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Conformational Defects Slow Golgi Exit, Block Oligomerization, and Reduce Raft Affinity of Caveolin-1 Mutant Proteins

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Caveolin-1, a structural protein of caveolae, is cleared unusually slowly from the Golgi apparatus during biosynthetic transport. Furthermore, several caveolin-1 mutant proteins accumulate in the Golgi apparatus. We examined this behavior further in this mutant study. Golgi accumulation probably resulted from loss of Golgi exit information, not exposure of cryptic retention signals, because several deletion mutants accumulated in the Golgi apparatus. Alterations throughout the protein caused Golgi accumulation. Thus, most probably acted indirectly, by affecting overall conformation, rather than by disrupting specific Golgi exit motifs. Consistent with this idea, almost all the Golgi-localized mutant proteins failed to oligomerize normally (even with an intact oligomerization domain), and they showed reduced raft affinity in an in vitro detergent-insolubility assay. A few mutant proteins formed unstable oligomers that migrated unusually slowly on blue native gels. Only one mutant protein, which lacked the first half of the N-terminal hydrophilic domain, accumulated in the Golgi apparatus despite normal oligomerization and raft association. These results suggested that transport of caveolin-1 through the Golgi apparatus is unusually difficult. The conformation of caveolin-1 may be optimized to overcome this difficulty, but remain very sensitive to mutation. Disrupting conformation can coordinately affect oligomerization, raft affinity, and Golgi exit of caveolin-1.

INTRODUCTION

Caveolin-1 is an important component of the membrane-embedded coat that surrounds caveolae (Rothberg et al., 1992; Fra et al., 1994; Smart et al., 1999; Fernandez et al., 2002). Although it is an integral membrane protein, caveolin-1 lacks conventional transmembrane domains. Instead, N- and C-terminal hydrophilic domains flank a central 33-residue hydrophobic domain (Smart et al., 1999). Both hydrophilic domains are located on the cytoplasmic side of the plasma membrane, and the hydrophobic domain has been suggested to form a helical hairpin embedded in the bilayer. A peptide corresponding to the last 20 residues of the N-terminal hydrophobic domain, a region named the caveolin scaffolding domain (CSD), can bind to membranes independently (Schlegel et al., 1999), at least in part because of the high number of aromatic residues in this domain (Arbuzova et al., 2000). The C-terminal domain also can associate with membranes independently, at least in part via three palmitate chains (Luetterforst et al., 1999).

Caveolin-1, alone or with the related protein caveolin-2, forms large (>400 kDa), SDS-stable oligomers (Monier et al., 1995; Sargiacomo et al., 1995; Scheiffele et al., 1998). These oligomers are thought to associate with each other to form the filamentous structure of the caveolar coat (Fernandez et al., 2002). Oligomerization occurs soon after synthesis (Scheiffele et al., 1998). It is not known where along the secretory pathway caveolin oligomers assemble into caveolae, although pits with the distinctive morphology of caveolae have only been reported at the plasma membrane (Rothberg et al., 1992; Denker et al., 1996). Residues 60–100 of caveolin-1 contain the oligomerization domain (Sargiacomo et al., 1995; Schlegel and Lisanti, 2000). However, changes outside this domain can block oligomerization (Machleidt et al., 2000; Lee et al., 2002), presumably through indirect effects.

Caveolin-1 has a high affinity for lipid rafts, membrane microdomains that have a high degree of lipid order and are concentrated in caveolae (Smart et al., 1999). Raft lipids and proteins are resistant to solubilization by nonionic detergents such as Triton X-100 (TX100) and 3-(3-cholamidopropyl)dimethylammonio)propanesulfonate, and can be isolated from cell lysates as detergent-resistant membranes (DRMs; London and Brown, 2000; Schuck et al., 2003; Shogomori and Brown, 2003). DRM association provides a useful tool for identifying proteins that have a high affinity for rafts (London and Brown, 2000; Shogomori and Brown, 2003). Acylation of raft proteins often aids in raft targeting. Caveo-
Caveolin-1 Mutant Proteins

lin-1 is unusual in that although it is triply acylated, prevention of acylation by mutation does not affect raft affinity (Dietzen et al., 1995). It is not known how caveolin-1 is targeted to rafts, although binding of the protein to cholesterol may play a role (Murata et al., 1995; Thiele et al., 2000).

