Caveolin-1 and -2 in the Exocytic Pathway of MDCK Cells

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Abstract. We have studied the biosynthesis and transport of the endogenous caveolins in MDCK cells. We show that in addition to homooligomers of caveolin-1, heterooligomeric complexes of caveolin-1 and -2 are formed in the ER. The oligomers become larger, increasingly detergent insoluble, and phosphorylated on caveolin-2 during transport to the cell surface. In the TGN caveolin-1/-2 heterooligomers are sorted into basolateral vesicles, whereas larger caveolin-1 homooligomers are targeted to the apical side. Caveolin-1 is present on both the apical and basolateral plasma membrane, whereas caveolin-2 is enriched on the basolateral surface where caveolae are present. This suggests that caveolin-1 and -2 heterooligomers are involved in caveolar biogenesis in the basolateral plasma membrane. Anti–caveolin-1 antibodies inhibit the apical delivery of influenza virus hemagglutinin without affecting basolateral transport of vesicular stomatitis virus G protein. Thus, we suggest that caveolin-1 homooligomers play a role in apical transport.

Caveolin-1, a 21–24-kD membrane protein, was originally isolated from two different sources: from trans-Golgi network–derived exocytic vesicles (hence its other name VIP21, vesicular integral membrane protein of 21 kD) (Kurzchalia et al., 1992), and from plasma membrane invaginations called caveolae (Rothberg et al., 1992). Caveolae can be internalized from the surface and the protein might cycle between different cellular compartments (Parton et al., 1994; Schnitzer et al., 1994), however, its functions are poorly understood thus far. Caveolin-1 plays an important structural role in forming caveolar invaginations on the plasma membrane (Fra et al., 1995), but as yet there are no studies addressing its function in the TGN. Caveolin-1 has two isoforms, α- and β-derived from the use of two alternative transcription initiation sites, the latter starting with an internal methionine at position 32 (Scherer et al., 1995). It has an unusual topology, forming a membrane-embedded hairpin structure with both the amino and carboxyl termini in the cytoplasm (Dupree et al., 1993). Furthermore, it has been reported to form high molecular weight homo- and heterooligomeric complexes (>104 kD) that resolve into 200-, 400-, and 600-kD oligomers when analyzed by SDS-PAGE without boiling (Monier et al., 1995; Sargiacomo et al., 1995). Caveolin-1 has also been shown to bind tightly to cholesterol (Murata et al., 1995; Li et al., 1996b). The primary determinants of oligomerization are protein domains of caveolin-1, but cholesterol binding promotes oligomerization that is further stabilized by the attachment of palmitoyl chains to the carboxyl-terminal region (Monier et al., 1996). The oligomers isolated from tissues or cultured cells are insoluble in detergents, such as Triton X-100 or CHAPS at 4°C (Kurzchalia et al., 1992; Sargiacomo et al., 1993). Oligomers can also be produced from in vitro–synthesized caveolin-1 after translocation into microsomal membranes. However, these oligomers are Triton X-100 soluble (Monier et al., 1995).

Recently, a related protein having 38% sequence identity to caveolin-1 was isolated from caveolin-enriched membranes and termed caveolin-2 (Scherer et al., 1996). Caveolin-2 was coexpressed with caveolin-1 in all the tissues analyzed, being most abundant in adipocytes. The epitope-tagged and overexpressed caveolin-2 colocalized with caveolin-1 in fibroblasts, as judged by immunofluorescence microscopy. However, in contrast to caveolin-1, caveolin-2 was found to exist mainly as a monomer (Scherer et al., 1996).

MDCK epithelial cells are a well-characterized model system for studying polarized membrane trafficking, and are one of the sources from which caveolin-1 was first isolated (Kurzchalia et al., 1992). These cells can form polarized monolayers with distinct apical and basolateral surfaces. Sorting of proteins destined for the two plasma membrane domains takes place in the TGN where sepa-
rate apical and basolateral vesicles form to deliver cargo to the correct destinations (Griffiths and Simons, 1986). In this paper we have analyzed the routing and biochemical characteristics of endogenous, newly synthesized caveolin-1 and -2 in MDCK cells. We provide evidence that the two caveolins can form homo- and heterooligomeric complexes in the ER, and that these complexes are modified during transport to the plasma membrane. We further demonstrate that large caveolin-1 complexes are present in apical transport vesicles. In contrast, smaller caveolin-1/-2 heterooligomers are routed to the basolateral membrane where caveolin-2 is enriched and caveolae structures are found. Apical but not basolateral exocytosis can be inhibited with caveolin-1 antibodies, suggesting that two different caveolin complexes function in TGN to surface transport.

Materials and Methods

Reagents

Unless otherwise stated, all chemicals were obtained from the sources described previously by Wandinger-Ness et al. (1990) and Ikonen et al. (1995). The polyclonal anti-caveolin-1 amino-terminal antibody has been characterized (Dupre et al., 1993) and the anti-caveolin-1 antibody designated N-20 was from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). To generate anti-caveolin-2 antibodies, polyclonal sera were raised against a synthetic peptide using the residues DFGDLEQLADS5GR of canine caveolin-2. Sera for biochemical experiments were affinity purified using a GST-His6-caveolin-2 fusion protein (see Fig. 1; Peränen, 1992). For immunofluorescence microscopy, sera were affinity purified on a column prepared by coupling the fusion protein to CNBr-activated Sepharose 4B or a protein A-Sepharose CL-4B column, according to the manufacturer’s instructions (Pharmacia Biotech, Inc., Piscataway, NJ). Antibodies were eluted at low pH (0.2 M glycine, pH 2.8). Molecular mass standards used in gradient centrifugation were from Sigma Chemical Co. (St. Louis, MO). Detergents were the following: SDS (Bio-Rad Laboratories, Hercules, CA), Triton X-100 (Serva, Heidelberg, Germany), CHAPS (Sigma Chemical Co.), and N-octyl-3-glucopyranoside (Calbiochem-Novabiochem, La Jolla, CA). Methyl-β-cyclodextrin was from Sigma Chemical Co. and lovastatin was from Merck, Sharp, and Dohme (Haar, Germany).

