lisanti et al., 1990*a*).

Cell Extraction

Cells were extracted for 30 min on ice at acidic pH with 1 ml of MBST (25 mM Mes, pH 6.5, 0.15 M NaCl, 1% Triton X-100) or at alkaline pH with 1 ml of TBST (10 mM Tris-HCl, pH 8.0, 0.15 M NaCl, 1% Triton X-100). Similarly, cells were re-extracted or directly lysed in the above buffers plus 60 mM octyl-glucoside. See figure legends for the details of individual experiments. This concentration of octyl-glucoside maximally solubilizes GPI-linked proteins (Hooper and Turner, 1988). All extraction solutions contained 1 mM PMSF.

Chemical Cross-linking

After biotinylation, cells were subjected to sequential detergent extraction and chemical cross-linking (on ice without agitation). Cells were first extracted for 30 min with 1 ml MBST, followed by cross-linking for 30 min with DTSSP (0.5–1 mg/ml in MBST; 1 ml). After cross-linking, the monolayer was extracted for 30 min with 1 ml TBST plus octyl-glucoside. This final extraction at alkaline pH was subjected to immunoprecipitation. All solutions contained protease inhibitors. A stock solution of DTSSP (200 mg/ml in DMSO) was aliquoted and stored frozen at −20°C.

In some experiments, cells were incubated after biotinylation with nystatin (50 μg/ml dissolved in PBS$^+$). After 5 min at 25°C, the nystatin solution was removed and the cells were extracted and cross-linked as described above. A stock solution of nystatin (50 mg/ml in DMSO) was stored at −20°C and diluted 1:1,000 with PBS$^+$ just before use. The nystatin concentration and incubation time/temperature were identical to those described previously for the selective morphological disruption of caveolae (Rothberg et al., 1992).

Immunoprecipitation

Cell lysates were precleared overnight with protein A-Sepharose at 4°C and then immunoprecipitated with a 1:150 dilution of rabbit polyclonal anti-gD-1 IgG or a 1:400 dilution of mouse monoclonal anti-caveolin IgG. Antibodies were pre-bound to protein A-Sepharose overnight in 1 ml TBST before addition to lysates. In the case of anti-caveolin, rabbit anti-mouse IgG (1:100 dilution; Cappel, Inc. Malvern, PA) was first pre-bound to protein

A-Sepharose. Immunoprecipitates were washed 6× with 1 ml MBST or TBST, depending on the pH at which the cell lysate was prepared, and analyzed by SDS-PAGE (10% acrylamide gels) under reducing conditions as described (Lisanti et al., 1989). It was not possible to elute gD-1-DAF immunoprecipitates with SDS and reprecipitate the eluates with anti-caveolin or visa versa, as immunoprecipitation with either gD-1 or caveolin IgG was prevented by even small amounts of residual SDS (not shown).

Streptavidin Blotting

After SDS-PAGE (10% acrylamide) and transfer to nitrocellulose, biotinylated proteins were detected by [^{125}I]streptavidin and autoradiography (Sargiacomo et al., 1989).

Immunoblotting

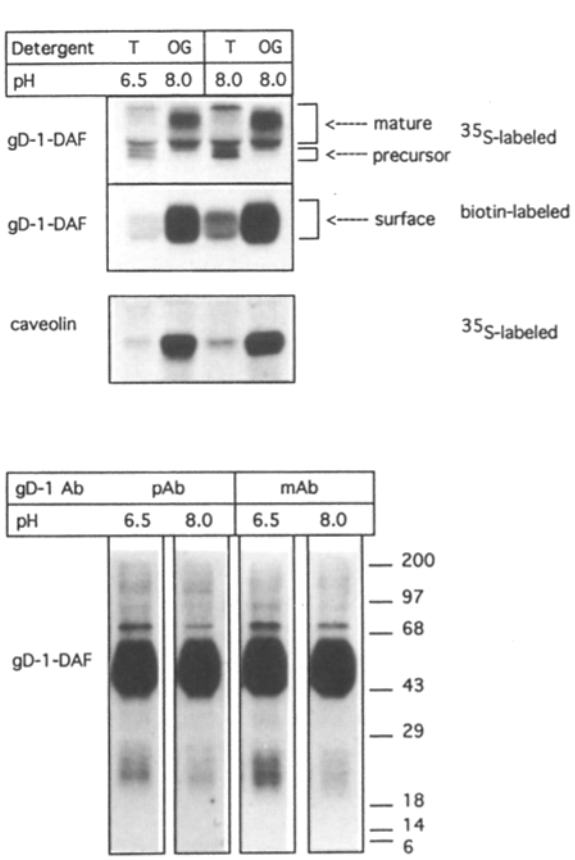
Blots previously probed with streptavidin were rehydrated and reblocked with BSA/non-fat dry milk solutions and incubated with a 1:500 dilution of anti-caveolin antibodies. Bound antibodies were visualized with alkaline phosphatase conjugated goat anti-mouse IgG and the appropriate substrates as described by the manufacturer (Promega Biotec, Madison, WI). These secondary antibodies did not cross-react with the rabbit anti-gD-1 IgG used for immunoprecipitation of gD-1-DAF.

Results and Discussion

Detergent Insolubility of a GPI-anchored Protein and Caveolin

To identify protein molecules that may be involved in the immobilization of GPI-linked proteins, we have used MDCK cells expressing a GPI-anchored fusion protein (gD-1-DAF) as a model system. We used the observation that GPI-anchored protein are predominantly insoluble in Triton X-100 under certain extraction conditions at 4°C, but are effectively solubilized by octyl-glucoside containing buffers (Hooper and Turner, 1988). In addition we investigated the effect of pH on insolubility and co-precipitation, as slightly acidic (pH 6.5) induces the immobilization of up to 90% of an apical GPI-anchored protein in FRAP experiments (Hannan et al., 1993).

