Signal Transducing Molecules and Glycosyl-phosphatidylinositol-linked Proteins Form a Caveolin-rich Insoluble Complex in MDCK Cells

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Abstract. GPI-linked protein molecules become Triton-insoluble during polarized sorting to the apical cell surface of epithelial cells. These insoluble complexes, enriched in cholesterol, glycolipids, and GPI-linked proteins, have been isolated by flotation on sucrose density gradients and are thought to contain the putative GPI-sorting machinery. As the cellular origin and molecular protein components of this complex remain unknown, we have begun to characterize these low-density insoluble complexes isolated from MDCK cells. We find that these complexes, which represent 0.4–0.8% of the plasma membrane, ultrastructurally resemble caveolae and are over 150-fold enriched in a model GPI-anchored protein and caveolin, a caveolar marker protein. However, they exclude many other plasma membrane associated molecules and organelle-specific marker enzymes, suggesting that they represent microdomains of the plasma membrane. In addition to caveolin, these insoluble complexes contain a subset of hydrophobic plasma membrane proteins and cytoplasmically-oriented signaling molecules, including: (a) GTP-binding proteins—both small and hetero-trimeric; (b) annexin II—an apical calcium-regulated phospholipid binding protein with a demonstrated role in exocytic fusion events; (c) c-Yes—an apically localized member of the Src family of non-receptor type protein-tyrosine kinases; and (d) an unidentified serine-kinase activity. As we demonstrate that caveolin is both a transmembrane molecule and a major phospho-acceptor component of these complexes, we propose that caveolin could function as a transmembrane adaptor molecule that couples luminal GPI-linked proteins with cytoplasmically oriented signaling molecules during GPI-membrane trafficking or GPI-mediated signal transduction events. In addition, our results have implications for understanding v-Src transformation and the actions of cholera and pertussis toxins on hetero-trimeric G proteins.

Ｉ N lieu of a transmembrane peptide anchor, many plasma membrane proteins contain a COOH-terminal glyco-lipid, glycosyl-phosphatidylinositol (GPI),[1] as their sole mechanism of attachment (Cross, 1990; Ferguson, 1991; Low, 1989). After transfer of GPI to protein in the lumen of the ER, the attached protein is transported to the cell surface and undergoes cholesterol-dependent clustering in microinvaginations, known as plasmalemmal vesicles or caveolae (Anderson et al., 1992; Rothberg et al., 1990, 1992; Simonescu et al., 1975).

Caveolin, originally identified as a major phosphoprotein in v-Src transformed cells (Glenney, 1989; Glenney and Zokas, 1989), appears to contribute to the characteristic striated cytoplasmic coat of caveolae (Rothberg et al., 1992).

Cloning and analysis of the caveolin cDNA predicts an unusual 30–40 amino acid membrane-spanning region (Glenney, 1992; Glenney and Soppet, 1992; Kurzchalia et al., 1992). It has been proposed that this hydrophobic region could form a hairpin structure within the membrane bilayer, allowing both the NH_2- and COOH-terminal domains of caveolin to remain entirely cytoplasmic. Immuno-localization studies reveal caveolin is present in cell surface caveolae, and intracellularly at the level of the TGN (Kurzchalia et al., 1992). As caveolin is present in roughly equal quantities in apically and basolaterally destined transport vesicles, caveolin may shuttle between the two epithelial surface domains and the TGN (Kurzchalia et al., 1992; Wandinger-Ness et al., 1990). In addition to caveolin, glycolipids, the plasma membrane Ca^{2+}-ATPase and a cell surface IP_3 receptor are clustered in caveolae (Montesano et al., 1982; Fujimoto, 1993; Fujimoto et al., 1993), suggesting that multiple factors (cholesterol, glycolipids, and Ca^{2+}) are involved in GPI–protein clustering.

Clues to the possible function of caveolar localization come from the study of GPI-linked proteins in T cells and polarized epithelial cells. In T cells, cross-linking of GPI-linked proteins with specific antibodies leads to proliferation

1. *Abbreviations used in this paper*: GPI, glycosyl-phosphatidylinositol; NRPTK, non-receptor type protein-tyrosine kinase; RSV, Rous sarcoma virus; VAC, vacuolar apical compartment.

(Robinson et al., 1989; Su et al., 1991) and tyrosine phosphorylation. Phosphorylation apparently derives from a physical association between cell surface GPI-linked proteins and cytoplasmically oriented nonreceptor type protein–tyrosine kinases (NRPTKs), including Lck or Fyn (Stefanova et al., 1991; Thomas and Samelson, 1992). As both classes of molecules do not span the membrane, it has been postulated that a transmembrane adaptor molecule that recognizes both GPI and NRPTKs would be needed to couple them physically.

In polarized epithelial cells, GPI-linked proteins are selectively transported to the apical surface and excluded from the basolateral domain (Lisanti et al., 1988, 1990b), with GPI acting as a dominant signal for apical transport (Brown et al., 1989; Lisanti et al., 1989). Apical recognition of GPI may occur intracellularly, via the TGN, (Le Bivic et al., 1990; Lisanti et al., 1990a) or at the level of the basolateral membrane (Gilbert et al., 1991; Schell et al., 1992). The latter route involves basolateral endocytosis and basolateral-to-apical transcytosis of GPI-linked proteins. As glycolipids are apically sorted in epithelial cells, it was postulated that GPI-linked proteins are sorted via co-clustering with glycolipids (Lisanti and Rodriguez-Boulan, 1990). However, as glycolipids and GPI-linked proteins do not span the membrane, we have more recently postulated the existence of a transmembrane adaptor molecule that recognizes GPI and transmits addressing signals to the cytoplasm (Hannan et al., 1993; Lisanti and Rodriguez-Boulan, 1992).

In support of both models, GPI-linked proteins become resistant to Triton X-100 solubilization (Hooper and Turner, 1988), during apical transport (Brown and Rose, 1992). Insolubility begins at the level of the Golgi complex and is thought to reflect a physical association between GPI-linked proteins and the putative apical sorting machinery. GPI-anchored proteins are also Triton insoluble in non-epithelial cells, indicating the generality of this phenomenon (Hoessli et al., 1985).

These low-density, Triton-insoluble complexes have been isolated from MDCK cells by flotation on sucrose density gradients and are selectively depleted of phospholipids, but are enriched in cholesterol, sphingomyelin and glycolipids and contain GPI-linked proteins (Brown and Rose, 1992). The insolubility of these complexes in Triton X-100 has been attributed to their high sphingolipid content as sphingolipids are Triton insoluble (Hagmann and Fishman, 1982; Streuli et al., 1981; Yu et al., 1973). In addition, these complexes are devoid of many well-known polarized transmembrane antigens (reviewed in Rodriguez-Boulan and Powell, 1992; Garcia et al., 1993). Although the lipid composition of these complexes has been studied in detail, nothing is known of their cellular origin or non-GPI-linked molecular protein components.

We report here that these complexes represent caveolae-enriched plasma membrane domains and contain a subset of hydrophobic membrane proteins and cytoplasmically oriented signalling molecules. The implications of our findings are discussed in relation to models of GPI trafficking and signal transduction.

Materials and Methods

Materials

Sulfo-NHS derivatives were from Pierce (Rockford, IL). Anti-caveolin IgG (mAb clone 2234) was as described (Glenney, 1989; Glenney and Zokas. 1989). Sources of commercially available antibodies were as follows: HSV gD-1 (Dakopatts, Denmark); c-Src, Fyn (Oncogene Sciences, Manhasset, NY); c-Yes (Cambridge Research Biochemicals, Chershire, UK); annexins, PLC-, Ras-GAP, ezrin (Biodesign International, Kennebunkport, ME) and anti-phosphotyrosine, PY-20 (Zymed, S. San Francisco, CA). A second specific antibody to c-Yes was as described (Zhao et al., 1990). Antibodies to human c-Yes were generated against antigens containing the unique NH_2-terminal region of c-Yes, and they do not cross-react with other NRPTKs (Cambridge Research Biochemicals; (Zhao et al., 1990)). Avid-AL was from UniSyn Technologies.

Cell Culture

MDCK cells were grown for 2–3 d at high density in 150 mm dishes in DME supplemented with antibiotics and 10% FBS (Rodriguez-Boulan, 1983b). For separate access to apical or basolateral membrane domains, MDCK cells were seeded at confluent levels on 24.5-mm tissue culture inserts (Transwells, Costar, Inc., Cambridge, MA) and harvested after 5 d. gD-1-DAF expressing MDCK cells were derived and cultured as described (Lisanti et al., 1989). Rat-1 cells were grown in DME supplemented with antibiotics, glutamine and 10% FBS, while FRT cells were grown in Ham's F12-Coon's modified media supplemented with antibiotics, glutamine, and 5% FBS.

Steady-state Metabolic Labeling

Cells were incubated 16–20 h in 10 mls of Hepes-buffered DME containing 1/10 the normal concentration of methionine and cysteine, supplemented with 10% dialyzed FBS, 0.2% BSA, glutamine, antibiotics, and 100 μCi/ml Expre^{35}S^{35}S-label (Dupont/NEN). Metabolically labeled proteins were detected after SDS-PAGE by fluorography with sodium salicylate (Chamberlain, 1979).

Surface Labeling

Plasma membrane proteins were surface labeled with the biotinylating reagent, sulfo-NHS-biotin, as described (Lisanti et al., 1988; Sargiacomo et al., 1989). After 10% SDS-PAGE and transfer to nitrocellulose, biotinylated proteins were detected by blotting with iodinated streptavidin and autoradiography. Cells were also surface labeled with a radio-iodinatable probe, sulfo-SHPP, analogous to sulfo-NHS biotin (Thompson et al., 1987). To calculate fold-enrichments in low-density complexes relative to total cell lysates, proteins recovered by immunoprecipitation were expressed as specific activities (cpm/μg total protein).

For domain-selective labeling, sulfo-NHS-biotin was added only to the apical or basolateral compartment of the filter chamber. Filters were excised from the chamber before lysis and immunoprecipitation, as described previously (Lisanti et al., 1988; Sargiacomo et al., 1989). This procedure efficiently labels the basolateral surface of filter grown cells, as evidenced by the following observations: (a) basolateral biotinylation occurs all the way up the level of the tight junction (Sargiacomo et al., 1989); (b) the polarity of numerous apical and basolateral markers has been estimated using this procedure and the results agree with those obtained from immunogold labeling (Sargiacomo et al., 1989; Le Bivic et al., 1990); and (c) cell surface proteins expressed in filter-grown non-polarized epithelial cells are equally and efficiently labeled from both the upper (apical) and lower (basolateral) compartments of the filter chamber (Lisanti et al., 1989).

Isolation of Low-density, Triton-insoluble Complexes

Low-density, Triton-insoluble complexes were prepared by our modification of a published protocol (Brown and Rose, 1992). Briefly, MDCK cell monolayers, grown to confluence on 150 mm dishes (5.9 × 10^7 cells), are extracted with 2 ml Mes-buffered saline (MBS; 25 mM Mes, pH 6.5, 0.15 M NaCl) containing 1% Triton X-100, and 1 mM PMSF. After collection, the extract is homogenized with 10 strokes of a Dounce homogenizer, adjusted to 40% sucrose, and placed at the bottom of an ultracentrifuge tube. A 5–30% linear sucrose gradient is formed above the lysate (in MBS, lacking Triton X-100) and centrifuged at 39,000 rpms for 16–22 h at 4°C in a rotor (SW41; Beckman Instruments, Palo Alto, CA). The opaque band (migrating at ~10–20% sucrose) is harvested, diluted with MBS and pelleted in the microfuge. Pellets are snap frozen in liquid nitrogen and stored frozen at −80°C. A 150 mm dish of MDCK cells representing 9–11 mg protein, yielded 4–6 μg of low-density, Triton-insoluble complexes, i.e., ~0.05% of the initial homogenate. Triton-extraction solubilized ~85% of

the protein (8.5 mg) the majority of which remained in the 40% sucrose layer, while ~1.5 mg formed an insoluble pellet below the 40% sucrose.

Organelle-specific membrane marker assays were performed by Jolanta Vidugiriene in the laboratory of Anant K. Menon (The Rockefeller University, New York) as described (Brada and Dubach, 1984; Vidugiriene and Menon, 1993). These assays systems were not affected by the presence or absence of 1% Triton X-100 in the initial homogenate. An ER membrane marker protein (mAb RFD6; Dako Corp., Carpenteria, CA) was also excluded from these complexes as determined by blotting (not shown).

When filter-grown MDCK cells were used to prepare these complexes (from two 6-well Transwells per gradient), one 100 mm dish of unlabeled MDCK cells was added as a carrier to facilitate efficient recovery of the complexes.

Detection of Endogenous GPI-anchored proteins

Triton-insoluble complexes were solubilized in TBS containing 1% Triton X-114 plus protease inhibitors (Lisanti et al., 1990b; Lisanti et al., 1988), followed by incubation at 37°C for 20 min. This condition effectively solubilizes GPI-linked proteins, as described previously (Brown and Rose, 1992). After solubilization, extracts were partitioned into detergent-rich and detergent-poor fractions by temperature-induced phase separation. GPI-linked proteins were detected by treatment with PI-specific phospholipase C (6–8 U/ml; from *Bacillus thuringiensis*) which releases them from the detergent into the aqueous phase. Our assay conditions were optimized using two exogenous GPI-anchored proteins expressed in MDCK cells and quantitatively convert these over-expressed proteins to soluble forms as assayed by immunoprecipitation of fractions before and after PI-PLC treatment (Lisanti et al., 1991a,b).