Unlike most plasma membrane proteins, a significant amount of caveolin-1, estimated at ~15%, is resolved by electron microscopy (Denker et al., 1996) is present in the Golgi apparatus at steady state (Rothberg et al., 1992; Dupree et al., 1993; Luetterforst et al., 1999). Although early studies suggested that caveolin-1 cycled constitutively between caveolae and the Golgi apparatus (Conrad et al., 1995), this was later shown not to occur (Nichols, 2002; Thomsen et al., 2002). Instead, as caveolin-1 is selectively cleared from the Golgi apparatus when protein synthesis is inhibited with cycloheximide (Nichols, 2002), the Golgi pool may be a biosynthetic intermediate in transit to the plasma membrane. If so, caveolin-1 exits the Golgi more slowly than most other cargo proteins, which are not detected in the Golgi apparatus at steady state.

Several caveolin-1 mutant proteins accumulate in the Golgi apparatus (Luetterforst et al., 1999; Machleidt et al., 2000; Lee et al., 2002), as does caveolin-2 when expressed without caveolin-1 (Mora et al., 1999; Parolini et al., 1999). Coexpression with caveolin-1 rescues cell-surface expression of caveolin-2. Golgi accumulation of mutant proteins is unusual, because most proteins accumulate in the endoplasmic reticulum (ER) through interaction with chaperone proteins if mutation blocks proper folding. It is not known whether mutation exposes cryptic Golgi retention signals in caveolin-1 (Luetterforst et al., 1999) or whether it destroys information required for efficient Golgi exit (Machleidt et al., 2000). We examined a panel of caveolin-1 mutant proteins to gain more insight into this unusual localization.

MATERIALS AND METHODS

Cells and Antibodies

Culture of COS-1, Fischer rat thyroid (FRT), and mouse melanoma ME4 cells has been described previously (Ostermeyer et al., 1999, 2001). The primary antibodies rabbit anti-caveolin-1 and mouse anti-GM130 antibodies were from BD Transduction Laboratories (San Diego, CA); rabbit anti-placental alkaline phosphatase (PLAP) antibodies were from DakoCytomation (Carpinteria, CA); and rabbit and mouse (12CA5) anti-hemaggulatinin (HA) epitope antibodies were from Santa Cruz Biotechnology (Santa Cruz, CA). The secondary antibodies fluorescein isothiocyanate- and Texas Red conjugated goat anti-rabbit IgG, Texas Red-conjugated goat anti-mouse IgG(+M), and horseradish peroxidase (HRP)-conjugated donkey anti-rabbit IgG were from Jackson ImmunoResearch Laboratories (West Grove, PA).

Plasmids

Plasmids encoding HA- and myc-tagged canine caveolin-1 (Dietzen et al., 1995), canine caveolin-1 with a C-terminal myc tag followed by the ER retrieval motif KSSL (Ostermeyer et al., 2001), human PLAP, and PLAP-HA, containing the extracellular domain of PLAP fused to the transmembrane and cytoplasmic domains of influenza hemagglutinin (Arreaza and Brown, 1995), have been described. pSAY2M2, encoding canine caveolin-1 with a C-terminal myc tag followed by green fluorescent protein (GFP), was the gift of S. Munro (Medical Research Council, Cambridge, United Kingdom). Plasmids encoding the caveolin-1 mutants 71A57, 76A80, 81A85, 86A90, 91A95, and 96A100 (Machleidt et al., 2000) were gifts of P. Liu and R.G. Anderson (University of Texas Southwestern, Dallas, TX). All other caveolin-1 mutants were made by mutagenesis of HA- and myc-tagged canine caveolin-1 (Dietzen et al., 1995), pSAY2M2, or using the QuickChange site-directed mutagenesis kit (Stratagene, La Jolla, CA). Amino acids altered in these mutants are referred to based on their position in wild-type canine caveolin-1a (Kurzchalia et al., 1992). Each of these mutants contains an N-terminal HA tag. Each contained a C-terminal myc tag, except for the following: Cav-NTD (ending with R101); Y148A5 (Ala substitutions for 13 residues of the residues deleted from the C terminus); and AC (lacking the entire C-terminal domain and ending with K135). Cav-ΔN1 lacked residues 3–48, Cav-ΔCSD lacked residues 81–100, and Cav-ΔN1/ΔC lacked residues 3–48 and 136–178. Cav-ΔN2/ΔC lacked residues 46–100 and 136–178. Hydrophobic domain Ala substitution mutants have been described previously (Ostermeyer et al., 2004). Other mutants are self-explanatory or are described in the figure legends. All mutations were verified by sequencing, and all mutant proteins migrated as expected on SDS-PAGE.