For these experiments, MDCK cells were sequentially extracted—first in Triton containing buffers at either acidic (6.5) or alkaline (8.0) pH, followed by a second round of extraction in octyl-glucoside containing buffers at alkaline pH. We find that the mature or cell surface form of gD-1-DAF (45–50 kD) is Triton insoluble, but is selectively solubilized by octyl-glucoside. In contrast, the precursor form of gD-1-DAF (42 kD) is completely Triton soluble (Fig. 1 *A*). Although these two forms differ in the addition of N-linked glycans, both are GPI-linked (Lisanti et al., 1990*a*). Qualitatively similar results were observed if the initial extraction was performed at slightly acidic or slightly alkaline pH. However, roughly twice as much of the mature or cell surface form of gD-1-DAF was solubilized at alkaline pH, suggesting that low pH enhances Triton insolubility. Similarly, we find that caveolin is Triton insoluble, but efficiently solubilized by octyl-glucoside, suggesting that the mature form of gD-1-



labeled amino acids) or surface labeled (with sulfo-NHS biotin) and subjected to sequential detergent extraction. Cells were first extracted in Triton-containing buffers at acidic (pH 6.5) or alkaline (pH 8.0) and then re-extracted with octyl-glucoside containing buffers at alkaline (pH 8.0). All extracts were then subjected to immunoprecipitation with either anti-gD-1 or anti-caveolin antibodies. Immunoprecipitates were washed at alkaline pH and metabolically labeled proteins visualized after SDS-PAGE by fluorography. Note that both the mature form of gD-1-DAF and caveolin are Triton insoluble, while the precursor form of gD-1-DAF is Triton soluble. In addition, insolubility in Triton is enhanced by extraction at acidic pH. (Note that in Triton-extracted samples, a small amount of the mature form of gD-1-DAF is present, but migrates anomalously because of the presence of co-migrating unlabeled IgG heavy chain used for immunoprecipitation. The IgG heavy chain appears as a negative band splitting the mature form of gD-1-DAF). Similarly, the cell surface form of gD-1-DAF was Triton insoluble and efficiently solubilized by octyl-glucoside, as visualized by [^{125}I]streptavidin blotting/autoradiography. (*B*) pH-dependent co-precipitation of MDCK proteins with gD-1-DAF. MDCK cells were surface biotinylated and extracted at acidic (6.5) or alkaline pH (8.0) in buffers containing octyl-glucoside. Extracts were then immunoprecipitated with either polyclonal (*pAb*) or monoclonal (*mAb*) antibodies directed against the ectodomain of gD-1. Immunoprecipitates were washed at the same pH at which the extract was prepared. Biotinylated proteins were detected after immunoprecipitation of gD-1-DAF by SDS-PAGE and blotting with [^{125}I]streptavidin/autoradiography. Note that endogenous MDCK cell surface proteins of 80 and 22–24 kD coprecipitate with gD-1-DAF. Co-precipitation was most efficient at acidic pH. Additional control experiments in which we attempted to immunoprecipitate gD-1-DAF from untransfected MDCK cells did not reveal these co-precipitating proteins (not shown). Furthermore, co-precipitation did not occur if cells were frozen at −20°C before lysis and immunoprecipitation at pH 6.5 (not shown).

Figure 1. Differential detergent extractability of gD-1-DAF and pH-dependent coprecipitation with endogenous MDCK cell surface proteins. (*A*) Differential solubility of gD-1-DAF and caveolin. MDCK cells were metabolically labeled overnight (with ^{35}S-

DAF and caveolin may be present in the same Triton-insoluble complex (Fig. 1 *A*).

Observations of differential detergent extractability have been made for other GPI-linked proteins (Brown and Rose, 1992; Hooper and Turner, 1988). However, these investigators did not examine the pH dependence of Triton insolubility or the solubility of caveolin.

pH-dependent Co-precipitation of 80- and 22–24-kD Cell Surface Components with a GPI-linked Protein

To detect cell surface molecules that may associate with the steady-state population of gD-1-DAF in a pH-dependent fashion, we performed cell surface labeling with sulfo-NHS-biotin followed by immunoprecipitation at pH 6.5 or 8.0 after lysis with octyl-glucoside. Biotinylated proteins were visualized by blotting with [^{125}I]streptavidin. Using these conditions, cell surface proteins of 80 kD and 22–24-kD co-precipitated with gD-1-DAF (Fig. 1 *B*). Virtually identical results were obtained with two different antibodies that recognize the gD-1 ectomain (polyclonal and monoclonal), indicating that co-precipitation is independent of antibody specificity. Co-precipitation was most dramatic at low pH, consistent with the notion that low pH stabilized the complex. In addition, these co-precipitating proteins were not detected when surface-labeled untransfected MDCK cells were used for gD-1-DAF immunoprecipitation (not shown).

Association of a GPI-linked Protein with a Hetero-oligomeric Caveolin Complex

We next combined the observations of differential detergent extractability and pH-dependent co-precipitation with a chemical cross-linking strategy. For chemical cross-linking we chose DTSSP—a water soluble, thiol-cleavable, homobifunctional reagent—that reacts with free amino groups. We reasoned that after chemical cross-linking, molecules that associate with gD-1-DAF would be covalently coupled, increasing their efficiency of co-precipitation at alkaline pH. After immunoprecipitation of gD-1-DAF, proteins that associate would be released by reduction of the complex.