Cell Culture

MDCK strain II cells were grown on 1.2- (Western blotting, microscopy, and transport assays), 2.4- (pulse-chase and metabolic labeling experiments), or 7.5-cm (immunoisolation of vesicles) Transwell filters (Costar, Cambridge, MA), as described previously (Pimplikar et al., 1994), plating 5 × 10^4 cells per filter, respectively. The filter cultures (with or without filters) were incubated for 20 min at 37°C with intermittent vortexing. After spinning for 10 min at 1400 g, the cell layer and other treatments performed in parallel with the [35S]methionine pulse-labeling were carried out with 180 μCi of [35S]methionine and immunoprecipitated with the respective antibody and recovered with protein A–Sepharose (Pharmacia Biotech, Inc.). The immunoprecipitates were washed five times with 20 mM Tris-HCl, pH 7.4, 100 mM NaCl, 0.4% SDS, 0.2% Triton X-100 in the presence of protease inhibitors (chymostatin, leupeptin, antipain, and pepstatin at 25 μg/ml each) and phosphatase inhibitors (200 μM sodium vanadate, 50 mM sodium fluoride). Lysates were incubated overnight at 4°C with the respective antibody and recovered with protein A–Sepharose (Pharmacia Biotech, Inc.). The immunoprecipitates were washed five times with 20 mM Tris-HCl, pH 7.4, 100 mM NaCl, 0.1% SDS, 0.5% Triton X-100, and phosphatase inhibitors (see above) and twice with 50 mM Tris-HCl, pH 7.5, before separation by SDS-PAGE.

For [3H]euclid MDCK cells from one 2.4-cm well of a six-well plate were washed twice with phosphate-free DME containing 0.1% FCS dialyzed against 20 mM Tris-HCl, pH 7.6, 137 mM NaCl (TBS). After incubating for 10 min in this medium the cells were labeled for 3 h at 37°C with 500 μCi of [3H]P]ATP in 1 ml of the same medium. Cells were lysed in 100 mM NaCl, 10 mM Tris-HCl, pH 7.4, 1% Triton X-100, protease inhibitors, 2 mM sodium vanadate, and 50 mM sodium fluoride by incubating for 20 min at 37°C with intermittent vortexing. After spinning at 3000 rpm in a microfuge (Eppendorf, Hamburg, Germany), the insoluble debris was discarded, the lysates precleared with protein A–Sepharose overnight at 4°C, and then precipitated with the anti–caveolin-1 antibody as described above.

[35S]methionine carried out overnight in an identical well, and other treatments performed in parallel with the [3H]P]ATP sample gave results similar to the steady-state [35S]methionine and immunoprecipitation on filter-grown cells as described above. [3H]P] and [35S]methionine was also carried out in J774 A1 cells, yielding results similar to MDCK cells.

SDS-PAGE, 2D Gel Electrophoresis, and Western Blotting

Immunoprecipitates were solubilized either in standard SDS-PAGE sample buffer or in alkaline buffer (Monier et al., 1995). The samples were incubated for 30 min at 25°C or heated to 95°C for 5 min as detailed in the figure legends. When the samples were not heated, 200-, 400-, and 600-kD standards used in gradient centrifugation were from Sigma Chemical Co. Oligomerization was monitored by SDSPAGE. The proteins were resolved on a 10% polyacrylamide gel and Coomassie blue-stained gels were photographed. The gels were stained, destained, dried, and subjected to autoradiography. The areas of the autoradiogram that corresponded to the bands that were previously identified as oligomers were cut out and counted in a scintillation counter to determine the relative amounts of 35S methionine and 32P P]ATP.

Abbreviations used in this paper: 2D, two-dimensional; HA, influenza virus hemagglutinin; SLO, streptolysin-O; VSV-G, vesicular stomatitis virus glycoprotein.

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Proteins were resolved as detailed by Wandelinger-Ness et al. (1990) in two dimensions by IEF and SDS-PAGE based on the method of Bravo (1984). For Western blotting, MDCK cells were lysed in the same buffer as for immunoprecipitations, incubated for 30 min at 25°C, and then proteins were separated on polyacrylamide gels. Proteins were transferred to a nitrocellulose membrane in 25 mM Tris-HCl, 190 mM glycine, and 20% methanol and after blocking and incubation with the respective antibodies detected with enhanced chemiluminescence (Amer sham Corp.).

Isolation of Budded Vesicles from Semintact Cells

A budded fraction was prepared essentially as described by Xu and Shields (1993) using a hypotonic swelling procedure to selectively disrupt the plasma membrane (Beckers et al., 1987). Briefly, confluent MDCK cells (passages 6–25) on 10-cm dishes infected with influenza virus (PR8) or VSV were pulse labeled with [35S]methionine 3 h after infection and then the radiolabeled glycoproteins were accumulated in the TGN by a 20°C temperature block for 2 h. After washing the cells three times with ice-cold swelling buffer (10 mM Heps [pH 7.2], 15 mM KCl) and incubation for 10 min on ice, cells were scraped in 3 ml of breaking buffer (50 mM Heps [pH 7.2], 90 mM KCl), pelleted at 800 g, and then washed once with breaking buffer. Cells were resuspended in GGA buffer (25 mM Heps, pH 7.4), 38 mM potassium glutamate, 38 mM potassium aspartate, 2.5 mM MgCl₂, 0.5 mM CaCl₂, 1 mM DTT, and protease inhibitors and complemented with ATP regenerating system, 0.1 mM GTP, 1 mg/ml rat liver cytosol. After incubation for 45 min at 37°C, cells were pelleted after a resuspension at 6,000 g for 5 min and then analyzed directly by Western blotting or fluorography, or the sample was adjusted to 30% Optiprep (Nycomed Pharma, Oslo, Norway), overlaid with 25% Optiprep, 5% Optiprep, all in 10 mM Heps (pH 7.4), 140 mM KCl, 2 mM EGTA, 1 mM DTT. The samples were centrifuged for 3 h at 55,000 rpm in a TLS55 rotor (Beckman, Munich, Germany) and then the vesicular fraction recovered from the 25%-50% interphase was analyzed by Western blotting, fluorography, or electron microscopy.