Other Reagents

Dipalmitoyl phosphatidylcholine (DPPC), diethanol phosphatidylcholine (DOPC), and cholesterol were from Avanti Polar Lipids (Alabaster, AL). N-Octyl-β-D-glucopyranoside (OG) was from Anatrace (Maumee, OH). Polyvinylidene difluoride (PVDF) membrane was from Millipore (Bedford, MA). X-ray film was from Eastman Kodak (Rochester, NY), and Western Lightning chemiluminescence reagent from PerkinElmer Life and Analytical Sciences (Boston, MA). Serva Blue G was from Serva (Heidelberg, Germany). Other reagents were from Sigma-Aldrich (St. Louis, MO).

Transfection and Immunofluorescence

Cells were transfected using Lipofectamine 2000 from Invitrogen (Carlsbad, CA), as described previously (Ostermeyer et al., 2004). All data were from transiently transfected cells, harvested 2 d after transfection, except immunofluorescence (IF) images of Ala-scan mutants in and near the CSD (71A57 and 76A80 shown in Figure 7B), where mixed populations of stably transfected FRT cells expressing each mutant protein were examined.

Myprotein was fractionated by paraformaldehyde-fixed, TX100-permeabilized, cover-slip-grown cells was as described previously (Ostermeyer et al., 2001). Where indicated, cells were incubated with 50 μg/ml cycloheximide for the indicated times and red images (every objective (numerical aperture 1.3), were captured separately into Adobe Photoshop from a SPOT cooled charge-coupled device 24 bit color digital camera (Diagnostic Instruments, Sterling Heights, MI) mounted on an Axioskop 2 microscope (Carl Zeiss, Oberkothen, Germany).

Reconstitution, Detergent Extraction, and DRM Analysis

For reconstitution of proteins and lipids from cell lysates into mixed liposomes, COS-1 or transfected MEB-4 cells in one 60-mm dish (expected to contain 400 nmol of membrane lipid; Brown and Rose, 1992) were harvested with 0.7 ml of TNE (25 mM Tris-HCl, 150 mM NaCl, and 5 mM EDTA, pH 7.4) containing 120 mM MgO and protease inhibitors (0.5 μg/ml leupeptin, 0.7 μg/ml pepstatin, and 0.2 mM phenylmethylsulfonyl fluoride). Liposomes were clarified by ultracentrifugation at 100,000 × g for 2 h in a Ti-100 table-top ultracentrifuge (Beckman Coulter, Fullerton, CA) for 10 min. Supernatant (200 μl) was mixed with 0.5 ml of lipids (4 mM, DPPC:Cholesterol 2:1 mol/mol or DOPC, as indicated) in TNE containing 180 mM GO. Mixtures were subjected to dialysis overnight against 5 l phosphate-buffered saline (PBS: 150 mM NaCl and 20 mM phosphate buffer, pH 7.4) to form mixed proteoliposomes containing cellular proteins and lipids and artificial lipids. Where indicated, the association of caveolin-1 with these liposomes was analyzed by flotation in sucrose gradients. To do this, an aliquot of liposomes was brought to 0.5 ml with PBS, mixed with 1.5 ml of TNE containing 53% sucrose, placed in a 12-ml ultracentrifuge tube, overlaid with 8 ml of TNE/38% sucrose and then 1 ml of TNE/50% sucrose, and subjected to ultracentrifugation at 100,000×g at 4°C for 18 h. Three fractions (3.6 ml each) were harvested from the bottom, and any pelleted material was collected and resuspended in 3.6 ml of TNE/1% TX100. Proteins in 0.5-ml aliquots of each fraction were collected by precipitation in trichloroacetic acid in the presence of 10 μg of bovine serum albumin as carrier. Protein pellets were washed once with acetone and once with diethyl ether, dissolved in gel loading buffer, and analyzed by SDS-PAGE and Western blotting.