MDCK cells were surface biotinylated, extracted and cross-linked at acidic pH in Triton and finally solubilized at alkaline pH in octyl-glucoside. These octyl-glucoside extracts were then subjected to immunoprecipitation with anti–gD-1-antibodies. Using this scheme we detected Triton-insoluble surface proteins of 80, 40, 22–24, and 14 kD co-precipitating with gD-1-DAF (Fig. 2). Increasing concentrations of cross-linker (0.5–8 mg/ml) did not lead to the detection of any additional co-precipitating components. However, it resulted in preferential co-precipitation of the prominent 22–24-kD component as the other components of the complex may have been extensively cross-linked and failed to enter the gel.

As caveolin is of a similar molecular weight to the 22–24-kD component of this complex, we suspected that it might represent the cell surface form of caveolin. To test this hypothesis, blots of gD-1-DAF immunoprecipitates previously probed with streptavidin (Fig. 3 *A*), were reprobed with anti–caveolin antibodies and bound antibodies visualized via alkaline phosphatase–conjugated secondary antibodies. These experiments indicate that a 22-kD species corresponding to caveolin is found in gD-1-DAF immunoprecipitates

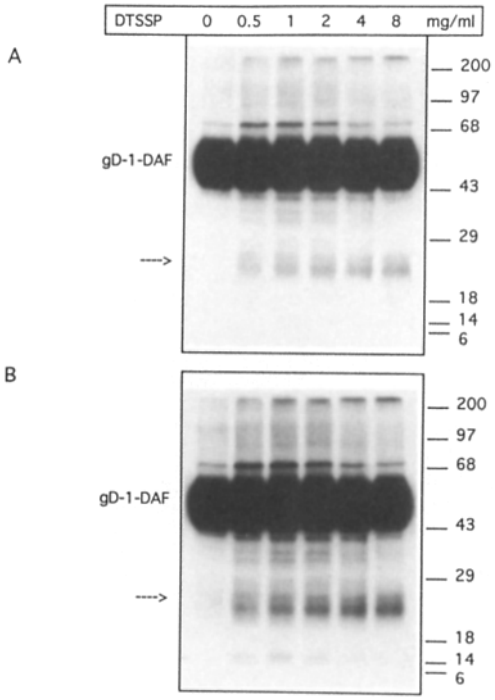


Figure 2. Chemical cross-linking of gD-1-DAF to endogenous MDCK surface proteins. MDCK cells were surface biotinylated and subjected to sequential detergent extraction and chemical cross-linking. Cells were first extracted at acidic pH (6.5) in Triton-containing buffers, followed by cross-linking with DTSSP (0–8 mg/ml) in the same buffer, and finally re-extracted at alkaline (pH 8.0) in octyl-glucoside containing buffers. The final extract was immunoprecipitated with antibodies to gD-1-DAF. Immunoprecipitates were washed at alkaline pH. Proteins cross-linked to gD-1-DAF were released by reduction immediately before SDS-PAGE and biotinylated proteins were visualized by streptavidin blotting. Note that endogenous MDCK surface proteins of 80, 40, 22–24, and 14 kD associate with gD-1-DAF during chemical cross-linking. Cross-linking was maximal with 0.5–1 mg/ml DTSSP. Two autoradiographic exposures are shown to better illustrate the relative abundance of the associated proteins.

previously cross-linked with DTSSP (Fig. 3 *B*). In addition, we subjected cross-linked cell extracts first immunoprecipitated with gD-1-DAF to a second round of immunoprecipitation with anti–caveolin antibodies (Fig. 3 *C*). Cross-linked samples reprecipitated with anti–caveolin antibodies were significantly depleted (~50–60%) of this 22–24-kD component, in accordance with the idea that it had been immunoabsorbed by initial precipitation with anti-gD-1. In contrast, uncross-linked controls reprecipitated with anti–caveolin IgG revealed a 22–24-biotinylated protein, corresponding to caveolin. Additional control experiments indicated that chemical cross-linking did not affect the ability of anti–caveolin IgG to recognize caveolin present in cross-linked samples (not shown). As up to 90% of cell surface gD-1-DAF can be immobilized by lowering the extracellular pH (Hannan et al., 1993), these results support our observation that caveolin is immunodepleted by gD-1-DAF antibodies after chemical cross-linking.

Taken together, these results identify caveolin as the 22–24-kD component associated with gD-1-DAF at the cell surface. As gD-1-DAF contains 12 extracellular free amino