To determine release of the trans-Golgi marker sialyltransferase from cells, a cell line expressing VSV-G-tagged human sialyltransferase was established and protein was detected by blotting with the monoclonal antibody 5D4 (Kreis, 1986) or immunoprecipitation.

Immunosolization of Apical and Basolateral Vesicles

Immunosolization of apical and basolateral vesicles from perforated MDCK cells was carried out as detailed by Wandelinger-Ness et al. (1990). Briefly, filter-grown MDCK cells were labeled overnight with [35S]methionine, infected with either influenza virus or VSV, and viral proteins were accumulated in the TGN by a 20°C chase for 30 min, and CHAPS-insoluble material was pelleted by centrifugation in a TLA45 rotor (Beckman) for 30 min at 45,000 rpm and 4°C. The apical or basolateral cell surface was then permeabilized with SLO, the endogenous cytosol was depleted, and transport to the intact surface was reconstituted. The amount of viral proteins reaching the surface was measured by trypsinization (HA) or surface immunoprecipitation (VSV-G).

To measure transport from the ER, the 20°C-chase was omitted and SLO permeabilization was carried out immediately after pulse labeling. The amount of viral proteins reaching the Golgi complex was determined by endoglycosidase H digestion. The anti–caveolin-1 amino-terminal antibody N-20 was used at a final concentration of 10 µg/ml. Before raising the temperature to 37°C to initiate transport, the cells were incubated for 30 min at 4°C with cytosol ± antibody and an ATP regenerating system. Quantitation of viral polypeptides resolved by SDS-PAGE was done with a Phosphor Imager (Molecular Dynamics, Sunnyvale, CA). Transport with added cytosol was defined as 100%, being 2.5–4.5-fold higher than without cytosol. The values obtained in the presence of antibodies were expressed as a percentage of those given without antibody addition. All transport assays were performed in triplicate and each condition was tested at least twice.

Immunofluorescence Microscopy

Filter-grown, confluent MDCK cells were rinsed once in PBS and then fixed for 10 min in 4% paraformaldehyde/PBS. The filters were rinsed briefly in PBS, cut from the holder, and then incubated for 30 min in 200 mM glycine/PBS. They were then incubated for 10 min in 0.1% Triton X-100/PBS and nonspecific antibody binding was blocked by incubating for 10 min in PBS containing 0.2% cold-water fish skin gelatin (PBSG). The filters were incubated for 2 h with either affinity-purified rabbit anti–caveolin-1 antibodies (N20) diluted 1:25 or rabbit anti–caveolin-2 antibodies diluted 1:10 in PBS-G. After extensive rinsing (more than six times at 5-min each), the filters were incubated for 1 h at 37°C with fluorescein isothiocyanate coupled anti-rabbit antibodies in PBS G. After extensive rinsing in PBS, the filter pieces were incubated in 50% glyceral, 100 µg/ml 1,4-diazobicyclo(2,2,2)-octane in PBS, and then examined under a confocal scanning light microscope (LSM410; Carl Zeiss, Inc., Thornwood, NY).

Immunoelectron Microscopy

Filter-grown confluent MDCK cells were rinsed once in PBS and fixed for 10 min in 4% paraformaldehyde/PBS. Triangular filter pieces in PBS were progressively infiltrated with gelatin at 37°C to a final concentration of 10%. They were put on ice and infiltrated with 2.1 M sucrose. The filter pieces were put on top of a cryosectioning stub and frozen in liquid nitrogen. Ultrathin cryosections were collected on grids and incubated face-down in PBS for 30 min at 37°C to dissolve the gelatin. The grids were transferred for 30 min to 10% FCS in PBS and then incubated for 1 h with affinity-purified rabbit anti–caveolin-1 antibodies (N20) diluted 1:20 or rabbit anti–caveolin-2 antibodies diluted 1:10 in PBS-G. After extensive rinsing (more than six times at 5-min each) the filterpieces were incubated for 1 h at 37°C with fluorescein isothiocyanate coupled anti-rabbit antibodies in PBS G. After extensive rinsing in PBS, the filter pieces were embedded in 50% glyceral, 100 µg/ml 1,4-diazobicyclo(2,2,2)-octane in PBS, and then examined under a confocal scanning light microscope (LSM410; Carl Zeiss, Inc., Thornwood, NY).

Sucrose Gradient Centrifugation

Cell lysates or immunosololated vesicles (300 µl) in 0.4% SDS, 0.2% Triton X-100, and protease inhibitors (see above) were loaded onto a linear 5–30% sucrose gradient in 20 mM Tris-HCl, pH 7.4, 100 mM NaCl, 0.4% SDS, 0.2% Triton X-100. After centrifugation in a SW60 rotor (Beckman) for 1 h at 45,000 rpm, 500 µl fractions were collected from the top. For Western blot analysis, proteins were TCA precipitated or immunoprecipitated with anti–caveolin-1 antibodies in the case of labeled samples. Proteins were resolved on gradient polyacrylamide gels.

CHAPS Extraction

After pulse-chase labeling, the cells were scraped on ice in 500 µl of 20 mM CHAPS in 50 mM Tris-HCl, pH 7.0, 100 mM NaCl, 1 mM EDTA (TNE) in the presence of protease and phosphatase inhibitors (see above). After resuspension 10 times with a 1-ml pipet tip, they were incubated on ice for 30 min, and CHAPS-insoluble material was pelleted by centrifugation in a TLA45 rotor (Beckman) for 30 min at 45,000 rpm and 4°C. The supernatant was collected and the pellet resuspended in CHAPS extraction buffer. 4 vol of 20 mM Tris-HCl, pH 7.4, 100 mM NaCl, 0.5% SDS, and protease and phosphatase inhibitors (see above) were added and caveolin complexes immunoprecipitated.