To analyze DRM association by using sucrose gradients, an aliquot of liposome suspension was adjusted to 1% TX100 in 0.5 ml by using PBS and a 10% TX100 stock and incubated on ice for 30 min. Sucrose step gradients were then formed as described above for detergent-free samples and subjected to sucrose gradient centrifugation, fractionation, SDS-PAGE, and Western blotting as described above. To analyze DRM association without sucrose gradients, after dialysis 0.2 ml of liposome suspension was mixed with 0.9 ml of TNE and collected by centrifugation at top speed in a Microfuge for 4 min. Supernatants were discarded and pellets resuspended in 0.5 ml of ice-cold PBS, adjusted to 1% TX100 with a 10% stock, incubated on ice for 30 min, and then subjected to ultracentrifugation in standard Microfuge tubes for 1 h at 4°C for 30 min in a TL-A rotor in a Ti-100 table-top ultracentrifuge. Supernatants were collected, and pellets resuspended in 0.5 ml of TNE/1% TX100. Equal volumes of supernatant and pellet fractions were subjected to SDS-PAGE and Western blotting to visualize the indicated proteins.

Gels and Blots

Blue native PAGE was performed as described previously (Schagger et al., 1991), with changes as noted next. Cells were lysed in lysis buffer (500 mM 6-aminocaproic acid, 2 mM EDTA, and 25 mM Bistris, pH 7.0) containing 120 mM MgO for 30 min at 4°C. Lysates were clarified by centrifugation for 10 min

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consisted of dispersed elements (Figure 1, G–L), as seen previously (Luetterforst et al., 1999). None of the mutant caveolin-1 proteins examined here had any obvious effect on Golgi morphology. Golgi staining of caveolin-1, relative to caveolar staining, gradually declined over a few hours of cycloheximide treatment (Figure 1, A–L), as reported previously (Nichols, 2002). By contrast, another exogenously expressed plasma membrane protein, PLAP-HA (Arreaza and Brown, 1995), was usually (≥90% of transfected cells) undetectable in the Golgi apparatus at steady state (Figure 1M; GM130 localization in the same cell shown in Figure 1N) and was never detected there after 1-h incubation with cycloheximide. Thus, caveolin-1 was cleared unusually slowly from the Golgi apparatus.

**Golgi Accumulation of Caveolin-1 Deletion Mutants**

Both the C-terminal domain of caveolin-1, expressed without the rest of the protein, and caveolin-1 lacking the C-terminal domain, accumulate in the Golgi apparatus (Luetterforst et al., 1999; Machleidt et al., 2000). These findings suggested that caveolin-1 might contain two Golgi-targeting signals; one in the C-terminal domain and the second in either the N-terminal or hydrophobic domain.

To localize the latter signal more precisely, we expressed the N-terminal domain of caveolin-1 alone (Cav-NTD). This protein was largely cytosolic, but it was concentrated in the Golgi apparatus when membrane association was seen (Figure 2A). To localize the putative signal further, we attempted to express constructs encoding smaller fragments of the N-terminal domain, but we could not detect the proteins. As an alternate approach, we made two new constructs, Cav-ΔN1/ΔC and Cav-ΔN2/ΔC. Each encoded half of the N-terminal domain, linked to the hydrophobic domain (Figure 2). Surprisingly, both proteins accumulated in the Golgi apparatus (Figure 2, D and G). In addition to the Golgi apparatus, Cav-ΔN2/ΔC also accumulated in the ER (Figure 2G), and sometimes in lipid droplets (Figure 2G, arrows), because it lacked a sequence needed for efficient ER exit (Machleidt et al., 2000). Caveolin-1 is partially redirected to lipid droplets when it overaccumulates in the ER (Ostermeyer et al., 2001, 2004). The pool of Cav-ΔN2/ΔC in the Golgi apparatus probably came from molecules that escaped lipid droplet targeting, and slowly left the ER via the secretory pathway. Despite the Golgi accumulation of ΔN1/ΔC, neither it nor any of the caveolin-1 mutant proteins examined here affected transport of a coexpressed plasma membrane protein, PLAP, to the cell surface (Figure 2, J–O).