Immunoprecipitation of Triton-insoluble complexes

Triton-insoluble complexes were solubilized for 45 min on ice with 1 ml of TBS (10 mM Tris, pH 8.0, 0.15 M NaCl) containing 1% Triton X-100, 60 mM octyl-glucoside, and 1 mM PMSF. After pre-clearing with protein A-Sepharose, samples were immunoprecipitated with a 1:150 dilution anti-gD-1 or a 1:400 dilution of anti-caveolin pre-bound to protein A-Sepharose. Rabbit anti-mouse IgG was used at a dilution of 1:100 pre-coupled to protein A-Sepharose to facilitate immunoprecipitation of mouse antibodies. For calculating fold enrichments, primary anti-bodies were pre-complexed to protein A-Sepharose as described above, however, they were used at a dilution of 1:100 to ensure optimal conditions for immunoprecipitation.

Electron Microscopy

Low-angle Rotary Shadowing. Triton-insoluble complexes (5 μg) were incubated for 30 min at 4°C in 200 μl MBS containing 60 mM octyl-glucoside. Samples were sandwiched between two 1-cm pieces of freshly cleaved mica. Mica sheets were then separated and placed on the stage of a Denton 502A vacuum evaporator and dried for 15–20 min at ~10⁻⁵ torr. Samples were shadowed with ~50 cm of platinum-paladium wire wrapped ten times around a tungsten wire at an angle of 4° while rotating at 50–60 rpms, and then coated with carbon at an angle of 90°. Replicas were removed from the mica by floating onto water, and picked up on uncoated 400-mesh copper grids. Micrographs of random fields were made at 80 kV with an electron microscope (1200 CX; JEOL USA, Peabody, MA).

Transmission. Pellets were fixed with 2.5% glutaraldehyde in 0.1 M Na cacodylate buffer (pH 7.2) for an hour on ice. Samples were then rinsed in 10% sucrose in 0.1 M Na cacodylate buffer, postfixed with 1% OsO₄ in cacodylate buffer and en bloc stained with 1% aqueous uranyl acetate. Samples were dehydrated in graded ethanol and embedded in polyBed 812 (Polysciences Inc., Warrington, PA). Thin sections were cut on a Ultracut E (Reichert JUNG, Vienna), stained with uranyl acetate and lead citrate, and examined under the Philips 410 TEM.

Detection of GTP-binding Proteins

GTP-binding proteins were detected by affinity labeling using an oxidation-reduction scheme that covalently couples radio-labeled GTP to the invariant lysine residue within the GTP-binding consensus sequence, NKXD (Low et al., 1992; Peter et al., 1992). Briefly, Triton-insoluble complexes were solubilized for 30 min on ice in MBS containing 60 mM octyl-glucoside. After solubilization, 5 μCi of [α-³²P] GTP was added and incubated for 10 min at 37°C. After binding, oxidation of protein bound nucleotides was initiated by addition of 4 mM NaIO₄ (1 min at 37°C), followed by reduction with 80 mM NaCNBH₃ (1 min at 37°C), and final treatment with 100 mM NaBH₄ (1 h at 4°C). Control samples were preincubated with 1 mM cold GTP for 30 min on ice before addition of radio-labeled GTP. Samples not

preincubated with cold GTP were adjusted to 1 mM cold GTP before analysis by SDS-PAGE.

The α subunits of heterotrimeric G proteins were detected by immuno-blotting with specific rabbit polyclonal antibodies (kind gift of Dr. Allen Spiegel, National Institutes of Health, Bethesda, MD; Schenker et al., 1991). Antibody specificities were as follows: QL (G_α q/11); AS/7 ($G_{\alpha i1}$, i2); RM/10 ($G_{\alpha s}$); GO/6 ($G_{\alpha o}$); and EC/2 ($G_{\alpha i3}$). As both $G_{\alpha i1}$ and $G_{\alpha o}$ are not expressed in renal or intestinal epithelial cells (Ercolani et al., 1990; Berghe et al., 1991), antibody AS/7 is specific for $G_{\alpha i2}$ in these cell types.

Immunoblotting

Triton-insoluble complexes (~20 μg) were solubilized in 150 μl sample buffer and separated by a preparative mini gel (10% SDS-PAGE). After transfer to nitrocellulose, sheets were longitudinally cut into 3 mm strips and transferred to multi-well trays for incubation with a variety of different primary antibodies. Bound primary antibodies were visualized with the appropriate alkaline-phosphatase conjugated secondary antibody. Incubation conditions were as described by the manufacturer (Promega Biotec, Madison, WI), except we supplemented our blocking solution with 1% non-fat dry milk (Carnation). Candidate antibodies were initially screened with total cell lysates to ensure that they reacted with a band of the appropriate molecular weight in MDCK cells. Total cell lysates were prepared by scrapping a 100 mm dish of confluent MDCK cells into 300 μl of sample buffer. Half of this solution was loaded per preparative mini gel and samples were treated as described above.

Immunofluorescence Microscopy

MDCK cells grown on glass coverslips were washed with PBS⁺ and subjected to methanol-acetone fixation (5 min methanol, 1 min acetone) at −20°C. After fixation, washes, and antibody incubations were done in PBS⁺ containing 0.2% BSA. Bound primary antibodies were visualized with the appropriate rhodamine-conjugated secondary antibody at a dilution of 1:200 (Cappel Laboratories, Malvern, PA).

In Vitro Kinase Reactions

Triton-insoluble complexes (5 μg) or immunoprecipitates were resuspended in 20 μl of kinase reaction buffer (20 mM Hepes, pH 7.4, 5 mM MgCl₂, 1 mM MnCl₂) and the reaction initiated by addition of 10 μCi [γ-³²P]ATP (2 μl). After incubation for 10 min at 25°C with intermittent mixing, the reaction was stopped by addition of 20 μl 2× Laemmli sample buffer and boiling for 2 min. In certain experiments, samples were pre-incubated for 30 min on ice with genistein at a concentration of 10 or 100 μM (in kinase reaction buffer). A 20 mg/ml stock of genistein was prepared in DMSO and stored frozen at −20°C.

Immunoprecipitation of Phosphorylated Proteins

After in vitro phosphorylation of Triton-insoluble complexes with [γ-³²P]ATP, samples were immunoprecipitated with anti-phosphotyrosine antibodies or antibodies to NRPTKs and their substrates. Samples were diluted to 1 ml with lysis buffer containing 10 mM Tris, pH 7.5, 5 mM EDTA, 50 mM NaCl, 30 mM sodium pyrophosphate, 50 mM NaF, 100 μM Na₃VO₄, 1% Triton, and 60 mM octyl-glucoside and extracted for 30 min on ice before preclearing and immunoprecipitation. Phosphotyrosyl-proteins were eluted from the anti-phosphotyrosine antibody by incubation with 50 mM phenyl-phosphate, dissolved in the above buffer containing phosphatase inhibitors (Zachary et al., 1991).

Phospho-amino Acid Analysis

In vitro phosphorylated proteins were separated by SDS-PAGE and localized by autoradiography. The 6-kD phosphoprotein, caveolin, and two phosphoprotein bands corresponding to the c-Yes protein were excised from the gel, digested with trypsin, and subjected to acid hydrolysis (6 N HCl at 100°C for 2 h). Hydrolysates were analyzed by one-dimensional electrophoresis in pH 1.9 buffer for 1 h and 45 min (Boyle et al., 1991; Luo et al., 1990).

Northern Blot Analysis of Caveolin Expression

Confluent monolayers from three 150 mm dishes were harvested by trypsinization and used to prepare mRNA via oligo (dT)-cellulose affinity chromatography (type III; Collaborative Biomedical Products, Bedford, MA).

mRNA (5–10 μg) was separated on formaldehyde–agarose (0.8%) gels, blotted on GeneScreen Plus membranes, UV cross-linked, and probed with the ^{32}P-labeled MDCK caveolin cDNA by standard procedures (Sambrook et al., 1989). Hybridization was carried out for 40 h at 60°C. Blots were washed sequentially with 2× SSC (10 min at room temperature), 2× SSC/1% SDS (30 min; 60°C), and 0.1× SSC/0.1% SDS (30 min at 68°C).

The full-length canine caveolin cDNA was cloned by PCR amplification of an MDCK cDNA library (gift of G. Apodacca and K. Mostov, University of California, San Francisco) using primers to the known sequence (Kurzchalia et al., 1992). PCR products of the expected size (0.6 kb) were gel purified, cloned into pBluescript II KS+ and subjected to double-stranded sequencing in both directions using Sequenase 2.0.

Results

Origin and Molecular Protein Composition of MDCK Triton-insoluble Complexes

Clustering and immobility of cell surface GPI-linked proteins is dramatically affected by extracellular pH (Hannan et al., 1993). To evaluate whether low pH stabilized low-density Triton-insoluble complexes, we prepared these complexes from MDCK cells using slightly acidic (pH 6.5) or slightly alkaline (pH 7.5) homogenization conditions. As a marker for recovery of these complexes, we used a recombinant GPI-linked protein (gD-1-DAF). Our results indicate that preparation at acidic pH increases the recovery of gD-1-DAF in Triton-insoluble complexes by 3–5-fold (Fig. 1 A). As other investigators have used slightly alkaline homogenization conditions (Brown and Rose, 1992), this critical modification should greatly facilitate the preparation of these complexes and the characterization of their protein components. Acidic pH may act to stabilize the complex, by mimicking the micro-environment of the TGN (Anderson and Pathak, 1985)—the putative sorting site of GPI-linked proteins.

As GPI-anchored proteins become insoluble at the level of the early Golgi complex, isolated Triton-insoluble complexes may represent Golgi or post-Golgi-derived membranous compartments, i.e., transport vesicles or elements of the plasma membrane. To test this hypothesis, we prepared Triton-insoluble complexes after surface labeling of MDCK cells with the membrane impermeant probe, sulfo-NHS-biotin (Fig. 1 B). After visualization of biotinylated proteins by blotting with iodinated streptavidin, we find that Triton-insoluble complexes reproducibly contain a discrete set of cell surface proteins of 100, 95, 80, 50, 42, 40, 35, 22–24, 16, and 14 kD. To investigate if these proteins are peripheral or integral components of the membrane, we solubilized biotinylated Triton-insoluble complexes with the detergent Triton X-114 by extraction at 37°C. After temperature-induced phase separation with Triton X-114, most biotinylated components of the Triton-insoluble complex partitioned with the detergent-rich phase (Fig. 1 B), consistent with the hypothesis that they represent integral plasma membrane components.

Detergent phases were next treated with PI-specific phospholipase C, to determine if these hydrophobic proteins represented GPI-linked components. Such treatment releases GPI-linked proteins to the aqueous phase, as it removes the hydrophobic lipid moiety of the GPI anchor. Using this strategy, we find that GPI-linked proteins represent a minor component of these complexes relative to other hydrophobic membrane proteins (Fig. 1 C), consistent with the idea that
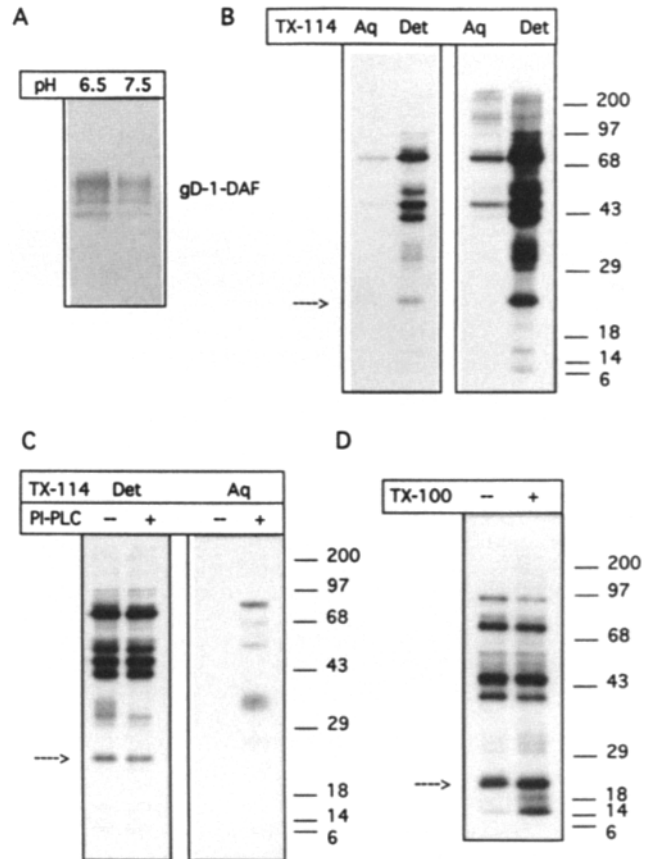

Figure 1. MDCK Triton-insoluble complexes contain cell surface proteins. (A) Slightly acidic pH enhances the recovery of Triton-insoluble complexes. Complexes were prepared from transfected MDCK cells using acidic or alkaline homogenization conditions and the recovery of gD-1-DAF (a recombinantly expressed GPI-linked protein) determined by immunoblotting. Note that recovery was greatly enhanced (3–5-fold) by the use of pH 6.5 buffers. (B) Triton-insoluble complexes contain multiple hydrophobic plasma membrane proteins. Complexes were prepared from surface biotinylated MDCK cells and the protein components fractionated using the detergent Triton X-114. After temperature-induced phase separation, proteins were recovered from the aqueous and detergent phases and analyzed by SDS-PAGE. Biotinylated proteins were detected by blotting with [^{125}I]streptavidin. Most components partition with the detergent phase, indicating hydrophobicity. Two exposures are shown. (C) GPI-linked molecules represent minor components of Triton-insoluble complexes. After fractionation with Triton X-114, detergent phases were collected and incubated in the absence or presence of PI-specific phospholipase C. Released GPI-anchored proteins subsequently partition with the final aqueous phase. Both detergent and aqueous phase are shown. GPI-linked proteins are released only from PI-PLC–treated samples and represent a minor component of the total biotinylated components. Biotinylated proteins were visualized as above. (D) Triton-insoluble components derive primarily from the plasma membrane. Complexes were prepared from MDCK cells after biotinylation in Mes-buffered saline in the absence or presence of 0.1% Triton X-100. In permeabilized cells, essentially the same pattern and extent of labeling is observed as compared as unpermeabilized controls. An increase in certain low molecular weight proteins is observed (35, 16, and 14 kD), suggesting they may be preferentially intracellular. In the above panels (B–D), an arrow denotes the position of a 22–24-kD cell surface protein we identified as caveolin in Fig. 4.