In Vitro Transport Assays

The transport of VSV-G and HA in streptolysin O (SLO)-permeabilized MDCK cells was carried out exactly as described previously (Pimplikar et al., 1994; Ikonen et al., 1995; for review see Lafont et al., 1995). Briefly, to measure TGN-to-surface transport, filter-grown MDCK cells were infected with VSV or influenza virus, viral proteins were pulse labeled with [35S]methionine, and then chased to the TGN using a 20°C incubation. The apical or basolateral cell surface was then permeabilized with SLO, the endogenous cytosol was depleted, and transport to the intact surface was reconstituted. The amount of viral proteins reaching the surface was measured by trypsinization (HA) or surface immunoprecipitation (VSV-G).

To measure transport from the ER, the 20°C-chase was omitted and SLO permeabilization was carried out immediately after pulse labeling. The amount of viral proteins reaching the Golgi complex was determined by endoglycosidase H digestion. The anti–caveolin-1 amino-terminal antibody N-20 was used at a final concentration of 10 µg/ml. Before raising the temperature to 37°C to initiate transport, the cells were incubated for 30 min at 4°C with cytosol ± antibody and an ATP regenerating system. Quantitation of viral polypeptides resolved by SDS-PAGE was done with a Phosphor Imager (Molecular Dynamics, Sunnyvale, CA). Transport with added cytosol was defined as 100%, being 2.5–4.5-fold higher than without cytosol. The values obtained in the presence of antibodies were expressed as percentage of those given without antibody addition. All transport assays were performed in triplicate and each condition was tested at least twice.

For immunolabeling isolated vesicles, small droplets (1–2 µl) of the vesicle preparation were put on top of formvar and carbon-coated copper grids for 5 min. They were then fixed on top of a drop of 4% paraformaldehyde (PFA) in PBS for 5 min. After blocking with 200 µl glycine in PBS for 10 min and a 5-min wash in PBS containing 0.5% BSA and 0.2% cold-water fish skin gelatin (PBG), the grids were incubated for 15 h with the first primary antibody in PBG at 4°C. After a 3–5-min rinse in PBG, the grids were incubated with protein A-coupled 15-nm gold particles in PBG for 1 h and then rinsed in PBG. In a second (5 h at room tempera-
tured and third (15 h at 4°C) round of immunoincubation, the sequence of labeling was repeated from the fixation to the washing in PBS. In the second and third round, protein A coupled to 10- and 5-nm gold particles were used, respectively. After the third round of labeling, the grids were rinsed in tridistilled water and then incubated for 5 min on ice in 0.3% uranyl acetate, 1.8% methylcellulose in tridistilled water. The excess fluid was removed and the grids were air-dried. Controls included the omission (of combinations) of primary antibodies after which the accompanying gold particles were not detected. For quantitations, routinely gold particles on 40 individual vesicles were counted. Vesicles were considered positive for the viral marker proteins when they contained at least two particles. In individual experiments similar results were obtained independently, in which the order of the antigens was labeled with the antibodies.

Results

A New Protein Coprecipitates with Caveolin-1 Antibodies

Antibodies raised against the first 20 amino-terminal amino acids of caveolin-1 recognize a single band of ~24-kD by Western blotting, corresponding to the α isoform of caveolin-1 (Dupree et al., 1993). However, these antibodies immunoprecipitate proteins that are resolved as two bands of ~21- and 24-kD, respectively, in SDS-PAGE (Dupree et al., 1993, Fig. 1 A). So far, these bands have been thought to represent α- and β-caveolin-1 (Monier et al., 1995). Surprisingly, when the immunoprecipitates were analyzed on a 2D gel, other spots in addition to the ones known to correspond to α- and β-caveolin-1 were revealed. By far, most abundant was a cluster of spots that corresponded in size to β-caveolin-1 but had a more acidic isoelectric point (Fig. 1 B). When comparing this pattern to the 2D gel maps of immunosolated apical and basolateral transport vesicles, we found that this elongated pattern corresponded exactly to a protein heavily enriched in basolateral transport vesicles, we found that this elongated pattern corresponded to a protein heavily enriched in basolateral transport vesicles, we found that this elongated pattern corresponded to a protein heavily enriched in basolateral transport vesicles, we found that this elongated pattern corresponded to a protein heavily enriched in basolateral transport vesicles, we found that this elongated pattern corresponded to a protein heavily enriched in basolateral transport vesicles, we found that this elongated pattern corresponded to a protein heavily enriched in basolateral transport vesicles. Fig. 3 A) round of immunoincubation, the sequence of sequences flanking this ATG conformed better to the Kozak consensus than those around the downstream ATG, suggesting that it may be used as the preferential initiator site. Moreover, database searches revealed a human expressed sequence tag (THC180343; GenBank/EMBL/DDBJ accession number AA367026) that also carries the upstream start codon. As the amino-terminal part was most divergent between caveolin-1 and -2, we used a peptide containing amino acids 26–40 as an immunogen to raise canine caveolin-2-specific antibodies (Fig. 2 D). In Western blotting this antibody was found to recognize two proteins of 19- and 21-kD, both distinct from α-caveolin-1 (Fig. 2, A and B). On 2D gels, the elongated group of spots representing B12 was detected (Fig. 2 C). In addition, the caveolin-2 antibodies cross-reacted with a cluster of spots of similar shape but of smaller size (~19 kD, Fig. 2 C). These were found to correspond to minor spots beneath the original band cut for microsequencing (refer to Fig. 1 B). Based on the sequence information, it is likely to represent the product of a transcript using methionine 14 as an alternative, less favored site for initiation. Clearly, the anti–caveolin-2 antibodies were specific for these two rows of spots and did not cross-react with α- and β-caveolin-1.