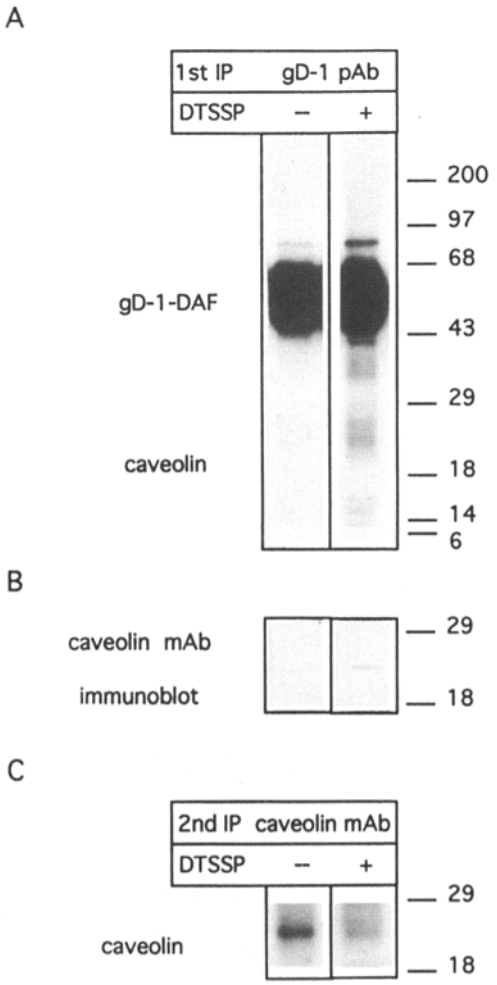


Figure 3. Identification of caveolin as the 22–24 kD component that associates with gD-1-DAF. (*A*) gD-1-DAF cross-linking. MDCK cells were surface biotinylated, subjected to chemical cross-linking, and gD-1-DAF immunoprecipitated as described in the legend of Fig. 2. Cross-linking was −/+ 1 mg/ml DTSSP. Note the coprecipitating proteins of 80, 40, 22–24, and 14 kD. (*B*) Caveolin immunoblotting. The blot of the corresponding autoradiograph shown in *A* was reprobed with a mAb to caveolin. Bound antibodies were visualized with an appropriate secondary antibody conjugated to alkaline phosphatase. Note that a single 22-kD species corresponding to the unmodified form of caveolin is observed only in samples cross-linked with DTSSP. The caveolin mAb preferentially recognizes the 22-kD species of caveolin by blotting (Glenney and Zokas, 1989), while additional larger forms may be visualized by immunoprecipitation. (*C*) Caveolin immunoprecipitation. The supernatants of gD-1-DAF immunoprecipitates shown in *A* were subjected to a second round of immunoprecipitation with anti-caveolin IgG and biotinylated caveolin visualized by streptavidin blotting. Note that uncross-linked samples reprecipitated with anti-caveolin identify a 22–24-kD component corresponding to surface-labeled caveolin. In contrast, cross-linked samples reprecipitated with anti-caveolin antibodies are substantially depleted of this 22–24-kD component, due to immunoabsorption by the first immunoprecipitation with anti-gD-1 IgG. Additional control experiments indicated that chemical cross-linking did not effect recognition of caveolin by anti-caveolin IgG.

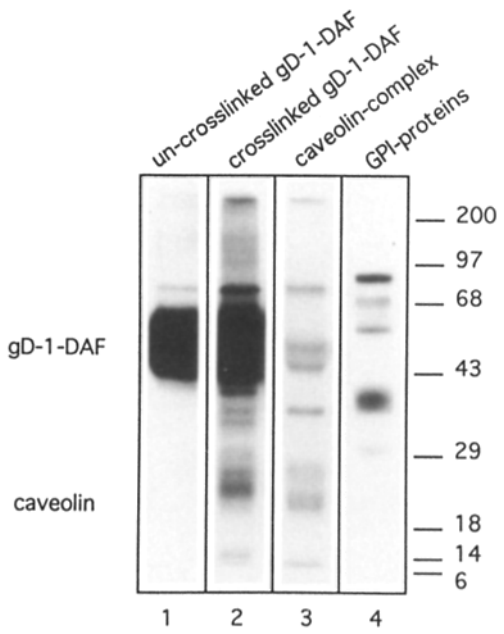


Figure 4. Caveolin is part of a hetero-oligomeric complex of MDCK cell surface proteins. gD-1-DAF cross-linking (lanes *1* and *2*). MDCK cells were subjected to biotinylation, extraction, and chemical cross-linking (−/+ 1 mg/ml DTSSP) as described in the legend of Fig. 2 and immunoprecipitated with antibodies to gD-1. caveolin hetero-oligomers (lane *3*). Untransfected MDCK cells were biotinylated, pre-extracted at acidic pH in Triton-containing buffers, and alkaline (pH 8.0) octyl-glucoside extracts immunoprecipitated with anti-caveolin IgG. Note that proteins of 80-, 50-, 40-, and 14-kD co-precipitate with caveolin without the aid of chemical cross-linking. Similar results were obtained if cells were directly extracted in alkaline octyl-glucoside containing buffers. Note that bands of similar molecular weights associate with gD-1-DAF, however, association with gD-1-DAF is dependent on chemical cross-linking. Control experiments in which the primary (rabbit anti-mouse IgG) or secondary antibody (anti-caveolin IgG) were omitted demonstrated that co-precipitation of these caveolin-associated components is specific (not shown). Similar controls with an irrelevant mouse mAb also demonstrated specificity. MDCK GPI-linked proteins (lane *4*). We compared the mobility of the elements of caveolin hetero-oligomers with endogenous MDCK GPI-anchored proteins (prepared after biotinylation through release with PI-specific phospholipase C). Note that the components of caveolin hetero-oligomers do not comigrate with endogenous MDCK GPI-linked proteins.

groups for potential biotinylation, while caveolin only contains three extracellular free amino groups—this difference must be taken into account when the relative abundance of gD-1-DAF and caveolin in these complexes is compared.

As caveolin appeared to represent one of the components complexed with gD-1-DAF, we wondered whether caveolin might form a hetero-oligomeric complex with the other cell surface components. To test this hypothesis, untransfected MDCK cells were surface labeled and immunoprecipitated with anti-caveolin antibodies. These experiments indicate that caveolin (22–24 kD) co-precipitates with proteins of 80, 50, 40, and 14 kD, without chemical cross-linking (Fig. 4, lane *3*). Association of this caveolin complex was independent of pH, as similar results were obtained by immunoprecipitation with anti-caveolin IgG at either acidic or alkaline