It is difficult to explain these and earlier findings (Luetterforst et al., 1999; Machleidt et al., 2000) by invoking specific Golgi retention signals in caveolin-1. Such a model would require that caveolin-1 contained three separate retention signals: one in the C-terminal domain, one in the N-terminal domain, and the third either in the N-terminal domain or the hydrophobic domain. All three signals would be cryptic in the intact protein, but they could be exposed by mutation.

An alternate model is that caveolin-1 must achieve the correct conformation for efficient Golgi exit. To determine whether Golgi accumulation might result from conformational defects, we examined a panel of caveolin-1 mutant proteins. In addition to localization, we examined two properties of caveolin-1 that might be sensitive to conformational changes: oligomerization and lipid raft targeting (Luetterforst et al., 1999; Machleidt et al., 2000; Lee et al., 2002).
A Reconstituted System for Assaying DRM Association of Caveolin-1 Mutant Proteins

Raf affinity of membrane proteins is usually examined by determining their association with DRMs after detergent extraction of cells. However, as a protein’s lipid environment at the time of extraction affects DRM association (Brown and Rose, 1992; Schroeder et al., 1998), we could not use this method to compare the raft affinity of proteins in different organelles. Instead, we examined DRM association in vitro, by using a liposome reconstitution assay. We mixed lysates of cells expressing the mutant proteins with excess raft-forming lipids, formed liposomes by dialysis, and determined whether the mutant proteins associated with the DRMs.

To test this system, we first showed that wild-type caveolin-1 derived from both COS and transfected MEB-4 cells associated efficiently with liposomes, as it floated with them in sucrose gradients (Figure 3A, top). MEB-4 cells were chosen because they produce very little endogenous caveolin-1 (Ostermeyer et al., 1999), but they express transfected constructs at high efficiency. About half the wild-type caveolin-1 associated with floating DRMs after Triton X-100 extraction (Figure 3A, middle). Very little caveolin-1 aggregated and pelleted in the gradients. To show that DRM association depended on liposome lipids, and not cellular lipids that remained associated with caveolin-1, we repeated the assay, mixing cell lysates with DOPC, a nonraft forming lipid. Caveolin-1 associated well with these liposomes (our unpublished data), but was efficiently solubilized when these liposomes were extracted with TX100 (Figure 3A, bottom).

For convenience, in later experiments, DRMs were collected as pellets rather than by flotation in sucrose gradients. We detected ~70% of both endogenous COS cell caveolin-1 and wild-type caveolin-1 expressed in MEB4 cells in DRMs by using this method (Figure 3B). We do not know why
from previous sucrose velocity gradient analysis (Monier et al., 1995) and was enriched in the Golgi apparatus than the wild-type protein (Figure 4).

We examined three additional hydrophobic domain mutant proteins. We made the first protein (G108A112L), after noticing that if the first half of the hydrophobic domain formed an α helix, then the helix face containing G108 and A112 would contain only small residues (Ostermeyer et al., 2002), suggesting that it might pack closely against another helix (Eilers et al., 2002). We changed these residues to Leu to determine whether their small size might be important in structure. (G108A112L) accumulated in the Golgi apparatus (Figure 5A, i), did not oligomerize properly (Figure 5B, lane 1), and associated poorly with DRMs in the reconstitution assay (Figure 5C), supporting the idea that these residues were structurally important.