GPI-linked proteins represent the "cargo" molecules and the other non-GPI-linked elements represent the more abundant "sorting machinery."

We evaluated whether intracellular membrane components contributed significantly to the composition of these complexes (not accessible to cell surface labeling), by performing the biotinylation reaction in MDCK cells permeabilized with 0.1% Triton X-100 (Fig. 1 D). Under these conditions, essentially the same labeling pattern and extent of labeling is observed as compared with unpermeabilized control cells, indicating that these complexes derive predominantly from the cell surface. However, an increase in labeling of certain low molecular weight components was observed (35, 16, and 14 kD), suggesting that these molecules may be located preferentially in an intracellular location.

To estimate what percentage of the cell surface these low-density insoluble complexes represent, MDCK proteins were surface-labeled with a radio-iodinatable probe, sulfo-SHPP, analogous to sulfo-NHS-biotin. After labeling, complexes were prepared and their recovery quantified using a gamma counter. We find that ∿0.4–0.8% of surface-labeled proteins are recovered in these complexes, indicating that they originate from only a small fraction of the cell surface. In addition, the specific activity of labeled cell-surface proteins in these complexes is ∿15–20 times greater than in total cell lysates, indicating that these complexes are enriched in a subset of plasma membrane components. Complexes were also prepared after steady-state metabolic labeling. Although low-density insoluble complexes represented only ∿0.005% of the total precipitable counts, they had a specific activity ∿5–7.5-fold higher than total cell lysates, indicating that they were enriched in newly synthesized proteins. These results are consistent with a role for these complexes in biosynthetic transport.

MDCK Triton-insoluble Complexes Behave as Integral Membrane Proteins

To investigate further whether the components of Triton-insoluble complexes behave as expected of integral membrane proteins, we explored other conditions that might solubilize these complexes. Metabolically labeled and surface biotinylated Triton-insoluble complexes were extracted using a variety of buffer compositions, separated into supernatant and pellet fractions by centrifugation and analyzed by SDS-PAGE. The results of these experiments are summarized in Table I. We find that Triton-insoluble complexes are resistant to solubilization by chelators, high salt, clathrin-dissociation buffer, detergents such as digitonin and saponin, and alkaline carbonate extraction. These observations independently suggest that the elements of this complex represent integral membrane components, and support our results from Triton X-114 extraction.

We next turned to conditions known to solubilize GPI-anchored proteins (Hooper and Turner, 1988). Extraction with octyl-glucoside effectively solubilized both cell surface and metabolically labeled samples (Fig. 2). In contrast, CHAPS extraction preferentially solubilized surface components, while sodium deoxycholate extraction preferentially solubilized metabolically-labeled components. This selective property of CHAPS could be exploited to further dissect the molecular components of these Triton-insoluble complexes. As similar differential detergent extractability of

Table I.

Solubilization of MDCK Triton-insoluble Complexes		
Conditions that do not effect solubilization		
Acidic pH		
Mes-buffered saline (25 mM Mes, pH 6.5, 0.15 M NaCl)		
Buffer alone		
Buffer plus		
5 mM EDTA		
1 M NaCl		
Detergents		
Triton X-100 (1%)		
Saponin (0.5%)		
Digitonin (0.5%)		
Neutral pH		
Clathrin dissociation buffer (0.5 M Tris, pH 7.0)		
Alkaline pH		
TBS (10 mM Tris, pH 8.0, 0.15 M NaCl)		
Carbonate extraction (100 mM sodium carbonate, pH 11.0)		

Conditions that effect solubilization	Surface biotinylated	Metabolically labeled
Mes-buffered saline plus detergents		
Octyl glucoside (60 mM)	+ +	+ +
CHAPS (30 mM)	+ +	±
Na deoxycholate (0.1%)	−	+ +

GPI-anchored proteins occurs in whole cells (octyl-glucoside > CHAPS > deoxycholate) (Hooper and Turner, 1988), our data suggest that insolubility of GPI-linked proteins may reflect an association with non-GPI-linked molecular components of Triton-insoluble complexes. These non-GPI-linked insoluble elements may represent lipids as these complexes were solubilized effectively only by detergents that resemble glycolipids (octyl-glucoside) and cholesterol (CHAPS and deoxycholate).

Triton-insoluble Complexes Represent Caveolin-enriched Membrane Domains

To ultrastructurally examine low-density Triton-insoluble complexes, we used the low-angle rotary shadowing technique. Briefly, these complexes were incubated with octyl-glucoside in pH 6.5 buffer (30 min at 4°C, without vortexing) to partially dissociate them before layering onto mica sheets and preparation of platinum replicas (see Materials and Methods). These studies reveal 50–100 nm spherical complexes, consisting of arrays of particles that are interconnected by strands and appeared in concentric rings (Fig. 3). Shadowing at an angle of 4° best demonstrates the presence of the numerous particles, while shadowing at an angle of 8° better illustrates the filamentous nature of this material. It is unlikely that the complexes we observe represent an artifact of octyl-glucoside incubation, as they are dispersed by vortexing during solubilization and are not observed when only the detergent solution was shadowed (not shown). In addition, they are not observed in FRT cells which lack detectable caveolin expression (see below).

As caveolae are of a similar size to the structures we visualized by rotary shadowing and contain a cytoplasmic striated coat structure consisting of four or more concentric strands of particulate material (Peters et al., 1985; Rothberg
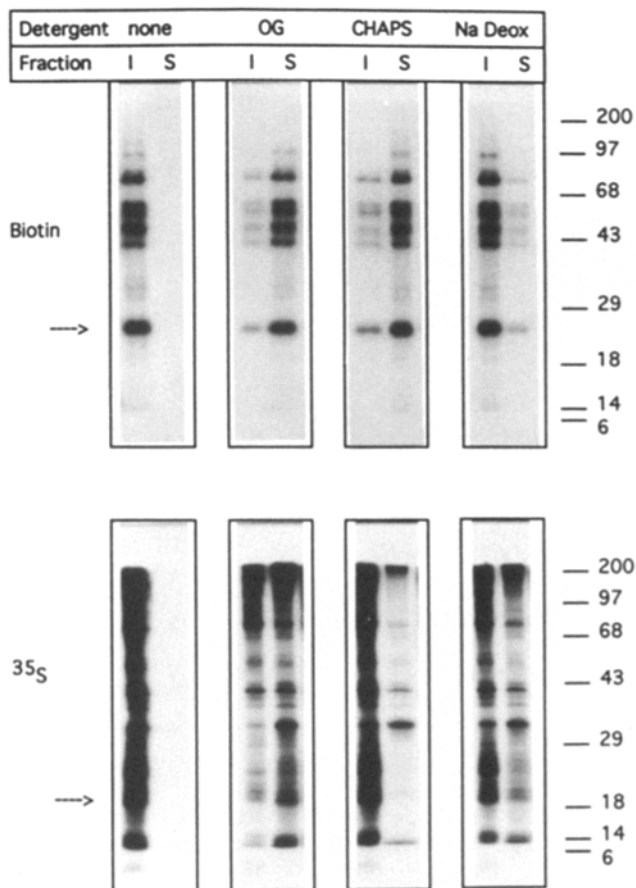
Figure 2. Differential solubilization of MDCK Triton-insoluble complexes. Complexes prepared from biotinylated or metabolically labeled MDCK cells were resuspended in 200 μl of Mes-buffered saline containing either octyl-glucoside (60 mM), CHAPS (30 mM), or sodium deoxycholate (0.1%) (Table I). After 30 min on ice, with intermittent vortexing, samples were centrifuged at 14,000 g for 15 min at 4°C. Proteins partitioning with the supernatant (*S*) were collected by acetone precipitation, pellets (*I*) were directly dissolved in sample buffer, and both fractions analyzed by SDS-PAGE. Extraction with octyl-glucoside best solubilizes both surface labeled and metabolically labeled proteins. In contrast, CHAPS preferentially solubilizes surface components. An arrow denotes the position of a 22–24-kD protein we identified as caveolin in Fig. 4.

et al., 1992), we suspected that these Triton-insoluble complexes may represent isolated caveolae. In support of this hypothesis, both caveolae (Montesano et al., 1982; Rothberg et al., 1990) and Triton-insoluble complexes (Brown and Rose, 1992) concentrate glycolipids, cholesterol, and GPI-linked proteins. To test this hypothesis further, we investigated whether Triton-insoluble complexes contain a 22-kD caveolar marker protein, namely caveolin. Blots of biotinylated Triton-insoluble complexes were re-probed with anti-caveolin IgG (Fig. 4 *A*). These experiments identify caveolin as the major 22–24-kD cell surface component of Triton-insoluble complexes. Similar results were obtained when these complexes were solubilized with octyl-glucoside and immunoprecipitated with anti-caveolin IgG (not shown). Biotinylated caveolin represented ∼10–20% of the total biotinylated proteins present in this complex. In accordance

with these observations, we find that the cell surface form of caveolin partitions with the detergent-rich phase of Triton-X-114 (see Fig. 1 *B*) and is insensitive to digestion with PI-PLC (see Fig. 1 *C*). In addition, caveolin is resistant to solubilization by all of the conditions listed in Table I and best solubilized by octyl-glucoside and CHAPS (Fig. 2), behaving as expected of an integral membrane protein.

To estimate whether caveolin was enriched in these complexes, we surface-labeled MDCK cells with the iodinatable probe, sulfo-SHPP. Caveolin was then immunoprecipitated from total cell lysates and from Triton-insoluble complexes and its relative enrichment compared (Fig. 5 *A*). In these experiments, we find that caveolin represents ∼0.18–0.2% of the labeled cell surface proteins. In contrast, caveolin represents ∼15–20% of the surface proteins present in Triton-insoluble complexes (in accordance with our estimates from biotinylation). When these results are expressed as specific activities (cpm/μg total protein), we find that caveolin is dramatically enriched (∼160-fold) in these complexes as compared to total cell lysates. Similarly, GPI-anchored gD-1-DAF represented 0.2% of labeled cell surface proteins and was ∼240-fold enriched in these complexes. In striking contrast, we find that organelle-specific marker enzymes are specifically excluded from these insoluble complexes (Fig. 5 *B*). Taken together, these results are consistent with the hypothesis that these complexes consist of caveolae-enriched membrane domains. Similarly, previous reports indicate that caveolin exists as a low-density, Triton-insoluble complex (Glenney, 1989), however, these complexes were not studied further.

The membrane topology of caveolin has not yet been experimentally determined. However, based on sequence analysis of the caveolin cDNA, it has been proposed that an unusual 30–40 amino acid hydrophobic region could form a hairpin structure within the membrane, allowing both the NH_2- and COOH-terminal domains of caveolin to assume an entirely cytoplasmic orientation (Kurzchalia et al., 1992). In addition, epitope tagging of the NH_2 terminus of caveolin reveals its cytoplasmic orientation (Kurzchalia et al., 1992). Taken together with our data that caveolin is accessible from the cell surface, it is likely that caveolin assumes a type II transmembrane orientation with a cytoplasmic NH_2 terminus and an exoplasmic COOH terminus. In accordance with this proposed membrane topology, the putative 44 amino acid caveolin ectodomain (residues 135–178) contains three lysine residues that would be accessible to cell surface biotinylation.