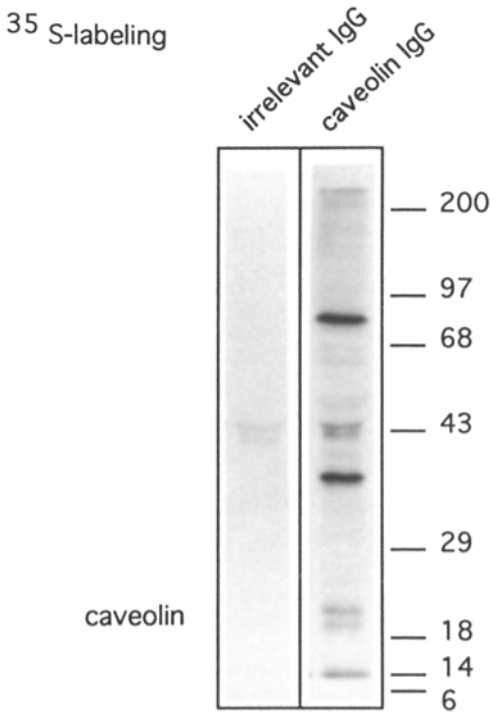


Figure 5. Caveolin hetero-oligomers form early after caveolin synthesis. Untransfected MDCK cells were pulse-labeled with ^{35}S-labeled amino acids for 15 min. Cells were extracted first at acidic (pH 6.5) in Triton-containing buffers and then re-extracted at alkaline (pH 8.0) in octyl-glucoside-containing buffers. Octyl-glucoside extracts were immunoprecipitated with anti-caveolin antibodies and labeled proteins visualized by fluorography. Note that newly synthesized caveolin co-precipitates as a complex with other proteins (80, 40, and 14 kD). Control experiments with an irrelevant IgG (gD-1 mAb) did not reveal any co-precipitating proteins.

pH. Note that the additional 50-kD species we detect by caveolin immunoprecipitation would have been masked by co-migrating gD-1-DAF. These results argue that caveolin exists as a hetero-oligomeric protein complex with other cell surface Triton-insoluble components.

As multiple GPI-anchored proteins are found in caveolae (Ying et al., 1992), the co-precipitating proteins we detect could represent endogenous GPI-anchored proteins. To test this hypothesis, we prepared surface biotinylated endogenous GPI-anchored proteins from untransfected MDCK cells through release with PI-specific phospholipase C and compared their mobility with the components that co-precipitate with gD-1-DAF or caveolin (Fig. 4, lanes 2–4). Note that none of the endogenous GPI-anchored co-migrated with these components, suggesting that the major cell surface components of this complex are non-GPI linked.

In some of our experiments, we detected a >200-kD species co-precipitating with gD-1-DAF or caveolin. As a 230-kD form of the IP_3 receptor has recently been shown to localize to cell surface caveolae and can be efficiently surface labeled with sulfo-NHS-biotin (Fujimoto et al., 1993), the band we observe may correspond to the IP_3 receptor. This possibility will be tested as specific antibodies become available.

Caveolin Forms a Hetero-oligomeric Protein Complex Soon after Caveolin Synthesis

In an attempt to identify caveolin hetero-oligomers early after caveolin synthesis, we immunoprecipitated caveolin after a 15-min pulse with ^{35}S-labeled amino acids. In accordance with our cell surface labeling studies, newly synthesized caveolin co-precipitates as a complex with Triton-insoluble proteins of 80-, 40-, and 14-kD molecular weights (Fig. 5). As is observed for the cell surface form of the complex, the metabolically labeled complex was stable at alkaline pH. These results indicate that caveolin hetero-oligomers are assembled intracellularly, before their arrival at the cell surface. As caveolin hetero-oligomers are Triton-insoluble soon after caveolin synthesis, while GPI-anchored proteins are soluble immediately after synthesis, the observed Golgi insolubility of GPI-linked proteins may be conferred by pH-dependent association with caveolin hetero-oligomers at the level of the Golgi.

Cholesterol-dependent Association of Caveolin Hetero-Oligomers with a GPI-linked Protein

To further test the hypothesis that these caveolin hetero-oligomers are part of a caveolar-protein complex, we performed cross-linking under conditions that selectively disrupt cell surface caveolae. Nystatin, a cholesterol binding antibiotic, selectively disrupts caveolae, but does not effect clathrin-coated pits, actin cables or other sub-membranous structures (Rothberg et al., 1992). After a brief pretreatment (5 min) of MDCK cells with nystatin, the nystatin solution was removed and cells were subjected to extraction and chemical cross-linking. Under these conditions, co-precipitation of the chemically cross-linked proteins with gD-1-DAF was dramatically inhibited (Fig. 6 *A*). In addition, such inhibition was time-dependent, i.e., undetectable at early times and maximal at 10 min of nystatin treatment. When caveolin was immunoprecipitated without cross-linking after nystatin pretreatment, the caveolin-associated proteins failed to co-precipitate (not shown), suggesting that nystatin acts through disruption of the interactions between members of this hetero-oligomeric protein complex. In this regard, the 3β-OH group of cholesterol that acts as its polar head group and protrudes from the exoplasmic leaflet of the lipid bilayer could form hydrogen bonds with adjacent glyco-sphingolipids or GPI, stabilizing the complex. Furthermore, as nystatin itself contains a derivative of D-mannose as its polar head group, making it a glyco-lipid, part of its effects may be mediated by interactions with glyco-sphingolipids or GPI.

Conversely, when cells were subjected to extended culture in lipoprotein-deficient serum (a condition that upregulates the synthesis of endogenous cholesterol), proteins of 80, 40, 22–24, and 14 kD efficiently co-precipitated with gD-1-DAF at alkaline pH without the aid of chemical cross-linking (Fig. 6 *B*). Additional components (100, 95, and 28–30 kD) also co-precipitated, suggesting that certain components of the complex are not detected by chemical cross-linking under our standard conditions. Co-precipitation under these conditions was also sensitive to inhibition by nystatin (not shown). Our results suggest that under these culture conditions the stability of the interaction between GPI-linked proteins and caveolin hetero-oligomers is dramatically increased.