P132L, which was detected in 16% of human breast tumors (Hayashi et al., 2001), colocalized with GM130 in the Golgi apparatus in FRT cells (our unpublished data), as reported previously (Lee et al., 2002). Those authors reported that coexpression with P132L caused retention of wild-type caveolin-1 in a perinuclear compartment in COS-7 and human mammary epithelial cells (Lee et al., 2002). By contrast, we saw no effect of coexpression on the localization of caveolin-1-GFP or P132L in FRT cells. Except in very highly

Correlation between Localization, Oligomerization, and DRM Association of Hydrophobic Domain Mutant Proteins

We next examined the behavior of a panel of caveolin-1 mutant proteins in the three assays. Results, shown in Figures 4–7 and summarized in Figure 8, are grouped according to the mutated protein domain. Almost all the mutant proteins showed a striking correlation between behavior in the three assays.

We first examined six mutant proteins in which sequential groups of five or six residues through the hydrophobic domain were changed to Ala (Figure 4A). Three of these proteins (102A5, 113A5, and 130A5) were similar to wild-type caveolin-1 in all three assays. First, they had similar localization patterns, although they were somewhat more enriched in the Golgi apparatus than the wild-type protein (Figure 4B). We examined oligomerization by using blue native gel electrophoresis. In this method, after gentle cell lysis with OG, detergent is replaced by Coomassie Blue dye, which supplies charge to allow migration in native acrylamide gels, but does not denature proteins and can preserve protein complexes (Schagger et al., 1994). Wild-type caveolin-1 expressed in MEB-4 cells migrated as a high-molecular-weight oligomer on blue native gels (Figure 4C), as expected from previous sucrose velocity gradient analysis (Monier et al., 1995; Sargiacomo et al., 1995; Machleidt et al., 2000). 102A5, 113A5, and 130A5 were largely oligomeric and comigrated with the wild-type proteins, although we also saw a fainter, faster-migrating form. This was probably a monomer, because it comigrated with mutant proteins lacking the oligomerization domain that migrate as monomers on velocity gradients (Sargiacomo et al., 1995). The same three proteins also associated well with DRMs isolated from mixed proteoliposomes (Figure 4D).

By contrast, the other three hydrophobic domain Ala-scan mutant proteins, 107A5, 118A5, and 123A6, were defective in all three assays: they were highly concentrated in the Golgi apparatus, migrated as monomers on blue native gels, and associated poorly with DRMs (Figure 4).
expressing cells, wild-type caveolin-1-GFP was present largely in caveolae (Figure 5A, iii), whereas P132L had a Golgi-like distribution (Figure 5A, vi).

P132L migrates as a monomer on sucrose velocity gradients (Lee et al., 2002). We saw two forms of P132L on blue native gels; oligomers that migrated more slowly than wild-type caveolin-1, and monomers (Figure 5B, lane 2). The relative amounts of the two forms varied between experiments. These results suggested that the slowly migrating form of P132L was an abnormal oligomer that dissociated more easily than wild-type caveolin-1. We could not detect P132L in reconstituted liposomes. Changing the second conserved Pro residue in the hydrophobic domain to Leu (P110L) did not affect cellular localization, oligomerization, or detergent insolubility in the reconstitution assay (Figure 5).

Correlation between Localization, Oligomerization, and DRM Association of C-Terminal Domain Mutants

A series of C-terminal truncation mutant proteins (Figure 6A, arrows indicate the last residue in each protein) showed good correlation between behavior in the three assays. Δ13, containing the longest C-terminal domain, had a wild-type distribution in cells (Figure 6B) and oligomerized and associated with DRMs normally (Figure 6, C and D). Δ21 was somewhat more concentrated in the Golgi apparatus than wild-type caveolin-1, although it was easily detectable in caveolae as well (Figure 6B). This protein was mostly oligomeric and associated well with DRMs (Figure 6, C and D). Δ31 was substantially more concentrated in the Golgi apparatus (Figure 6B), although not as completely as some other mutants and did not oligomerize or associate well with DRMs (Figure 6, C and D). Finally, Δ39 and ΔC were concentrated in the Golgi apparatus (ΔC, Figure 6B; Δ39, our unpublished data), as reported previously for a C-terminal deletion mutant in Chinese hamster ovary cells (Machleidt et al., 2000), failed to oligomerize and associated poorly with DRMs (Figure 6, C and D). We sometimes saw a second form of Δ39 and ΔC that migrated slightly more slowly than the monomer, as seen for ΔC in Figure 6C.