Apical/Basolateral Distribution of MDCK Caveolin-rich Insoluble Complexes

To determine the polarized distribution of the surface components of Triton-insoluble complexes, we grew MDCK cells on permeable supports that allow separate access to the apical and basolateral domains. After apical or basolateral biotinylation, we prepared these complexes and visualized their biotinylated proteins by streptavidin blotting (Fig. 4 *B*, *left*). Caveolin and other surface-labeled Triton-insoluble components were not restricted to the apical or basolateral surface, but were present mainly in a non-polarized or slightly basolateral (65%) distribution. A few components also appeared basolateral specific (70 and 40 kD), while none were confined to the apical domain. As the sorting of
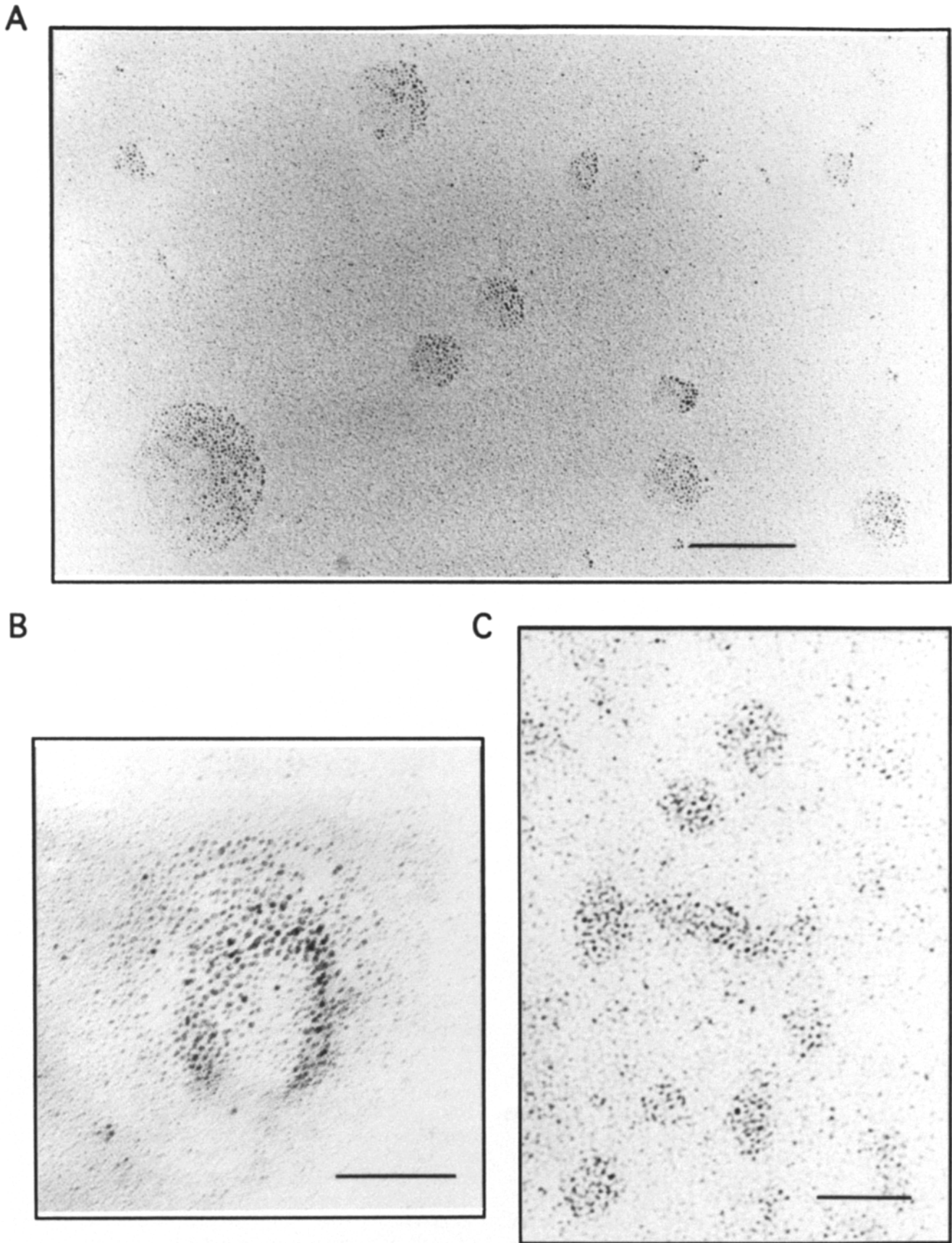
Figure 3. Low-angle rotary shadowing of the MDCK Triton-insoluble complex. After incubation with octyl-glucoside, partially dissociated complexes were visualized by platinum replica-rotary shadowing (see Materials and Methods). Note 50–100-nm spherical structures reminiscent of caveolae, consisting of globular components interconnected by strands and arranged in concentric rings. The shadowing angle and bar length are listed for each micrograph. (*A*) 4°, 0.1 μm; (*B*) 4°, 0.05 μm; (*C*) 8°, 0.05 μm.

A

Triton-insoluble complex caveolin blot

— 200
— 97
— 68
— 43
— 29
— 18
— 14
— 6

caveolin

B

Fraction	Triton-insoluble		Total	
Membrane domain	Ap	Bl	Ap	Bl

200 —
97 —
68 —
43 —
29 —
caveolin
18 —
14 —
6 —

— 200
— 97
— 68
— 43
— 29
— 18
— 14
— 6

Figure 4. Caveolin is a major cell surface component of Triton-insoluble complexes. (*A*) Complexes were prepared from surface-labeled M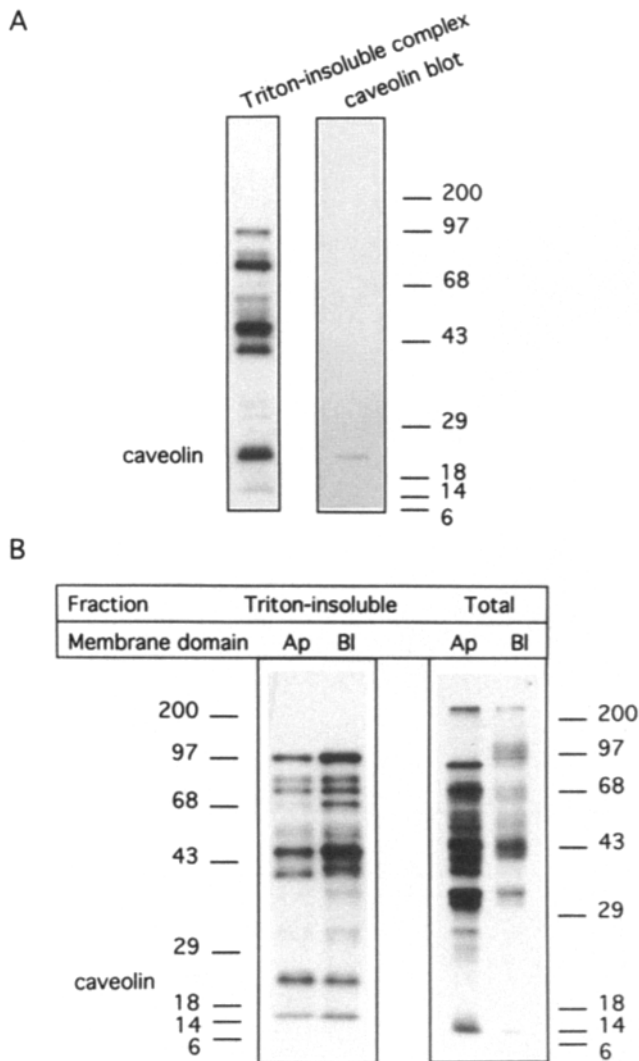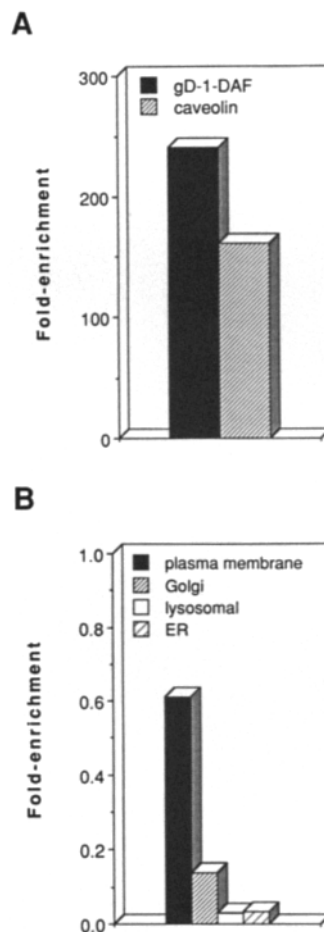DCK cells. After probing with [¹²⁵I]streptavidin, blots were re-probed with anti-caveolin IgG. Bound antibodies were visualized with alkaline phosphatase conjugated secondary antibodies. (*B*) Filter-grown MDCK cells were apically or basolaterally biotinylated and used to prepare Triton-insoluble complexes. Caveolin and other major surface-labeled components appear to be unpolarized, while certain minor components are confined to the basolateral (70 and 40 kD) surface (*left*). This is in striking contrast to total biotinylated proteins obtained from apically or basolaterally labeled MDCK cells (*right*).

plasma membrane proteins occurs both at the level of the TGN and the cell surface (Lisanti and Rodriguez-Boulan, 1992; Simons and Wandinger-Ness, 1990), the non-polarized distribution of the Triton-insoluble complex may reflect the existence of sorting machinery that shuttles between the apical and basolateral domains and the TGN. These results are consistent with the proposed transcytotic role of caveolae in endothelial cells (Severs, 1988; Simonescu et al., 1975) and the observation that caveolae are not confined to the apical or basolateral domains of epithelial cells (Rothberg et al., 1992; Dupree et al., 1993).

In accordance with our observations, caveolin and many other protein components of transport vesicles are equally

A

gD-1-DAF
caveolin

Fold-enrichment
300
200
100
0

B

plasma membrane
Golgi
lysosomal
ER

Fold-enrichment
1.0
0.8
0.6
0.4
0.2
0.0

Figure 5. Triton-insoluble complexes represent caveolae-enriched domains. (*A*) Enrichment of caveolin and GPI-anchored gD-1-DAF. MDCK cells were surface-labeled with the radio-iodinatable probe, sulfo-SHPP. Caveolin and gD-1-DAF in Triton-insoluble complexes and total cell lysates were quantitated by immunoprecipitation and expressed as specific activities (cpm/µg total protein) to calculate fold enrichments. Our results indicate that both caveolin and GPI-anchored gD-1 are over 150-fold enriched in Triton-insoluble complexes. In a typical experiment, we recovered a total of 55,000 cpms in Triton-insoluble complexes from 7.5 × 10⁶ cpms representing total cell surface proteins (0.7–0.8%). (*B*) Depletion of organelle-specific marker enzymes. Triton-insoluble complexes and total cell lysates were assayed for compartment-specific membrane markers: plasma membrane (alkaline phosphodiesterase, non-GPI-linked); Golgi (mannosidase II); lysosomal (β-hexosaminidase); and ER (glucosidase II). Specific activities (enzyme U/µg total protein) were used to calculate fold enrichments. Note that all marker enzymes were depleted from the complexes.

distributed between isolated apically and basolaterally destined TGN transport vesicles, while few are apical or basolateral specific (Kurzchalia et al., 1992; Wandinger-Ness et al., 1990). This is in striking contrast to the polarized steady-state distribution of the majority of plasma membrane proteins expressed in MDCK cells, which assume an apical or basolateral distribution (Sargiacomo et al., 1989) (Fig. 4 *B, right*).

MDCK Triton-insoluble Complexes Contain Multiple GTP-binding Proteins

As hetero-trimeric and small GTP-binding proteins are involved in directing intracellular membrane traffic (Bomsel and Mostov, 1992), we suspected that GTP-binding proteins would be present in these Triton-insoluble complexes. To efficiently detect GTP-binding activity, we used an oxidation reduction scheme that covalently couples [α-³²P] GTP to the lysine residue within the GTP-binding consensus sequence, NKXD. An advantage of this method is that it does not rely on the renaturation of G proteins, as in GTP-blotting assays which preferentially detect Ras-related GTP-binding proteins. The specificity of this labeling scheme has been extensively tested using a variety of known GTP-binding proteins, both in purified form and complex mixtures (Low et al., 1992; Peter et al., 1992).

Figure 6. MDCK Triton-insoluble complexes contain small, large and heterotrimeric GTP-binding proteins. (A) Affinity labeling of GTP-binding proteins. Complexes were solubilized in Mes-buffered saline containing octyl-glucoside and affinity labeled with [α-^{32}P] GTP. We detect GTP-binding proteins of 85, 46, 40–42, 38, 25, and 6 kD that are undetectable in control samples pre-incubated with cold GTP. Two exposures are shown. (B) Detection of the α subunits of hetero-trimeric G proteins. Complexes were separated by preparative SDS-PAGE and transferred to nitrocellulose. Blots were cut into strips and incubated with antibodies specific for the α subunits of the different subclasses of trimeric G proteins (see Materials and Methods). Bound antibodies were visualized with an alkaline-phosphatase–conjugated secondary antibody. Immunoreactive bands of the appropriate molecular weight are seen by blotting with antibodies to: $G_{q/11}$ (42 kD); G_{i2} (40 kD), G_s (42 and 46 kD), and G_{i3} (41 kD). The two G_s bands correspond to alternatively spliced forms of the protein that are resolved by SDS-PAGE. An additional band of 38 kD has been observed previously with antibodies to $G_{q/11}$ (Schenker et al., 1991), and may

Using this scheme, we identified a discrete set of GTP-binding proteins of 85, 46, 40–42, 38, 25, and 6 kD (Fig. 6 A). We speculate that the 25-kD protein may represent a member of the Ras-related family of small GTP-binding proteins ($\sim$20–30 kD), while the 46, 40–42, and 38-kD proteins may represent the α subunits of hetero-trimeric G proteins ($\sim$40–50 kD). In addition, as the 100-kD microtubule motor protein dynamin (involved in membrane traffic) has recently been identified as a GTP-binding protein (Shpetner and Vallee, 1992), it is possible that the 85–90-kD GTP-binding protein we detect is a candidate motor protein.