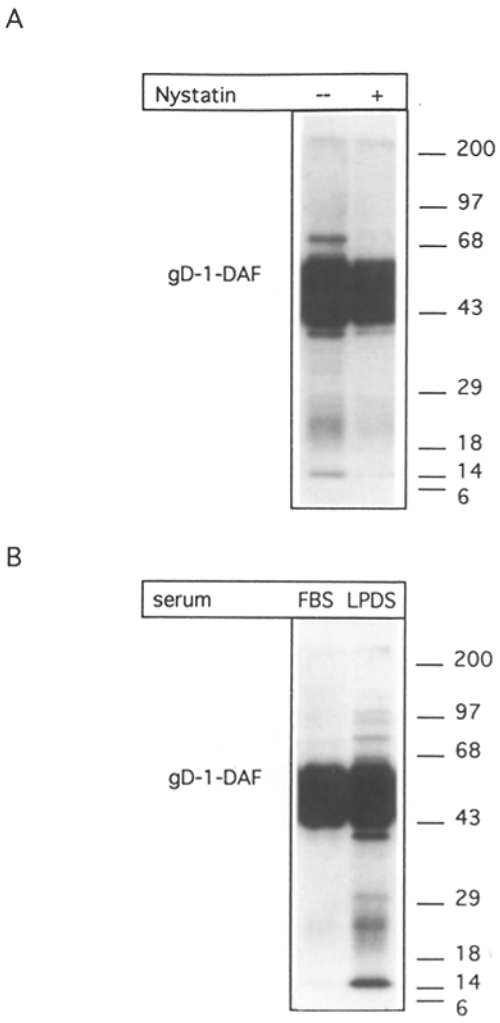


Figure 6. Cholesterol-dependent association of caveolin hetero-oligomers with gD-1-DAF. (*A*) Nystatin inhibits the association of gD-1-DAF with caveolin hetero-oligomers. MDCK cells were subjected to chemical cross-linking as described in Fig. 2. For these experiments, MDCK cells were cultured in normal medium containing fetal bovine serum (*FBS*). However, cells were pretreated with nystatin (−/+ 50 μg/ml) for 5 min at 25°C before extraction and chemical cross-linking. Extracts were immunoprecipitated with antibodies to gD-1-DAF. Note that treatment with nystatin, a cholesterol binding antibiotic that selectively disrupts caveolar ultrastructure, also inhibits the association of gD-1-DAF with the caveolin hetero-oligomers. (*B*) Culture in LPDS promotes association of gD-1-DAF with caveolin hetero-oligomers. MDCK cells were cultured in normal medium containing fetal bovine serum (*FBS*) or lipoprotein deficient fetal bovine serum (*LPDS*). After cell surface biotinylation, extracts were immunoprecipitated with antibodies to gD-1-DAF. Note that culture in LPDS, a treatment that upregulates the endogenous synthesis of cholesterol, also promotes the association of gD-1-DAF with caveolin hetero-oligomers—without the need for chemical cross-linking.

As total cellular cholesterol levels are known to remain relatively constant (Brown and Goldstein, 1980), one possible interpretation of these results is that formation of this complex may be related to the cellular transport of newly synthesized cholesterol to the cell surface. Currently, the mechanism for cholesterol transport remains unknown, however, cholesterol is depleted from ER membranes and is highly concentrated in the TGN and plasmalemmal caveolae (Orci et al., 1981; Lange et al., 1989). In addition, at steady-state about 90% of cellular cholesterol is localized to the plasma membrane and is thought to reside in its luminal aspect (Lange et al.,1989; Simons and van Meer, 1988; van Meer, 1989). Caveolin is also specifically localized to these two cellular compartments (TGN and plasma membrane) as well as TGN-derived transport vesicles (presumably intracellular caveolae) (Kurzchalia et al., 1992; Rothberg et al, 1992).

Steady-state Polarity of Caveolin Hetero–oligomers in Wild-type and in Mutant MDCK Cells Which Fail to Immobilize GPI-linked Proteins

To determine the apical/basolateral polarity of caveolin hetero–oligomers, we immunoprecipitated caveolin after selectively labeling untransfected MDCK cells at the apical or basolateral surface. We find that certain components of this complex are preferentially basolateral (80-, 50-, and 40-kD components; 65% basolateral) and non-polarized (caveolin; 22–24-kD component) or are apically localized (14-kD component; 90% apical) (Fig. 7). The observation that caveolin is non-polarized was anticipated from the results of Simons and co-workers. They found that caveolin, like the many other components of transport vesicles, is present in roughly equal quantities in both apically and basolaterally destined TGN transport vesicles (Kurzchalia et al., 1992; Wandinger-Ness et al., 1990). The explanation for the non-polarized distribution of caveolin and the other components may be related to the observation that caveolae are not restricted to the apical or basolateral domains of epithelial cells and are present on both epithelial surfaces (Rothberg et al., 1992; Dupree et al., 1993), as is also true for clathrin-coated pits. As has been observed for clathrin-coated vesicles (Puszkin et al., 1989), many different populations of caveolae may exist with distinct accessory proteins that function to target caveolae to a variety of cellular locations.

We next explored the polarity of caveolin hetero–oligomers in ConA^r MDCK cells which fail to immobilize and selectively missort GPI-linked proteins (Hannan et al., 1993; Lisanti et al., 1990*b*). Although the defect in this cell line is unknown, it presumably involves mannosylation. Using untransfected ConA^r MDCK cells, we find that many components of caveolin hetero–oligomers (especially the 80-, 40-, 14-kD components) no longer efficiently co-precipitate with caveolin and caveolin appears to be slightly more basolaterally localized (Fig. 7). However, the 50-kD component still efficiently co-precipitated. These results suggest that this complex is unstable or does not form correctly in these mutant MDCK cells.