Caveolin-2 Is Present in a Large Oligomeric Complex

Based on their sequence similarity, caveolin-2, like caveolin-1, would be predicted to form high molecular weight complexes. Unexpectedly, the epitope-tagged overexpressed human caveolin-2 was found to exist preferentially as a monomer (Scherer et al., 1996). To find out if endogenous canine caveolin-2 is present in stable oligomers, we applied the same technique as used previously for human caveolin-1 (Monier et al., 1995), taking advantage of the stability of the oligomers in SDS and 2-mercaptoethanol. The high molecular weight proteins in the MDCK cell lysate were separated by SDS-PAGE and then analyzed by Western blotting using caveolin-1 and -2-specific antibodies. Fig. 3 A) shows that both caveolins resolved into 200-, 400-, and 600-kD complexes. To further characterize the oligomers, we developed a fractionation technique that
separated the complexes into several size classes. This was based on sedimentation velocity centrifugation in a sucrose gradient in the presence of SDS and Triton X-100 (refer to Materials and Methods). When a MDCK cell lysate was analyzed by this method and the caveolins visualized by immunoblotting with both anti–caveolin-1 and -2, we found that some of the oligomers migrated to the middle of the gradient (corresponding to ~200–400 kD), whereas others sedimented as very large complexes (>600 kD) (Fig. 3 B). All complexes contained both caveolin-1 and -2. However, the large complexes contained significantly less caveolin-2 than the 200–400-kD oligomers. The vast majority of the other cellular proteins were detected in the first four fractions of the gradient (data not shown).

To estimate the stoichiometry of caveolin-1 and -2 in the caveolin complexes, the immunoprecipitations were performed after an overnight metabolic labeling (Fig. 4). The precipitated proteins were analyzed by 2D gelelectrophoresis and then the intensities of the 2D gel spots were quantitated by Phosphor Image analysis (as caveolins-1 and -2 contain five or six methionine residues, respectively, incorporated radioactivity should represent approximately equivalent amounts of each protein). In the case of anti–caveolin-1 antibodies, the ratio was about 2:2:1 for α–caveolin-1/β–caveolin-1/caveolin-2 (summing the major 21- and minor 19-kD spots of caveolin-2) (Fig. 4, top). On the other hand, anti–caveolin-2 antibodies precipitated the proteins in the ratios of about 1:1:1 (one molecule of α– and one of β–caveolin for every caveolin-2 molecule) (Fig. 4, bottom). These findings demonstrate that there is about two times more caveolin-1 than -2 in the complexes at steady state. This could result from two different complexes, one type containing only caveolin-1 and another containing both caveolins.

**Modification of the Caveolin Complexes during Biosynthetic Transport**

Our data suggest that caveolin-1 and -2 form oligomeric complexes in MDCK cells. To follow the formation and intracellular transport of these complexes, we decided to analyze the properties of the newly synthesized caveolins in a pulse-chase protocol using anti–caveolin-1 antibodies. As shown above, this protocol also brings down caveolin-2. The proteins were labeled with a 7-min pulse of [35S]methionine and (a) directly cooled on ice and processed for analysis, (b) chased for 1 or 2 h at 20°C, or (c) chased for 1 or 2 h at 37°C before analysis. The 20°C samples represent the complexes present in the Golgi complex after the 20°C chase. The bulk of the pulse-labeled caveolins were localized in a density gradient fraction containing the trans-Golgi marker sialyltransferase (data not shown).

**Figure 3.** Caveolin-2 forms high molecular weight complexes. Western blotting using anti–caveolin-1 antibody N-20 or anti–caveolin-2 antibodies was performed after separating MDCK cell lysate on SDS-PAGE without boiling (A). Alternatively, the cell lysate was first fractionated by sedimentation velocity in a 5–30% sucrose gradient, and TCA proteins precipitated from the fractions were separated by 13% SDS-PAGE after boiling in alkaline sample buffer (B). The migration of molecular mass standards in the gradient is indicated.

**Figure 4.** Composition of the caveolin oligomeric complex at steady state. MDCK cells were labeled overnight with [35S]methionine, proteins were immunoprecipitated with caveolin-1 (top) or -2 antibodies (bottom), and then analyzed by 1D (left) or 2D gel electrophoresis (right) after boiling in alkaline sample buffer. The anode is to the right. For quantification of the relative amounts of caveolin-1 and -2, gels were scanned by a Phosphor Imager.
Figure 5. Analysis of the caveolin complex by 2D gel electrophoresis during biosynthetic transport. MDCK cells were pulse labeled with 

$[^{35}S]$methionine for 7 min and either lysed immediately (A), chased for 1 h at 20°C (B) or chased for 1 h at 37°C (C) before lysis. Alternatively, cells were labeled for 3 h with $[^{32}P]$ATP (D). The caveolin complex was immunoprecipitated by anti-caveolin-1 antibodies and then analyzed on 2D gels.

Protein Composition of the Complexes. The results shown in Fig. 5 suggest that a heterooligomeric complex is formed already in the ER, as both anti-caveolin-1 (Fig. 5, A–C) and -2 antibodies (data not shown) brought down both of the proteins in all conditions tested. The relative proportions of the two proteins did not seem to change significantly during biosynthetic transport, except for slightly more caveolin-2 being precipitated without chase (in the ER) than in the other conditions (compare Fig. 5 A to B and C).

This data also demonstrates that the efficiency of immunoprecipitation is low in the ER (~10%, assuming that a constant amount of protein is present throughout the pulse-chase protocol), whereas the antibody brings down the proteins quantitatively at steady state. This was also evident in a previous report (Monier et al., 1995), and could be because of conformational changes in the epitope or more efficient precipitation of higher oligomers versus monomers or dimers (see below).