To determine whether the 38–46-kD GTP-binding proteins we detect represent the α subunits of hetero-trimeric G proteins, we screened these complexes by immunoblotting with antibodies that distinguish between the different α subunit sub-classes (see Materials and Methods). We find that the α subunits of G_s (42 and 46 kD), G_{i2} (40 kD), G_{i3} (41 kD), and $G_{q/11}$ (42 kD) are indeed components of these insoluble complexes and are enriched from 25-fold in the case of G_s, to 175-fold for G_{i3}—as compared to total cell lysates (Fig. 6, B and C). As G_{i2} is confined to the basolateral membrane, while G_{i3} is localized to the Golgi complex, and G_s is present in apical, basolateral and Golgi locations (Ercolani et al., 1990; Berge et al., 1991), our results suggest that caveolin-rich insoluble complexes may represent caveolae or TGN-derived vesicles that shuttle between the Golgi complex and the two surface domains possibly along both endocytic and exocytic routes.

Caveolin and c-Yes Are the Major Phosphoproteins of Triton-insoluble Complexes

Two independent lines of evidence suggest that GPI-anchored proteins may interact with NRPTKs: (a) In T cells, cross-linking of GPI-linked proteins leads to proliferation and tyrosine phosphorylation via endogenous members of the Src family of NRPTKs (Stefanova et al., 1991; Thomas and Samelson, 1992); and (b) caveolin, was first identified as a major v-Src substrate in Rous sarcoma virus (RSV) transformed cells (Glenney, 1989).

In light of the above findings, we wondered whether MDCK Triton-insoluble complexes contain NRPTKs or their substrates. To test this hypothesis, we screened Triton-insoluble complexes via immunoblotting. All antibodies were first tested against MDCK extracts containing total cell proteins to ensure that the antibodies reacted with an MDCK cell protein of the appropriate size. Results of our antibody screening are shown in Table II, with pertinent positive immunoblots illustrated in Fig. 7 A. Caveolin and an additional v-Src substrate, i.e., annexin II (light and heavy chains), are present in these complexes, while other annexins tested and certain tyrosine kinase substrates (PLC-γ, Ras-GAP, and ezrin) are excluded. Of the NRPTKs known to be expressed in epithelial cells, only c-Yes (62 kD) is present in this complex, while c-Src and Fyn were excluded. This result was

represent an uncharacterized G protein α subunit. G_o is not detected as its expression is restricted to brain and neuro-endocrine tissues. (C) Enrichment of hetero-trimeric G protein α subunits. Specific activities were estimated as in Fig. 5 A, except MDCK cells were metabolically labeled for 20 h.

Table II. Identification of NRPTKs and Tyrosine Kinase Substrates in MDCK Caveolin-rich Insoluble Complexes

Antibody	SH domain	Molecular weight	Associated kinase	Immunoreactivity to complexes	Polarity
		(kD)			
Marker protein					
gD-1-DAF	–	45–50	–	+	Ap
Non-receptor tyrosine kinases					
c-Src	SH2/SH3	60	–	–	?
c-Yes	SH2/SH3	62	–	+	Ap
Fyn	SH2/SH3	60	–	–	?
Annexins					
I	–	35	EGF	–	?
II, LC	–	11	–	+	Ap
II, HC	–	39	v-Src/pkc	+	Ap
IV	–	32	–	–	Bl
Tyrosine kinase substrates					
PLC-gamma	SH2/SH3	150	EGF/FGF/PDGF	–	?
Ras-GAP	SH2/SH3	120	v-Src/EGF/PDGF/CSF-1	–	?
Caveolin	–	22–24	v-Src	+	Ap/Bl
Ezrin	–	81	EGF	–	Ap

All antibodies listed were found to be immunoreactive with a band of the expected molecular weight in total MDCK cell lysates prepared with SDS-PAGE sample buffer.

validated with two independent antibodies to c-Yes. In addition, we estimate that caveolin, c-Yes, and annexin II are 50–400-fold-enriched in low-density–insoluble complexes, relative to total cell lysates (Fig. 7 B).

We next performed in vitro phosphorylation reactions with Triton-insoluble complexes. Several groups of phosphoproteins were detected that fell into four distinct molecular weight ranges: 200–85, 62–45, 24–22, and 14–6 kD (Fig. 8 A). The 62–45-kD phosphoproteins were most sensitive to inhibition by genistein (10 and 100 μM), a specific tyrosine kinase inhibitor (Akiyama et al., 1987). We later identified these components as c-Yes (see below).

We specifically identified caveolin as the 24–22-kD phosphoprotein component by immunoprecipitation. (Fig. 8 B). Although caveolin was originally identified as a v-Src substrate, this is the first demonstration that caveolin can undergo phosphorylation in vitro. We were unable to detect a phosphorylated form of annexin II heavy chain by immunoprecipitation with antibodies either directed against the heavy or light chains. This may be related to the presence of phosphatase activity in these complexes, although we included the appropriate phosphatase inhibitors (see Materials and Methods) during immunoprecipitation.

Similarly, we identified c-Yes as the 62–45-kD phosphoprotein using two independently generated polyclonal antibodies directed against different domains of the amino terminal portion of the human Yes protein (see Materials and Methods). Triton-insoluble complexes were solubilized with octyl-glucoside at high pH, c-Yes immunoprecipitated, and in vitro phosphorylation reactions performed on the immunoprecipitates (Fig. 8 C). Two major protein bands of 62 kD and 55 kD and two minor bands of 50 and 45 kD were detected in the immune complex kinase assay. The 62-kD band represents the intact c-Yes protein; the other protein species represent products of proteolytic degradation. We frequently observe this pattern of c-Yes immunoprecipitation when cell lysates are subjected to multiple steps of fractionation, specifically in alkaline conditions. MDCK cells lysed

in RIPA buffer (Brugge and Erickson, 1977) and directly precipitated with the anti-c-Yes antibodies show one protein product of 62 kD that is active as a protein tyrosine kinase (Zhao, Y.-H., and M. Sudol, unpublished results).

To evaluate whether tyrosine-specific phosphorylation had occurred, samples were solubilized with octyl-glucoside at high pH with vortexing (a condition that effectively dissociates these complexes) and immunoprecipitated with PY 20 IgG (Fig. 8 D). The specificity of immunoprecipitation was demonstrated by competition with phenyl-phosphate. We find that many of the same phosphoproteins seen in total in vitro reactions are immunoprecipitated with anti-phospho-tyrosine IgG and are efficiently competed with phenyl-phosphate. These included the proteins we identified as caveolin and c-Yes, as well as other phospho-protein components of 200–85 and 6 kD.

We performed phospho-amino acid analysis to unequivocally determine the nature of the phosphorylation (Fig. 8 E). Although, c-Yes, caveolin and the 6-kD substrate all contained detectable levels of phospho-tyrosine (P-Y), they were predominantly phosphorylated on serine (P-S). A P-S/P-Y ratio of 5:1 was estimated for c-Yes, while a ratio of 10–20:1 was observed for caveolin and 20:1 for the 6-kD substrate. These observations indicate that this complex contains an unidentified serine-kinase activity, in addition to the c-Yes kinase. This is in accordance with the observation that in v-Src transformed cells, caveolin in vivo phosphorylation corresponds to a P-S/P-Y ratio of 10–20:1 (Glenney, 1989). Similarly, in fibroblasts the c-Yes protein was shown to be phosphorylated in vivo predominantly on serine and to a lesser degree on tyrosine, whereas in vitro exclusively on tyrosine (Sudol and Hanafusa, 1986). However, c-Yes precipitated from some hematopoietic cells displays in vitro tyrosine and serine phosphorylation (Zhao, Y.-H., and M. Sudol, unpublished results). From our data, we cannot exclude the possibility that the high level of serine phosphorylation of c-Yes and other proteins reflects a functional in vivo interaction with a serine kinase.
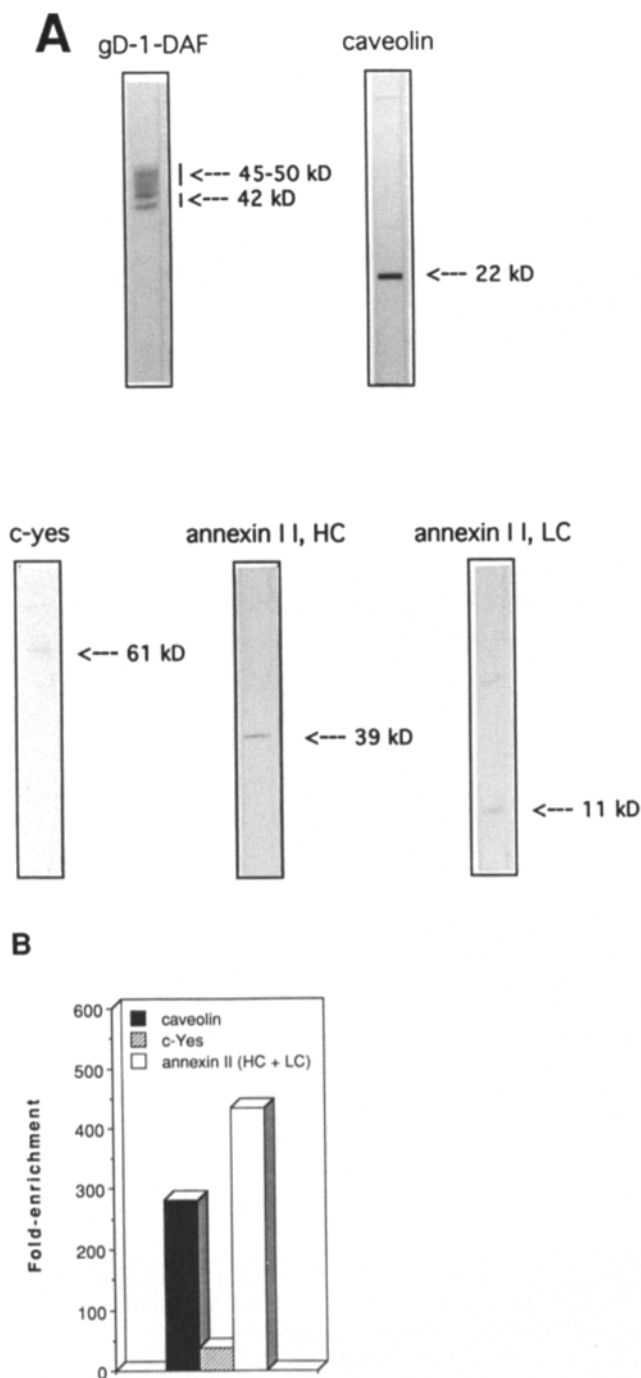
Figure 7. Caveolin, annexin II, and c-Yes are components of MDCK Triton-insoluble complexes. (A) Immunoblotting. Complexes were solubilized in sample buffer, separated by preparative SDS-PAGE and transferred to nitrocellulose. Blots were cut into strips and incubated with a variety of specific antibodies. Bound antibodies were visualized with alkaline-phosphatase conjugated secondary antibodies. Immunoreactive bands of the appropriate molecular weights are seen by blotting with antibodies to gD-1-DAF (45–50 kD), caveolin (22 kD), c-Yes (61 kD), annexin II heavy chain (39 kD), and annexin II light chain (11 kD). (B) Enrichment of caveolin, c-yes, and annexin II. Specific activities were calculated as in Fig. 5 A, except MDCK cells were metabolically labeled for 20 h.

To visualize the distribution of the proteins we identified as phosphoprotein components of Triton-insoluble complexes, we performed indirect immunofluorescence microscopy on MDCK cell monolayers. Immunolocalization revealed differing patterns of cytoplasmic punctate fluorescence and varying degrees of juxta-nuclear staining (Fig. 9), with caveolin and phosphotyrosine yielding the most similar patterns. These observations indicate that caveolin-rich-insoluble complexes may represent a discrete point of overlap in the overall cellular distribution of these components. In the case of caveolin, immunogold labeling studies have previously identified these structures as caveolae at both the level of the plasma membrane and the trans-Golgi network (Kurzchalia et al., 1992). Previous immunolocalization studies indicate that both annexin II (Gerke and Weber, 1984) and c-Yes (Zhao et al., 1990) are located beneath the apical plasma membrane in epithelial cells and that annexin II (Glenney, 1990; Emans et al., 1993) is also present in TGN tubulo-vesicular structures.