Caveolin Hetero–oligomers Are Associated with a GPI-linked Protein during Transport to the Cell Surface

The polarized cell surface delivery of gD-1-DAF can be followed by masking the existing cell surface population and detecting the newly arrived population by cell surface labeling with sulfo-NHS-biotin and blotting with [^{125}I]streptavidin (Lisanti et al., 1990*a*). This assay for cell surface transport

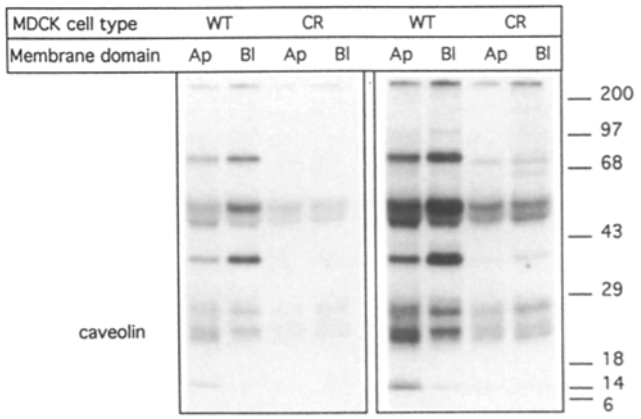


Figure 7. Apical/basolateral polarity of caveolin hetero-oligomers in wild-type and mutant MDCK cells which missort GPI-linked proteins. Wildtype (*WT*) or ConA-resistant (CR) MDCK cells were apically or basolaterally biotinylated and extracted at alkaline pH (8.0 in octyl-glucoside containing buffers. Cell extracts were immunoprecipitated with anti-caveolin antibodies. Biotinylated proteins were visualized after SDS-PAGE by streptavidin blotting. Note that in wild type cells, caveolin (22–24 kD) is nonpolarized and other members of the complex are apically (14 kD; 90% apical) or basolaterally (80, 50, and 40 kD; 65% basolateral) distributed. In ConA^r cells, note that caveolin is slightly more basolateral and does not efficiently co-precipitate with all the other members of the complex. As the latter cell type fails to immobilize and missorts GPI-linked proteins, these results may imply that complete caveolin hetero-oligomers are necessary for correct apical sorting of GPI-linked proteins. As the cell surface expression of this complex also appeared reduced (by ~50%) in the ConA^r cells, two exposures are shown for better illustration. To correct for these differences in relative cell surface expression, compare WT lanes of the left panel with CR lanes of the right panel.

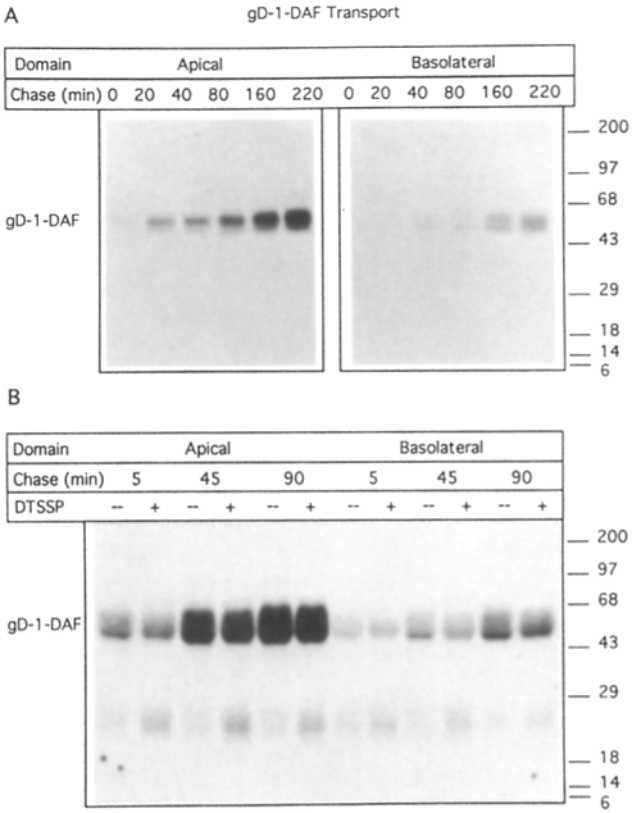


Figure 8. Caveolin is associated with gD-1-DAF during transport to the cell surface. (*A*) Polarized cell surface delivery of gD-1-DAF. The steady-state population of gD-1-DAF was masked by quenching with sulfo-SHPP and MDCK cells recultured at 37°C. The arrival of newly delivered gD-1-DAF was detected by biotinylation of either the apical or basolateral cell surface. Biotinylation was performed at 4°C in PBS$^+$, after various times of reculture at 37°C in normal medium. Delivery of gD-1-DAF occurred in an apically polarized fashion, as described previously (Lisanti et al., 1990*a*). (*B*) Chemical cross-linking during cell surface transport of gD-1-DAF. Filter-grown MDCK cells were subjected to the cell surface transport assay described in *A*, followed by chemical cross-linking as in Fig. 2. Samples were −/+ DTSSP (1 mg/ml). Cell surface delivery was for 5, 45, and 90 min. Note that in cross-linked samples gD-1-DAF associates with a major 22–24 kD species we identified as caveolin. Other components of caveolin hetero-oligomers (80, 40, and 14 kD) are detected on over-exposure of the autoradiograph (See Fig. 9 *A*).

detects the cell surface delivery of gD-1-DAF, but no other associated proteins are observed (Fig. 8 *A*).