Size of the Complexes. To monitor changes of the complex sizes, pulse-labeled proteins were subjected to fractionation in the SDS–Triton X-100 sucrose gradient followed by immunoprecipitation with anti-caveolin-1 antibodies. The newly synthesized caveolins were monomeric (Fig. 6 A, fractions 2 and 3), but a sizeable fraction had already oligomerized forming a SDS–Triton X-100 stable complex of ~200 kD (Fig. 6 A, fractions 5 and 6). Taking into account the inefficient precipitation of the monomers in the ER, a cautious quantitative assessment would be that in the ER at least ten times more of the protein exists as a monomer than as oligomer. Nevertheless, oligomerization obviously starts early in the biosynthetic route. When the proteins were chased for 1 h at 20°C, i.e., when most of the newly synthesized proteins resided in the Golgi apparatus, the caveolin complex increased in size, peaking at ~400-kD with some already extending to the 600-kD range (Fig. 6 B). When performed at 37°C, most of the protein was chased to the cell surface; the caveolin oligomers matured further to higher molecular weight complexes, reaching the size range of the very large structures detected at steady state (Fig. 6 C). It is noteworthy that the choice of detergent in the gradient is critical to observe the size changes. N-octyl-3-$

\mbox{D}-glucopyranoside (octylglucoside) has previously been used in comparable size fractionation schemes and reported to detect oligomers between 200–400-kD, irrespective of the conditions (Li et al., 1996b; Monier et al., 1996). In our experience, when SDS and Triton X-100 were replaced with octylglucoside, a similarly sized 200–400-kD oligomer was detected either in the ER or on the plasma membrane (data not shown). Therefore, the observed complex sizes do not necessarily reflect the molecular weight of the oligomers in vivo; however, they clearly demonstrate changes in size and nature of the complexes during biosynthetic transport. The maturation is likely to be achieved not only by protein–protein interactions, but might also be influenced by the lipid environment. This could explain why the choice of detergent and its capability to dissociate or preserve such protein–lipid complexes is critically influencing the resolved complex sizes.

Cholesterol has been demonstrated to promote homooligomerization in micromes (Monier et al., 1996) and could regulate the oligomer size in vivo. The membrane cholesterol level is low in the ER and increases along the exocytic route towards the plasma membrane. We chased the pulse-labeled proteins to the cell surface (1 h at 37°C) and then incubated the cells with methyl-$\beta$-cyclodextrin to extract plasma membrane cholesterol. Indeed, this treatment (causing the removal of $\geq$50% of cellular cholesterol; Rietveld, A.G., and K. Simons, personal communication) resulted in a decrease in size of the caveolin oligomers from the one typical for the plasma membrane to a size corresponding to the Golgi form (Fig. 6 D). It is important to note that under these conditions, the intracellular localization of caveolin-1 is not significantly changed as judged by immunofluorescence analysis (data not shown).

Phosphorylation of the Complexes. One striking observation from the 2D gel analysis of the caveolin complexes is that the number of spots corresponding to caveolin-2 in-
creases during transport to the cell surface, with one extra, more acidic spot in the Golgi complex (Fig. 5 B) and two after 1 h at 37°C (delivery to the cell surface) (Fig. 5 C). Furthermore, this modification is extensive at steady state, producing up to four adjacent spots of the longer and three of the shorter form of caveolin-2 (Fig. 4). We assumed that these modifications causing an increase in negative charge without changing the apparent molecular weight could be phosphorylations, and therefore, we performed metabolic labelings with [32P]ATP in parallel with [35S]methionine. After [32P] and immunoprecipitation with anti–caveolin-1, antibodies samples were separated on 2D gels. This analysis showed that in steady-state conditions, caveolin-2 is indeed heavily phosphorylated, whereas caveolin-1 is not (Fig. 5 D).

**Detergent Solubility.** Caveolin-1 is known to be insoluble in the detergents Triton X-100 and CHAPS at 4°C, both in TGN-derived transport vesicles and in plasma membrane caveolae, whereas it is soluble when inserted into microsomes in vitro (Kurzchalia et al., 1992; Melkonian et al., 1995; Monier et al., 1995). To find out when this change occurs during biosynthetic transport, we analyzed the solubility of the caveolin complexes during a pulse-chase experiment. The oligomer was found to be entirely soluble immediately after the pulse labeling, and two-thirds of it still remained soluble after a 1-h chase at 20°C (Fig. 7). When the chase at 20°C was extended to 2 h, three-fourths of the complex had become CHAPS insoluble. If the protein was allowed to proceed along the biosynthetic route at 37°C, a 1-h chase was sufficient to convert it to a form resistant to detergent CHAPS solubilization as efficiently as the protein did at steady state (≃90% being CHAPS insoluble). Similar changes in the detergent solubility of the caveolin complex were observed when Triton X-100 was used at 4°C instead of CHAPS (data not shown). These data suggest that only after reaching the Golgi complex do caveolins become increasingly resistant to detergent extraction.

**Different Caveolin Complexes Exist in Apical and Basolateral Vesicles**

The selective recovery of caveolin-2 in immunosolated basolateral carriers (Fig. 1) suggests that the caveolin complexes exiting the TGN along the apical and the basolateral routes may have different compositions. We fractionated metabolically labeled apical and basolateral vesicles after immunosolation by SDS–Triton X-100 sucrose gradient centrifugation and determined the anti–caveolin-1 precipitable radioactive in each fraction. Most of the caveolin oligomers in the basolateral vesicles were found in the middle fractions, reflecting the size range seen after the 20°C block whereas the apical vesicles, which are enriched in caveolin-1 (Fig. 1), had a significant proportion of oligomers migrating to the bottom of the gradient (Fig. 8). Thus, the caveolin complexes incorporated into the apical carriers are larger than those in the basolateral ones, as judged by sedimentation velocity centrifugation.