Triton-insoluble Complexes in a Fibroblastic Cell Line

To further investigate whether low-density insoluble complexes represent caveolae-enriched membrane domains, we explored their ultrastructure and biochemical composition using a fibroblastic cell line, i.e. Rat-1, as caveolae are better morphologically distinguished in fibroblasts than in epithelial cells (Rothberg et al., 1992). Rat-1 complexes were processed for transmission electron microscopy and compared with MDCK complexes (see Fig. 11 A). MDCK complexes contained 50–100 nm vesicular structures, but the majority of membranous structures were 5–10–fold larger in diameter. These membrane sheets contained numerous microinvaginations of the size and shape expected of caveolae (arrowheads). In striking contrast, Rat-1 complexes appeared to consist almost exclusively of 50–100 nm vesicular structures that resemble plasmalemmal vesicles (caveolae) seen in transmission micrographs of whole cells. As the morphology of caveolae may vary from predominantly membrane micro-invaginations (in epithelial cells) to individual or collections of vesicular structures (in fibroblasts and endothelial cells) (Rothberg et al., 1992; Severs, 1988), our results correlate well with the expected caveolar morphology in these different cell types. We also compared these complexes biochemically via cell surface labeling, metabolic labeling, GTP-affinity labeling (not shown), and in vitro phosphorylation. We find that the Rat-1 complexes contain caveolin and other cell surface proteins, possess GTP-binding proteins and undergo phosphorylation as in MDCK cells (see below). However, Rat-1 caveolin appeared to migrate with a slightly higher apparent molecular weight. These morphological and biochemical results provide further evidence that these low-density, Triton-insoluble complexes represent caveolar-derived membrane components.

An Epithelial Cell Line That Fails to Express Caveolin

FRT, a rat thyroid epithelial cell line, is highly polarized and correctly sorts a variety of apical and basolateral transmembrane antigens as compared with MDCK cells; however, FRT cells fail to recognize GPI as an apical sorting signal and missort glycolipids to the basolateral domain (Zurzolo et al., 1993a,b). The putative defect in these cells that alters
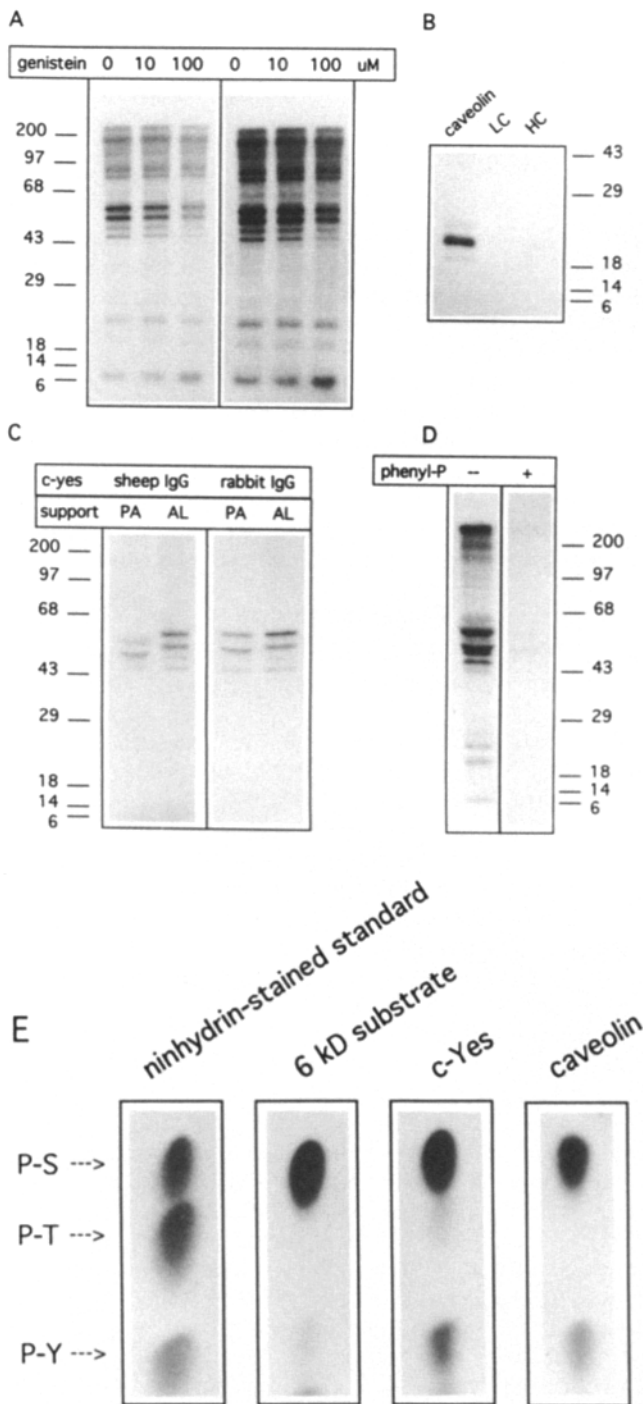
both the polarity of GPI-linked proteins and glycolipids (as compared with MDCK cells) is unknown.

As our current results suggested a role for caveolin in the sorting of GPI-linked proteins in MDCK cells, we examined the expression of caveolin in FRT cells. We used Rat-1 cells as a positive control to ensure that the anti-caveolin IgG recognize the rat protein product. All three cell lines were metabolically labeled overnight and cell lysates immunoprecipitated with anti-caveolin IgG. A 22–24-kD species corresponding to caveolin was specifically immunoprecipitated from both Rat-1 and MDCK cells, but was absent from FRT cells (Fig. 10 A). Identical results were obtained when cells were pulsed-labeled (for 15 or 30 min), indicating that a 22–24-kD form of caveolin is not synthesized in FRT cells (Fig. 10 B).

The sequence of caveolin is highly conserved, with 86% amino acid sequence identity from chicken to human (Glenney, 1992). Using the canine caveolin cDNA, we performed Northern analysis on mRNA from all three cell lines (Fig. 10 C). Our results indicate that a 3-kb transcript of the expected size is present in Rat-1 and MDCK cells, but undetect-

precipitation with anti-caveolin identified the 24–22-kD phosphorylated species as caveolin. No phosphorylated bands were immunoprecipitated with antibodies to annexin II. (C) In vitro phosphorylation of immuno-isolated c-Yes. Complexes were solubilized as in B and immunoprecipitated with two different polyclonal antibodies to c-Yes (sheep anti-c-Yes; Cambridge Research Biochemicals and rabbit anti-c-Yes; as previously described (Zhao et al., 1990)). After c-Yes immunoisolation, samples were subjected to in vitro phosphorylation. Only two major species (62 and 55 kD) and two minor species (50 and 45 kD) undergo phosphorylation. The 62-kD band represents the intact c-Yes protein, while the other protein species are c-Yes degradation products frequently observed when processing the c-Yes protein in alkaline buffers. Two different supports were also used for binding the different primary antibodies: protein A-Sepharose (PA) or Avid-AL (AL). In the case of the sheep antibody, rabbit anti-sheep IgG was used as a bridge for coupling to protein A-Sepharose. On the other hand, Avid-AL strongly binds all IgG forms without the need for a secondary antibody. Note that the mobility of c-Yes is altered by the presence of co-migrating unlabeled rabbit anti-sheep secondary antibodies used to facilitate immunoprecipitation. Experiments in which the primary antibodies were omitted or use of irrelevant IgG demonstrated specificity (not shown). (D) Tyrosine-specific phosphorylation occurs. After in vitro phosphorylation, samples were solubilized at alkaline pH in buffers containing octyl-glucoside and phosphatase inhibitors and immunoprecipitated with anti-phosphotyrosine antibodies (PY-20). As a control for specificity, certain samples were eluted by treatment with phenylphosphate. Phosphoproteins that remained bound to protein A-Sepharose were analyzed. The 62–45-kD species and 24–22-kD species correspond to c-Yes and caveolin, respectively (see B and C). Note that the mobility of c-Yes is altered by the presence of co-migrating unlabeled rabbit anti-mouse secondary antibodies used to facilitate immunoprecipitation of PY-20. (E) Phosphoamino acid analysis of c-Yes, caveolin and the 6-kD protein phosphorylated in an in vitro kinase assay. All three Phosphoproteins are phosphorylated predominantly on serine residues and to a lesser degree also on tyrosine. (P-Y) Phosphotyrosine; (P-T) phosphothreonine; (P-S) phosphoserine. The phosphoamino acids were resolved by one-dimensional electrophoresis on a TLC plate. Visualization of the phosphoamino acid standards was by ninhydrin reaction (see Materials and Methods).
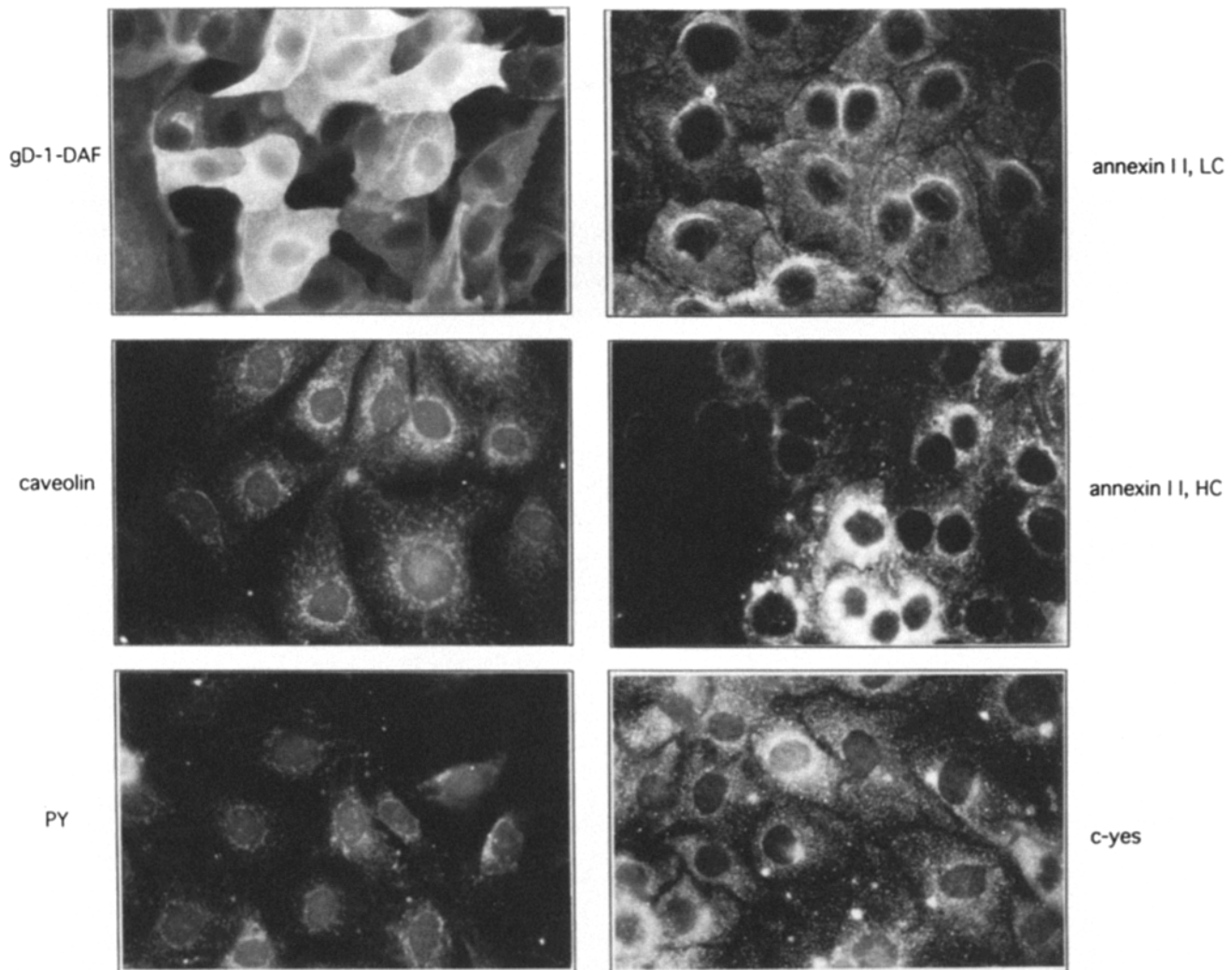

Figure 8. Caveolin and c-Yes are the two major phosphoprotein components of MDCK Triton-insoluble complexes during in vitro phosphorylation. (A) Triton-insoluble complexes possess kinase activity. Complexes were resuspended in kinase reaction buffer and the reaction initiated by the addition of [γ-^{32}P]ATP. Reactions were terminated by boiling in sample buffer. Certain samples were pre-incubated with genistein (10 and 100 μM), a protein–tyrosine kinase inhibitor. Phosphoproteins fall into four molecular weight ranges: 200–85, 62–45, 24–22, and 14–6 kD. The 62–45-kD species were most sensitive to inhibition by genistein. These proteins correspond to phosphorylated forms of c-Yes (see C below). Two exposures are shown. (B) Identification of known phosphoproteins. Complexes were in vitro phosphorylated, solubilized at alkaline pH with octyl-glucoside, and immunoprecipitated with antibodies to caveolin and annexin II (heavy chain or light chain). Immuno-

Figure 9. Immuno-localization of the phosphoprotein components of Triton-insoluble complexes. gD-1-DAF expressing MDCK cells were seeded on glass coverslips and prepared by methanol/acetone fixation for immunostaining. After incubation with primary antibodies, bound antibodies were visualized with rhodamine-conjugated secondary antibody. Primary antibodies were as follows: gD-1, caveolin, anti-phosphotyrosine *(PY)*, annexin II light chain, annexin II heavy chain, and c-Yes. All antibodies tested yield distinct fluorescent dotted patterns and peri-nuclear staining, with caveolin and anti-PY appearing most similar.

able in FRT cells. In addition, Southern analysis revealed a genomic copy of caveolin in all three cell lines (not shown). One interpretation of these results is that a lack of caveolin mRNA in FRT cells reflects tissue-specific expression of the caveolin gene. In this regard, caveolin is expressed at high levels in heart, skeletal muscle, placenta, and lung, while barely detectable in other tissues (Glenney, 1989, 1992).