To detect proteins that may associate with gD-1-DAF during its transport to the apical cell surface, we combined our cell surface delivery assay with chemical cross-linking under our newly established conditions. Using this approach, the most prominent protein co-precipitating with gD-1-DAF is the 22–24-kD protein we identified as the cell surface form of caveolin (Fig. 8 *B*). We can detect this association as early as 5 min of chase to the cell surface. The amount of caveolin detected as a co-precipitating protein does not increase with increasing delivery of gD-1-DAF, but remains similar to the amount detected after 5 min of chase. These results argue that caveolin is preferentially associated with the fraction of gD-1-DAF that has just arrived at the cell surface.

Over exposure of the autoradiograph of cross-linked gD-1-DAF molecules, reveals the other components (80, 40, and 14 kD) of the caveolin-complex (Fig. 9), in accordance with our steady-state experiments. However, these components were preferentially associated with apically delivered gD-1-DAF molecules, while they were depleted or absent from the pool of basolaterally delivered gD-1-DAF. As these results suggested that complete caveolin hetero-oligomers may be selectively delivered to the apical domain, we studied the polarized cell surface delivery of caveolin in the absence of chemical cross-linking. Although caveolin was delivered in a non-polarized fashion, apically delivered caveolin co-precipitated with 80-, 50-, 40-, and 14-kD components, while basolaterally delivered caveolin did not co-precipitate with these components, except for the 50-kD species. These results suggest that the apical routing of caveolin may depend on interactions with other components of this hetero-oligomeric protein complex.

Conclusions and Significance

Using the MDCK epithelial cell line, we have identified a complex of cell surface protein molecules that interacts in a pH- and cholesterol-dependent fashion with a model recombinant GPI-linked protein. These caveolin hetero-oligomers form intracellularly soon after caveolin synthesis and are transported to the cell surface. Complete caveolin het-

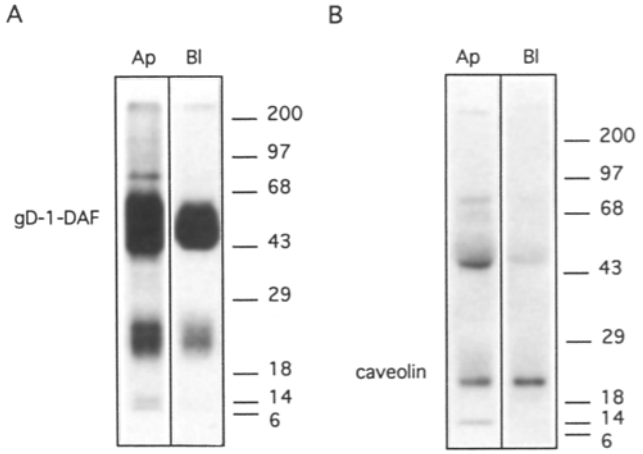


Figure 9. Polarity of caveolin hetero-oligomers during transport to the cell surface. (*A*) Over-exposures of chemically cross-linked samples from Fig. 8 *B*, representing the 45 min time point are shown. Note that apically delivered gD-1-DAF co-precipitates with 80-, 40-, 22-, 24-, and 14-kD components, while basolaterally delivered gD-1-DAF co-precipitates only with the 22–24 kD component we identified as caveolin (by blotting and immunoprecipitation as in Fig. 3). (*B*) Polarized cell surface delivery of caveolin. The surface delivery of caveolin was monitored as described in Fig. 8 for gD-1-DAF (without chemical cross-linking). A representative time point (45 min) is shown here for comparison with *A*. The delivery of caveolin was nonpolarized – in accordance with the observation that caveolin is present in roughly equal quantities in apically and basolaterally destined TGN transport vesicles (Kurzchalia et al., 1992). However, apically delivered caveolin co-precipitated with other members of the caveolar-protein complex, while basolaterally delivered caveolin only co-precipitated with the 50-kD species. These results suggest that the apical delivery of caveolin may depend on interactions with the other components of the caveolin hetero-oligomeric complex.

ero-oligomers are apically routed, while caveolin and a 50-kD component are basolaterally sorted. In contrast, at steady-state caveolin hetero-oligomers are present both on the apical and basolateral surfaces of epithelial cells, consistent with the observation that caveolae are present on both epithelial surfaces. Caveolin is non-polarized both during transport and at steady state.

These results have implications for the biogenesis of caveolae, if we assume that the formation of caveolin hetero-oligomers represents the assembly of caveolae. Our results would then suggest that apical caveolae form intracellularly during transport to apical cell surface, while basolateral caveolae form at the level of the basolateral membrane. Therefore, basolateral transport of caveolin might occur in non-caveolar vesicular carriers. This working hypothesis would explain the differences we observe between the existence of caveolin hetero-oligomers at steady state and during cell surface transport assays. The intracellular formation of apically destined caveolae might also explain the observation that both GPI-linked proteins and glycosphingolipids (components of plasmalemmal caveolae at steady state) are preferentially sorted to the apical plasma membrane of epithelial cells. Further experiments will be necessary to more directly explore this working hypothesis.

In summary, our results relate several seemingly disparate observations on the behavior of GPI-linked proteins. Detergent insolubility, pH-dependent clustering and immobility, and cholesterol-dependent caveolar localization all appear to be different manifestations of the formation of a complex between GPI-linked proteins and a caveolar-protein complex, that includes the type II transmembrane protein caveolin. It remains to be elucidated which member, if any, of this protein complex recognizes the free GPI moiety in the absence of an attached protein ectodomain.

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Note Added in Proof. We have recently developed a simple, rapid one-step procedure for isolating caveolae from cultured cells (Sargiacomo, M., M. Sudol, Z. Tang, and M. P. Lisanti. 1993. *J. Cell Biol.* 122:789–807). As these isolated caveolae contain cell surface proteins of molecular weights identical to those seen in immuno-isolated caveolin hetero-oligomers, it is likely that these hetero-oligomers represent the assembly units of caveolae.

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