Because Δ21 was more severely affected than Δ31 in all three assays, we changed either the first or second five residues in the 10-residue sequence that distinguished these proteins to Ala, in the context of the full-length protein. Both of these mutant proteins (Y148A5 and H153A5, Figure 6A) had a wild-type localization in cells (our unpublished data), oligomerized efficiently (Figure 6C), and associated well with DRMs (Figure 6D). For unknown reasons, oligomers of Y148A5 and H153A5 migrated slightly faster than those of the wild-type protein, an effect that did not depend on their lack of a myc tag (Figure 6C). We conclude that residues 148–157 did not contain a specific motif that directly affected localization, oligomerization, or raft affinity. Finally, Cys-, a nonpalmitoylated caveolin-1 mutant, was indistinguishable from wild-type caveolin-1 in all three assays (localization not shown; oligomerization and detergent-insolubility in Figure 6, C and D), in agreement with previous reports (Dietzen et al., 1995; Monier et al., 1996).

Localization, Oligomerization, and DRM Association of N-Terminal Domain Mutants

The first three N-terminal domain mutants that we examined were defective in all three assays. ΔCSD, which lacked F81-Y100 (Figure 7A, left), accumulated in the Golgi apparatus (Figure 7B), failed to oligomerize, (Figure 7C, i), and did not associate well with DRMs (Figure 7D, left). Aromatic residues, which are abundant in the CSD, were previously shown to aid in membrane association of a CSD peptide (Arbuzova et al., 2000). We made two mutant proteins, WFF/AAA and 97/SASA (Figure 7A, right), to examine the importance of these residues. Both proteins showed the same behavior: they accumulated in the Golgi apparatus (97/SASA, Figure 7B; WFF/AAA, our unpublished data), failed to oligomerize (Figure 7C, i), and associated poorly with DRMs in the reconstitution assay (Figure 7D, left). Thus, the CSD, and especially aromatic residues in that domain, were important in caveolin-1 structure.

We next examined six mutant proteins in which sequential groups of five residues from the CSD and the region just upstream of it were changed to Ala (71A75–86A100: Figure 7A, right). Our results were largely similar to those of Machleidt et al. (2000), who made these constructs. All the proteins were concentrated in the Golgi apparatus, but they...
were also detectable in punctate structures. 71A75 and 76A80 illustrate the range of localization patterns (Figure 7B). We saw both monomeric and oligomeric forms of all the proteins except 96A100, which was entirely monomeric (Figure 7C, ii). 76A80 and 86A90 were largely oligomeric, whereas a higher fraction of 71A75, 81A85, and 91A95 was monomeric.

Oligomers of these proteins migrated more slowly than wild-type caveolin-1 (Figure 7C, ii). To determine whether this resulted from the respective epitope tags, we introduced the changes present in either 71A75 or 76A80 into our caveolin-1 construct. The new mutants 71* and 76* had the same oligomer:monomer ratio as 71A75 and 76A80, respectively (our unpublished data). Furthermore, they co-migrated with the original mutant proteins and migrated more slowly than wild-type caveolin-1, although they migrated faster than P132L (Figure 7C, iii and iv, only oligomer bands shown). 71A75 and 76A80 associated well with DRMs (Figure 7D).

81A85 and 86A90 showed intermediate and variable behavior, and 91A95 and 96A100 were mostly TX100 soluble. By contrast, Machleidt et al. (2000) found that all these mutants were detergent soluble when transfected cells were extracted with Triton X-100, probably because the mutants accumulated in raft-poor Golgi membranes.

Although 91A95 and 96A100 were defective in all three assays, behavior of the other four proteins was less consistent. We do not know the significance of the slow migration of their oligomers. These proteins may have been borderline cases, with slightly altered conformations that affected their behavior in unpredictable ways.

By contrast, ΔN1, which lacked the first half of the N-terminal domain (G3-E48), was the only mutant protein that showed clearly discordant behavior in the three assays. ΔN1 accumulated in the Golgi apparatus (Figure 7B), consistent with previous reports of similar mutant proteins (Lüttetforst et al., 1999; Machleidt et al., 2000). Nevertheless,
ΔN1 oligomerized and associated with DRMs as well as wild-type caveolin-1 (Figure 7C, v and D). Thus, oligomerization and DRM association were not sufficient for efficient Golgi exit. Results of the behavior of the mutant proteins in the three assays are summarized in Figure 8.