Cells That Fail to Express Caveolin Form Incomplete Triton-insoluble Complexes

To further understand the missorting phenotype of FRT cells, we examined the morphology and composition of Triton-insoluble complexes prepared from FRT cells. Ultrastructurally, FRT complexes consisted primarily of 50–100 nm vesicular structures, some appearing interconnected or in bunches, as previously observed for caveolae (Severs, 1988) (Fig. 11 *A, inset*). These morphological observations demonstrate that FRT cells can form Triton-insoluble complexes. We next analyzed these complexes biochemically

(Fig. 11 *B*). No cell surface proteins were detected in FRT complexes even on radiographic overexposures. Identical results were obtained when filter-grown FRT cells labeled apically or basolaterally with sulfo-NHS-biotin (not shown), indicating that our results were not due to selective basolateral localization of these complexes. Similarly, we find that FRT complexes are depleted of metabolically labeled components. However, certain metabolically labeled components can be observed on radiographic over-exposures. Note that a metabolically labeled 22–24-kD band we have identified as caveolin in MDCK and Rat-1 cells is conspicuously absent from FRT complexes. This is in accordance with our observation that FRT cells do not express detectable levels of caveolin (see above). We also examined the phospho-protein profile of FRT complexes. Only a 14–6-kD component underwent in vitro phosphorylation, while bands we identified as phosphorylated caveolin and c-Yes were absent from these complexes. In addition, visualization of gently solubilized FRT complexes by low-angle rotary shad-

A steady-state

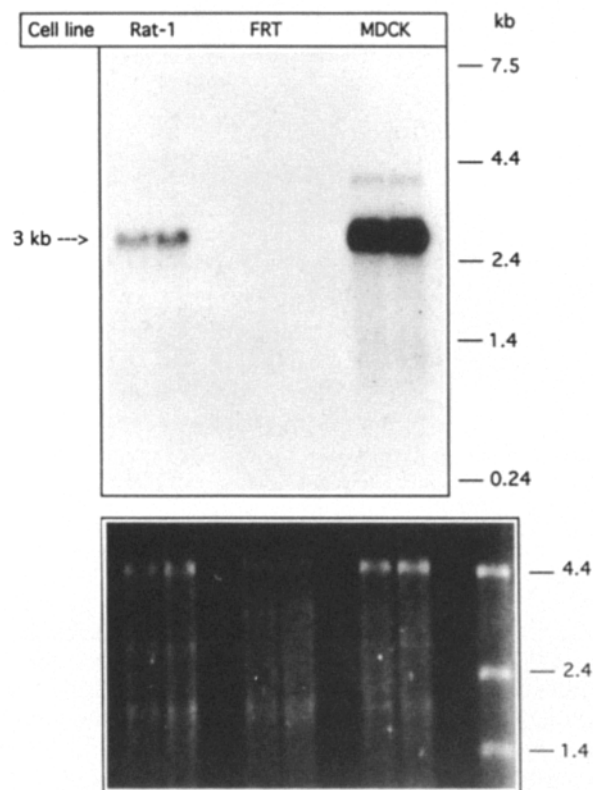| Cell line	Rat-1		MDCK		FRT	
Triton-fraction	S	I	S	I	S	I
caveolin						

— 29

— 18

B pulse

Cell line	Rat-1		MDCK		FRT	
Triton-fraction	S	I	S	I	S	I
caveolin						

— 29

— 18

C Northern analysis

Cell line	Rat-1	FRT	MDCK	kb

— 7.5

— 4.4

3 kb --->

— 2.4

— 1.4

— 0.24

— 4.4

— 2.4

— 1.4
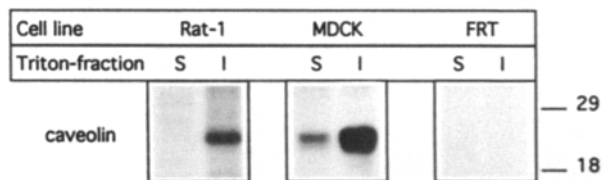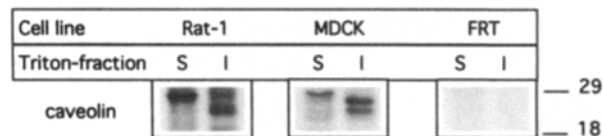
Figure 10. FRT cells fail to express detectable levels of caveolin. (*A* and *B*) Immunoprecipitation. FRT cells were metabolically labeled either overnight (*A*) or pulsed for 15 min (*B*). Cells were extracted first at acidic pH (6.5) in Triton-containing buffers and then re-extracted at alkaline pH (8.0) in octyl-glucoside containing buffers. Both extracts were immunoprecipitated with anti-caveolin antibodies and labeled proteins visualized by fluorography. MDCK cells and Rat-1 cells were processed in parallel and served as positive controls. (*S*) Triton-soluble fraction; (*I*) Triton-insoluble fraction. A 22–24-kD species corresponding to immuno-isolated caveolin is easily detected both in steady-state or pulse-labeled MDCK and Rat-1 cells. In striking contrast, FRT cells fail to express detectable levels of caveolin, even on overexposure of the autoradiograph.

owing did not reveal any discernable structures (not shown), in contrast to MDCK cells (Fig. 3). As caveolin appears to contribute to the cytoplasmic coat of caveolae (Rothberg et al., 1992) and is not expressed in FRT cells, these results were not unexpected.

Taken together, these results provide supporting evidence that Triton-insoluble complexes normally contain putative components of the GPI protein sorting machinery—as they appear biochemically incomplete in a cell line that fails to express caveolin and selectively missorts GPI-linked proteins and glycolipids. These results also suggest that caveolin may be necessary to recruit other components to the complex.

Discussion

Are Low-density, Triton-insoluble Complexes Caveolae?

Caveolae are present at the cell surface of a wide variety of cells. Glycolipids, cholesterol, GPI-linked proteins, and caveolin cluster in these micro-invaginations (Montesano et al., 1982; Rothberg et al., 1992, 1990*b*; Severs, 1988) and are all resistant to Triton X-100 extraction (Glenney and Zokas, 1989; Hagmann and Fishman, 1982; Hooper and Turner, 1988; Streuli et al., 1981; Yu et al., 1973). Morphologically caveolae appear as 50–100-nm spherical structures, with a striated cytoplasmic coat consisting of concentric rings of globular subunits linked together as strands (Peters et al., 1985; Rothberg et al., 1992). In addition, caveolae are not restricted to the apical or basolateral domains of endothelial or epithelial cells (Rothberg et al., 1992; Dupree et al., 1993).

Low-density, Triton-insoluble complexes prepared from MDCK cells are enriched in cholesterol and glycolipids, and contain GPI-linked proteins (Brown and Rose, 1992). In this report, we demonstrate that these complexes: (*a*) morphologically appear by low-angle rotary shadowing as 50–100-nm spherical structures, consisting of globular subunits aligned in concentric rings; (*b*) that they contain caveolin as a major transmembrane phospho-protein component; (*c*) that similar complexes prepared from the fibroblastic cell line, Rat-1, appear morphologically indistinguishable from authentic caveolae (plasmalemmal vesicles) seen in transmission electron micrographs of intact fibroblasts; and (*d*) caveolin and other components of Triton-insoluble complexes are present on both apical and basolateral domains,

(*C*) Northern Blot Analysis. Isolated mRNA from Rat-1 (8–10 μg), FRT (8–10 μg), and MDCK cells (5 μg) was separated using formaldehyde–agarose gels, transferred to hybridization membranes and probed with the ³²P-labeled MDCK caveolin cDNA. A prominent ∼3-kb transcript of the expected size of caveolin mRNA was detected in Rat-1 cells and MDCK cells, but was absent from FRT cells. Note that the probe hybridizes most efficiently with the MDCK species as they are identical. The ethidium-stained agarose gel before transfer is shown below the autoradiogram for comparison. As expected, a small amount of contaminating rRNA was present in all the samples. Molecular sizes were derived from ethidium bromide-stained marker RNA.
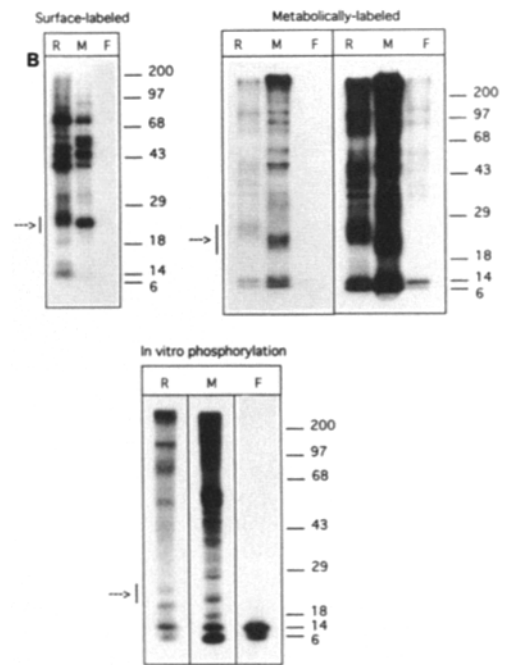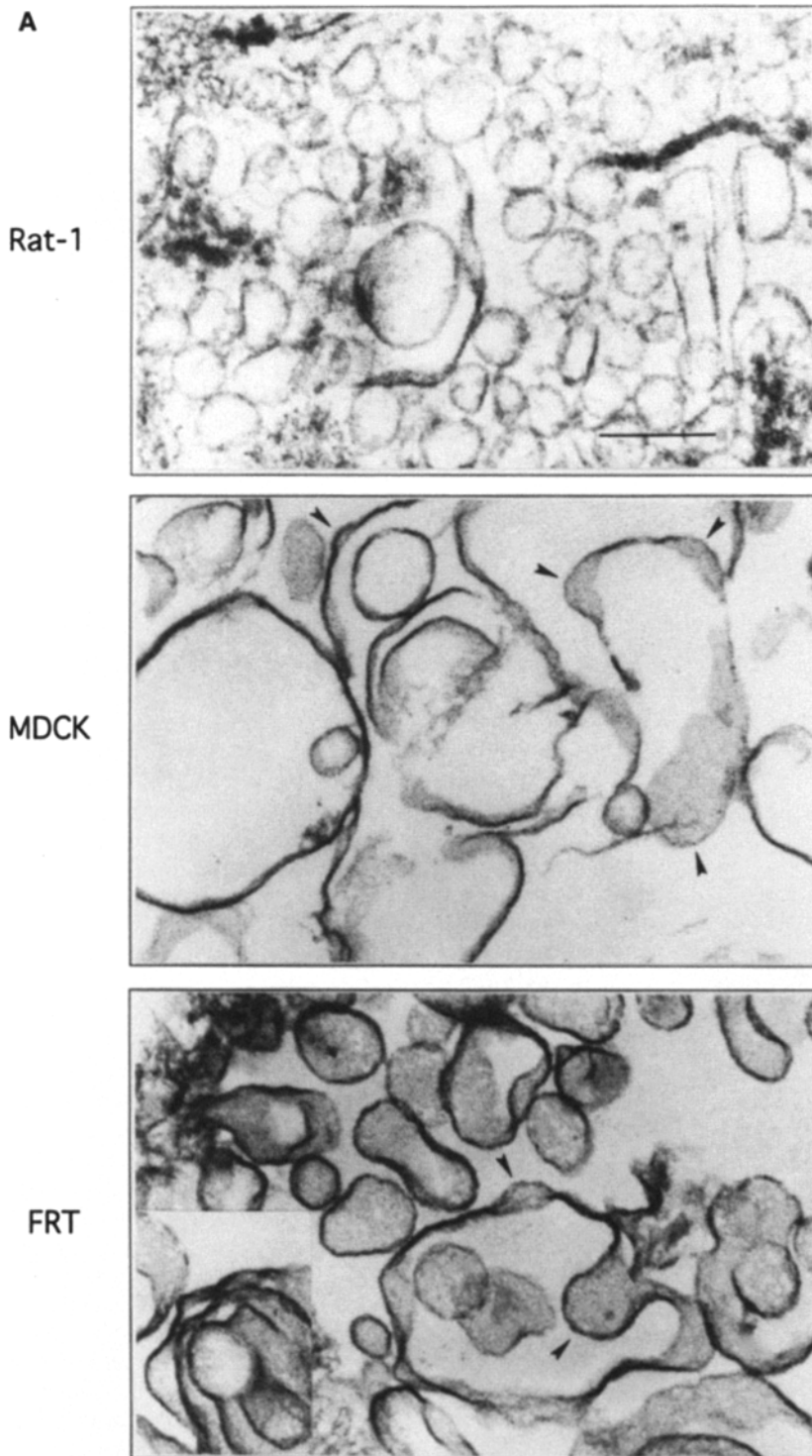
A

Rat-1

MDCK

FRT

Figure 11. Morphological and biochemical comparison of Triton-insoluble complexes prepared from fibroblastic (Rat-1) or epithelial (MDCK and FRT) cell lines. (*A*) Morphological. Complexes were prepared from Rat-1, MDCK and FRT cells and examined by transmission EM. In the case of Rat-1, studies reveal a preponderance of 50–100 nm vesicular structures. Similar 50–100 nm vesicles were observed for MDCK cells, however, larger (5–10×) vesicular structures were more numerous; arrowheads indicate membrane invaginations that are of the same size and shape expected for caveolae. In FRT cells, these complexes consisted predominantly of 50–100 nm vesicular structures as seen with Rat-1 cells and often appeared linked or in bunches (*inset*). This dendritic appearance has previously been ascribed to caveolae which often appear linked together in bunches (like clusters of grapes). Bar, 0.2 μm. (*B*) Biochemical. Complexes were prepared from equivalent cell numbers of Rat-1, MDCK and FRT. Cells were surface-biotinylated, metabolically labeled, or left unlabeled for in vitro phosphorylation. Note that Rat-1 and MDCK complexes contain caveolin and other cell surface proteins, are easily metabolically labeled, and undergo phosphorylation. Bands we have identified as caveolin are indicated by arrows. Note that caveolin migrates with a slightly higher molecular mass in Rat-1 cells. Major differences were observed between FRT complexes and complexes prepared from the other cell lines. These differences may reflect their unusual sorting phenotype. Two exposures of metabolically labeled samples are shown.

unlike most MDCK surface proteins which assume a polarized distribution.