The evidence for a differential distribution of caveolin-1 and -2 in apical and basolateral TGN–derived vesicles so far is based on the immunosolation of vesicles and subsequent mapping of the protein components by 2D electrophoresis. To obtain larger amounts of vesicles that would allow a more quantitative analysis by Western blotting and immunoelectron microscopy, we modified the procedure previously established by Wanding-Ness et al., 1990. TGN–derived vesicles were released from semintact cells after disruption of the plasma membrane by hypotonic swelling as previously described by Xu and Shields (1993). The release of the viral glycoproteins from MDCK cells under these conditions was reported to be regulated in the same manner as surface transport in SLO-permeabilized cells (Pimplikar and Simons, 1993; Misch et al., 1996). 15–25% of the viral marker proteins were released depending on cytosol and the addition of energy (2.5–5.5-fold stimulation, data not shown). The glycoproteins acquired terminal oligomeric modifications and release of proteins was not due to fragmentation of the Golgi complex, since the trans-Golgi marker sialyltransferase was efficiently retained in the cells (data not shown). Released vesicles were separated from the cytosol and heavy membrane fragments by flotation through an Optiprep step gradient. The obtained fraction predominantly contained vesicles of 80–100 nm in diameter as analyzed by negative staining and ≃50% of all vesicles could be labeled with the antibodies against the cytoplasmic tail of the viral proteins. We next analyzed the distribution of the caveolins in the vesicle population by triple labeling with antibodies against caveolin-1, -2, and antibodies against the viral glycoproteins (Fig. 9). VSV-G– and HA-containing vesicles were both labeled with caveolin-1 antibodies, whereas significant caveolin-2 labeling was restricted to basolateral (VSV-G–containing) vesicles. Apical vesicles showed significantly higher labeling for caveolin-1 than the basolateral ones (Table I). Similar results were obtained indepen-

![Figure 7](https://example.com/figure7.jpg)

**Figure 7.** Detergent solubility of the caveolin complexes during biosynthetic transport. MDCK cells were pulsed for 7 min with [35S]methionine and then chased. Cells were lysed in 20 mM CHAPS in TNE and the caveolin complex was immunoprecipitated from the supernatant (S) and the pellet (P) and then analyzed by 3–17% SDS-PAGE after boiling. Note that the complex is precipitated increasingly efficiently during the chase. The autoradiograph had to be exposed longer to detect pulse-labeled caveolin in the ER (no chase, data not shown).

![Figure 8](https://example.com/figure8.jpg)

**Figure 8.** Size of the caveolin complexes in immunosolated apical and basolateral transport vesicles. Apical and basolateral vesicles were isolated from virally infected MDCK cells as described in Materials and Methods. The immunosolated vesicle preparations were fractionated by sedimentation velocity centrifugation and the caveolin complex in each fraction immunoprecipitated using anti-caveolin-1 antibodies. The radioactivity recovered from each fraction is indicated.
The differential localization of caveolin-1 and -2 in polarized MDCK cells was investigated. We found caveolin-2 to be strongly enriched in basolateral vesicles, whereas basolateral vesicles were similarly tested in the transport assays but were not reproducibly inhibited by adding specific antibodies to the permeabilized cells. These transport steps can be selectively inhibited by adding specific antibodies to the permeabilized cells (for review see Lafont et al., 1995).

**Table 1. Quantitative Analysis of Caveolin Distribution in Exocytic Vesicles from MDCK Cells**

<table>
<thead>
<tr>
<th></th>
<th>cav-1</th>
<th>cav-2</th>
<th>cav-1/cav-2</th>
</tr>
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<tbody>
<tr>
<td>HA</td>
<td>10.0 ± 0.9</td>
<td>11.0 ± 0.8</td>
<td>0.9 ± 0.5</td>
</tr>
<tr>
<td>VSV-G</td>
<td>3.8 ± 0.4</td>
<td>6.9 ± 0.7</td>
<td>1.6 ± 0.2</td>
</tr>
</tbody>
</table>

The table shows one representative experiment. Routinely, gold label on 40 HA and 40 VSV-G-positive vesicles was quantitated. The number of gold particles counted per vesicle is shown. The ratio of label for caveolin-1 and -2 was calculated for each individual vesicle and is listed under cav-1/cav-2. Standard errors are given.

Anti–caveolin-1 Antibodies Inhibit Apical Transport of HA

To test whether the caveolins are functionally involved in exocytic transport from the TGN to the basolateral membrane, we used anti-caveolin-1 and -2 antibodies in the transport assays. The anti–caveolin-1 amino-terminal antibody had no effect on ER-to-Golgi transport of HA, nor on the basolateral transport of VSV-G. However, apical transport of HA was reproducibly inhibited by ~50% (Fig. 12). This finding suggests that antibody binding to the amino-terminal part of caveolin-1 specifically interferes with the function of the caveolin complex in the apical pathway. However, binding to caveolin-1 present in basolateral vesicles is either not possible or does not interfere with transport. Anti–caveolin-2 antibodies were similarly tested in the transport assays but were found to be without effects (data not shown). These data do not prove that caveolin-1 has an essential function in apical transport, but they clearly demonstrate that caveolin-1 is in a different state in the apical vesicles as compared to the basolateral vesicles.

**Discussion**

Our analysis of the anti–caveolin-1 immunoprecipitate by 2D gel electrophoresis revealed, for the first time, that the caveolin complex is actually a heterooligomer of two caveolins, -1 and -2, with similar molecular weights but differing isoelectric points. This contradicts the previous result by Scherer and co-workers (1996) who failed to detect ca-
Caveolin-2 in a high molecular weight complex. The fact that they analyzed only the shorter form of the overexpressed epitope-tagged caveolin-2 may account for the differences to our results. It is possible that the use of detergent in immunoprecipitation may lead to artificial association of proteins after cell lysis and coperipitation of complexes formed in vitro. However, we demonstrated that caveolin-1 and -2 reside in the same basolateral vesicles (Fig. 9, B and C) and caveolin-2 was strongly enriched also in caveolar immunopurifications performed with anti-caveolin-1 antibodies in the absence of detergents (Fra, A.M., and K. Simons, unpublished data), corroborating our immunolocalization data that demonstrated both caveolin-1 and -2 were present in caveolae on the basolateral membrane (Fig. 11).

Another fact that has previously escaped attention is that caveolin-2 exists in two forms. Like caveolin-1, caveolin-2 seems to use alternative translation initiation sites to produce α and β isoforms. In caveolin-2, the longer alpha form is clearly more abundant, whereas the α and β isoforms of caveolin-1 are equally prevalent.