These numerous parallels are striking and lead us to believe that Triton-insoluble complexes represent caveolae-enriched membrane domains. However, further proof will be necessary before low-density, Triton-insoluble complexes (biochemical) and caveolae (morphological) can be considered interchangeable.
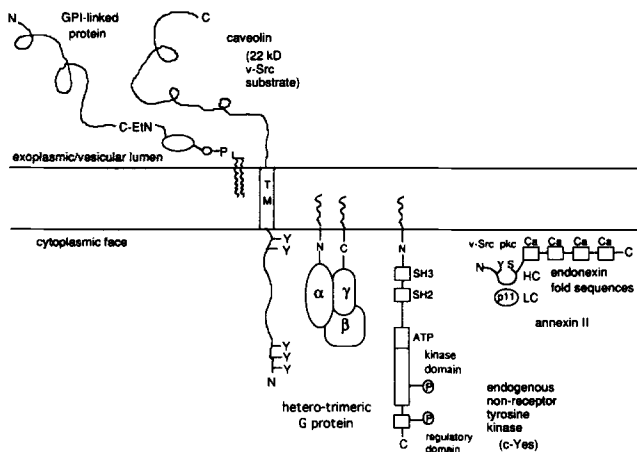
Figure 12. Diagrammatic representation of the known plasma membrane topology of identified components of the Triton-insoluble complex. The salient features and domain organization of a generic GPI-linked protein, caveolin, a hetero-trimeric G protein, c-Yes, and annexin II are illustrated where known. Note that annexin II is actually a hetero-tetramer containing two light and two heavy chains. Both GPI-linked proteins and c-Yes contain lipid modifications for membrane attachment. G-proteins are lipid modified with α subunits containing NH_2-terminal myristoylation, and γ subunits containing COOH-terminal sites for prenylation (not to scale).

Caveolae, Hetero-trimeric G Proteins, and Epithelial Protein Sorting

Recent experimental observations indicate a new role for hetero-trimeric G proteins as regulators of the constitutive secretory pathway. Various hetero-trimeric G proteins have been localized to the TGN where their GTPase activity regulates vesicle budding and release (Melancon, 1993). In polarized epithelial cells, G_s is involved in directing apical vesicular traffic, while members of the G_i class are involved in basolateral transport (Stow et al., 1991; Bomsel and Mostov, 1992; Pimplikar and Simons, 1993). In support of these observations, we report here that multiple hetero-trimeric G proteins (both G_s and G_i classes) are enriched in caveolin-rich insoluble complexes that form during the apical transport of GPI-linked proteins. Our results may also imply an association between caveolae and hetero-trimeric G proteins during signal transduction.

Evidence for the latter association stems from experiments with cholera toxin. The B subunit of cholera toxin binds to a ganglioside and caveolar component, GM_1, at the apical surface of intestinal epithelial cells (Montesano et al., 1982). As it enters cells via caveolae, its hydrophobic A_1 subunit pierces the membrane and activates G_s via ADP-ribosylation. Activated apical G_s must then be transported across the cell to the basolateral membrane to reach and stimulate adenylate cyclase (Dominguez et al., 1987). As caveolae have been long been implicated in transcytosis (Severs, 1988), transcellular transport of activated G_s could be mediated by caveolae. Caveolar localization of G_s would be consistent with our observation that G_s is enriched in caveolin-rich insoluble complexes and the recent observation that caveolin and the β-adrenergic receptor, a G_s-coupled receptor, co-localize in caveolae (Dupree et al., 1993). Similarly, the B subunit of pertussis toxin recognizes gangliosides

at the apical surface of respiratory epithelium (Saukkonen et al., 1992), and inactivates the G_i class of α subunits via ADP-ribosylation. Although the mechanism for pertussis toxin internalization is currently unknown, it is likely to occur via caveolae as tetanus and cholera toxin both recognize gangliosides and are internalized by caveolae (Montesano et al., 1982). In light of the above associations, we expect that many G protein coupled receptors will be found to signal through a regulated affiliation with caveolae.

Interestingly, activation of G_s by cholera toxin stimulates the tumorigenicity of RSV-transformed cells (Gottesman et al., 1984). As caveolin was first identified as the major v-Src substrate in RSV-transformed cells (Glenney, 1989), caveolin or v-Src could interact with activated G_s to influence tumorigenicity.

GPI Protein Sorting and NRPTKs

The c-*yes* proto-oncogene encodes a 62-kD N-myristoylated member of the Src family of NRPTKs (Sudol, 1993). Elevated levels of c-Yes protein are expressed in neural tissues, epithelia (i.e., kidney, intestine, liver, and lung) and spermatids (acrosomes) (Sudol and Hanafusa, 1986; Zhao et al., 1990)–all polarized cells. In epithelia, c-Yes appears confined to the microvillus border of the apical plasma membrane (Zhao et al., 1990). Based on this location, it has been suggested that c-Yes might be involved in regulating apical exocytic–endocytic trafficking events (Sudol, 1990). Our results are consistent with this hypothesis, and suggest that c-Yes may be involved in regulating the intracellular apical recognition of GPI-linked proteins. In addition, c-Yes is often found as a complex with a 38-kD phosphoprotein (Grandori et al., 1991). As the heavy chain of annexin II has a reported molecular mass of 36–39 kD, the 38-kD c-Yes-associated protein could represent annexin II. This is consistent with our observation that annexin II and c-Yes are present in MDCK Triton–insoluble complexes.

Caveolin is the major v-Src substrate in RSV-transformed chick embryo fibroblasts and its phosphorylation depends on the membrane association of v-Src via NH_2-terminal myristoylation (Glenney, 1989; Glenney and Zokas, 1989). However, whether caveolin is a substrate for endogenous NRPTKs remains unknown. It contains five revolutionary conserved cytoplasmic tyrosine residues as potential sites for phosphorylation. Here we show that caveolin undergoes phosphorylation on tyrosine in vitro when present in Triton-insoluble complexes. Given that caveolin is a major v-Src substrate and c-Yes is an active NRPTK present in these complexes, we suggest that caveolin may represent an endogenous substrate for c-Yes. In addition to being a substrate for c-Yes, caveolin may play the role of an adaptor molecule that communicates extracellular signals from GPI-linked proteins to the c-Yes kinase. One of the best examples for such an interaction comes from studies of adaptor molecules, MB-1 and B29, that integrate signals from the IgM receptor to Lyn kinase in B cells (Cambell et al., 1991). A similar adaptor molecule has been postulated to link CD36 receptor on platelets with Fyn, Lyn, and c-Yes kinases (Huang et al., 1991). Future studies will determine whether c-Yes, caveolin, and a GPI-linked protein form a functional signal transduction complex at the apical surface of epithelia.

The annexins represent a growing family of twelve distinct calcium-dependent phospholipid-binding proteins. Each an-

nexin contains a unique NH₂-terminal region and four-to-eight repeated calcium-binding domains. The function of the annexins remains unknown, although different annexins have been shown to possess calcium channel activity, 1,2 cyclic inositol-2 phosphohydrolase activity, are required for the budding of clathrin-coated vesicles, or have been suggested to represent exocytic fusion pores (Creutz, 1992).

Annexin II exists as a hetero-tetramer as a complex of two 39-kD heavy chains and two 11-kD light chains. Light chains bind to the unique NH₂-terminal region of the heavy chain and this association is regulated by phosphorylation at sites present with the unique NH₂ terminus. This heavy chain region contains phosphorylation sites both for v-Src and protein kinase C. Extensive evidence has been presented that implicates annexin II in the regulation of exocytic fusion events (Ali et al., 1989; Drust and Creutz, 1988; Nakata et al., 1990). Most studies have explored its association with the calcium-regulated secretion of chromaffin granules. In these studies, annexin II is localized on the cytoplasmic face of chromaffin granules and beneath the plasma membrane at sites of exocytic fusion. However, in epithelial cells it is located on the cytoplasmic face of the apical cell surface (Gerke and Weber, 1984). Given this apical localization, it is interesting that in MDCK cells apical secretion can also be calcium-dependent (Rodriguez-Boulan and Nelson, 1989). Culture of MDCK cells in low-calcium medium prevents the formation of a mature apical surface as it is retained intracellularly as a post-Golgi compartment, termed vacuolar apical compartment (VAC). VACS contain apical antigens (including GPI-linked proteins [Lisanti et al., 1990a]), but exclude basolateral antigens. Exocytosis of VACS occurs within minutes upon return to normal extracellular calcium levels. Rises in intracellular calcium levels are highest at the sites of VAC fusion with the plasma membrane (Nigam et al., 1992). Thus, the association of annexin II with Triton-insoluble complexes reported here is consistent with its proposed role in exocytic fusion and further suggests a role in the apical transport of GPI-linked proteins. In support of our observations, an early report demonstrated that annexin II co-fractionates with the GPI-linked protein, 5' nucleotidase, in a low-density membrane preparation (Radke et al., 1983). However, these results preceded the identification of this protein as a member of the annexin family and the discovery of the GPI anchoring mechanism.

In light of the calcium-dependent membrane association of annexins, it is interesting to note that caveolae are thought to be involved in regulation of intracellular calcium stores and as such contain a Ca²⁺-ATPase (Fujimoto, 1993) and a 230-kD cell surface form of the IP₃ receptor (Fujimoto et al., 1993).

Membrane Protein Sorting as a Form of Intracellular Signal Transduction

Like many cellular membrane compartments, Triton-insoluble complexes can be visualized as containing three classes of protein components: solely exoplasmic/luminal (GPI-linked proteins), transmembrane (caveolin and other cell surface components), and solely cytoplasmic (GTP-binding proteins, annexin II, c-Yes, and a serine-kinase activity). Given this membrane topology (Fig. 12), transmembrane protein components could be expected to recognize GPI-linked proteins (caveolin or another cell surface molecule)

and transfer destination signals to the cytoplasm via their cytoplasmic tails. These cytoplasmic tail(s) could then interact with signal transducing molecules (GTP-binding proteins or cellular kinases), to transmit these destination signals to other proteins involved in vesicular budding, transport, or membrane fusion (annexin II). This scheme superficially resembles a model for signal transduction, complete with ligand (GPI), receptor (caveolin and others), and cytoplasmic signal transducing molecules (G-proteins and cellular kinases/substrates). Similarly, others have recently proposed that sorting during membrane trafficking may represent a form of intracellular signal transduction (Bomsel and Mostov, 1992). Our working hypothesis for GPI sorting agrees with this "signal transduction model" of membrane trafficking.

A prediction of this working hypothesis is that a cell surface molecule acts as a transmembrane adaptor molecule, simultaneously recognizing GPI-anchored proteins and interacting with downstream cytoplasmic addressing molecules. We propose that caveolin could represent this transmembrane adaptor based on several experimental observations: (a) caveolin spans the membrane and exists as a type II transmembrane protein; (b) it is part of complex containing GPI-linked molecules and cytoplasmic signaling molecules; (c) it undergoes phosphorylation in the absence of v-Src transformation; (d) it localizes to the TGN—the site of segregation of apical from basolateral proteins (Kurzchalia et al., 1992); and (e) epithelial cells that fail to express caveolin missort GPI-linked proteins and contain incomplete Triton-insoluble complexes.

In conclusion, our findings clarify several seemingly unrelated observations regarding GPI-linked proteins. Insolubility in non-ionic detergents, caveolar localization, association with NRPTKs, and apical sorting in epithelial cells, all appear to be different manifestations of the formation of a multi-subunit lipid–protein complex. Further studies will be necessary to elucidate the spacial relationship between the members of this putative GPI-sorting complex.

Note Added in Proof: The serine kinase activity we observe is unaffected by activators or inhibitors of protein kinase A, protein kinase C, or CaM-kinase, suggesting that this activity represents a kinase that is distinct from

these classes of well-known protein kinases. In addition, we find that paxillin (another v-Src substrate) is associated with isolated caveolar membrane domains.

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