**Biosynthetic Maturation of the Caveolin Complex**

One of the aims of this work was to analyze the changes taking place in the physical state of the caveolins during their passage from the ER to the cell surface. Earlier work has led to the following findings: a high molecular weight oligomer forms already in the ER and an oligomeric structure is maintained when the protein complex is transported along the biosynthetic route. Caveolin isolated from tissues, which represents the steady-state distribution of the protein in the TGN and caveolae, also exists as a large complex. This oligomer is detergent-insoluble and binds cholesterol (Monier et al., 1995; Murata et al., 1995). The caveolin-cholesterol complex is a key structural element in the formation of caveolae and is also potentially important in the organization of glycosphingolipid-cholesterol microdomains (rafts) involved in protein, and lipid sorting (Fra et al., 1995; Parton and Simons, 1995; Ikonen and Simons, 1997). Because neither caveolae nor rafts exist in the ER, the caveolin complex should change its behavior during intracellular transport. We now have systematically analyzed the biochemical characteristics of the caveolin oligomers in pulse-chase experiments and found the following changes.

First, although caveolin oligomers are partially formed early in the biosynthetic route, they increase in size on their way to the cell surface. Monomeric forms can also be detected in the ER (although accurate quantitation of the ratios between monomer and oligomer is not possible because the monomer is immunoprecipitated less efficiently). We used velocity sedimentation in SDS–Triton X-100 sucrose gradients to follow the changes during maturation of the caveolin complex. We found that the newly synthesized oligomer migrates as a 200-kD complex under these conditions. After 1 h at 20°C, the structures are between 200–400-kD and after 1 h at 37°C their size increases even further. Second, the complex becomes gradually detergent-insoluble from a completely soluble ER form to an insoluble form after transport to the Golgi complex. Third, caveolin-2 was found to become modified by the addition of increasing numbers of phosphate groups, the most extensive phosphorylation being found at steady state and therefore likely to be related to the trafficking of the complex between the TGN and the cell surface. Caveolin-1, on the other hand, was not phosphorylated under these conditions, although the protein can function as a substrate for kinases (Glenney and Soppet, 1992; Mastick et al., 1995; Li et al., 1996a). Fourth, the antibody reactivity of the complex changes during the chase period. There is not only a difference in the immunoprecipitation efficiencies between the monomeric and oligomeric forms, but also between different sizes of oligomers; the bigger the oligomer the more easily it is brought down by the anti–caveolin-1 amino-terminal antibody (Fig. 5). The same is true for the anti–caveolin-2 amino-terminal antibody (data not shown). Interestingly, we found that an anti–caveolin-1 carboxy-terminal antibody failed to immunoprecipitate the mature complex. However, this antibody recognized the ER form of the oligomer equally effectively as the amino-terminal antibody (data not shown). Similarly, the myc epitope that we introduced in the carboxy terminus of caveolin-2 is also less accessible at the plasma membrane (data not shown), and antibodies against the carboxy terminus of caveolin-1 fail to detect the protein on the cell surface (Dupree et al., 1993). This suggests that a carboxy-terminal epitope available for antibody recognition in the ER becomes hidden during maturation of the complex. This is in agreement with the report that the carboxy terminus of caveolin is involved in oligomerization (Song et
al., 1997). Also, palmitoylation of cysteine residues in the carboxy-terminal domain might interfere with antibody recognition (Dietzen et al., 1995).

Specialized Complexes of Caveolins Exist in the Apical and Basolateral Traffic Routes

These data demonstrate that the newly synthesized caveolins undergo dramatic changes during their transport to the cell surface. However, the most startling finding was the preferential routing of caveolin-2 to the basolateral cell surface as judged by its enrichment in basolateral carriers and its localization on the basolateral plasma membrane domain. Thus, the distributions of caveolin-1 and -2 are different over the polarized cell surfaces, caveolin-1 being present on both the apical and the basolateral sides. Our biochemical data and the electron microscopy analysis of apical and basolateral vesicles suggests that heterooligomers of caveolin-1 and -2 are incorporated into the basolateral transport vesicles in the TGN, whereas caveolin-1 homooligomers are included into the apical vesicles and transported to the apical plasma membrane domain. This notion is in accordance with our quantitation of the stoichiometry of the two proteins. Caveolin-1 was more abundant than caveolin-2 and caveolin-2 antibodies failed to coprecipitate caveolin-1 quantitatively. Moreover, we provided biochemical evidence for two differentially behaving caveolin oligomers. Size fractionation of the apical and basolateral caveolin complexes from immunoisolated vesicles revealed that the apical complexes formed larger oligomers than the basolateral ones. This, together with the fact that the >600-kD complexes contained less caveolin-2 (Fig. 3), further supports the presence of caveolin-1 oligomers in the apical vesicles. Finally, we found that apical protein transport can be disturbed by the addition of caveolin-1 antibodies, as reflected by reduced apical delivery of HA, whereas the transport of VSV-G protein recruited to basolateral vesicles occurred normally. Transport of HA was, however, not inhibited by the antibody earlier in the biosynthetic route, as ER-to-Golgi transport was not affected.

Working Model for the Differential Roles of Caveolin-1 and -2 in MDCK Cells

Based on these results we envisage caveolins-1 and -2 to participate in the two post-Golgi membrane traffic routes in MDCK cells as follows. The apical vesicles and the apical plasma membrane domain contain mostly caveolin-1 in a homooligomeric complex. The basolateral vesicles and the basolateral plasma membrane domain, on the other hand, contain heterooligomers of caveolin-1 and -2. The putative homooligomers of caveolin-1, formed in the ER, polymerize to larger structures by interactions with sphingolipid–cholesterol raft components during the formation of apical transport vesicles. How the heterooligomers of caveolin-1 and -2 become excluded from the apical vesicles and included in the basolateral carriers is an open question. They could become bound to another protein with basolateral sorting determinants, with binding possibly regulated by phosphorylation of caveolin-2.

One problem arising from the postulated differential sorting of caveolin-1 and -2 complexes concerns the biogenesis of caveolae. Caveolae are present in many cell types but in MDCK epithelial cells they are observed only on the basolateral but not on the apical cell surface (Fig.
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References