Persistence of Caveolin-1 Mutants in the Golgi Apparatus

We next determined the localization of selected mutant proteins in cycloheximide-treated cells. 97/SASA and 118A5 remained highly concentrated in the Golgi apparatus after prolonged cycloheximide treatment (Figure 9, A–F). Golgi localization of 102A5 and ΔZ1, which were not as highly concentrated in the Golgi apparatus at steady state, was reduced but still detectable after 3-h cycloheximide treatment (Figure 9, G–I). By contrast, Golgi staining of Δ31 (the C-terminal truncation mutation that was more Golgi-enriched than ΔZ1 at steady state) was still pronounced in the Golgi after 3-h cycloheximide treatment (Figure 9, M–O). Thus, the concentration of caveolin-1 mutants in the Golgi apparatus at steady state was at least partially explained by their reduced rate of Golgi exit.

Transport through the Secretory Pathway Is Not Required for Oligomerization or Acquisition of Raft Affinity

Transport to the late Golgi apparatus or plasma membrane might be necessary for acquisition of raft affinity, for instance, through a posttranslational modification or conformational change that occurred only in a late compartment. To test this possibility, we examined Cav-KKSL, wild-type caveolin-1 with an appended ER retrieval signal (Ostermeyer et al., 2001). As reported previously (Ostermeyer et al., 2001), Cav-KKSL was only detectable in the ER and in ER-derived lipid droplets (Figure 10, top), presumably through efficient retrieval to the ER in COPI-coated vesicles. Cav-KKSL oligomerized and associated with DRMs as well as wild-type caveolin-1 (Figure 10, middle and bottom). Thus, transport to late compartments of the secretory pathway was not required for oligomerization or acquisition of raft affinity.

DISCUSSION

Golgi exit, oligomerization, and raft affinity correlated strikingly among a broad spectrum of caveolin-1 mutant pro-
Of 26 mutant proteins examined, nine were close to normal in all three assays, and 14 were defective in all three. Only two mutant proteins (71A75 and 76A80) showed abnormal oligomerization and Golgi exit, but they retained raft affinity. H9004 also convincingly broke the correlation: it oligomerized normally and associated with DRMs, but it was defective for Golgi exit. Based on these data, normal oligomerization and raft affinity might be required for Golgi exit, although H9004 showed that neither is sufficient. Alternatively, conformational defects might affect the three properties independently.

The clearest conclusion of this work is that the conformation of caveolin-1 is highly optimized, and extremely sensitive to small changes. Even caveolin-2, which accumulates in the Golgi apparatus and does not oligomerize normally without caveolin-1, cannot achieve this optimized conformation on its own (Mora et al., 1999; Parolini et al., 1999). Sequences in caveolin-1 that were sensitive to mutation were spread among all three domains of the protein. Thus, most of the changes probably acted indirectly, by affecting overall conformation, and not by disrupting specific motifs.

We can speculate on how changes in a few of the mutant proteins might have affected conformation. (G108/H11001 A112)L

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![Figure 8](image-url)  
**Figure 8.** Summary of localization, oligomerization, and TX100-insolubility results. The caveolin-1 mutant proteins are diagrammed schematically as in Figure 2A. Mutants are grouped according to the domain altered. Mult., deletions in two domains. HydD, CTD, and NTD, changes in the hydrophobic, C-terminal, or N-terminal domains, respectively. Asterisks indicate substitutions. Four or more substituents are indicated by a single larger asterisk. Column 3 (PM): efficiency of Golgi-to-plasma membrane transport. +++, wild-type localization; ++, slightly enhanced Golgi localization; +, Golgi accumulation. None of these proteins except Cav-ΔN2/ΔC accumulated predominantly in the ER, except in highly expressing cells. Column 4 (Olig): oligomerization on blue native gels. +++, wild-type oligomerization, with very little monomer; ++, predominantly oligomeric; +, some oligomer; +/−, substantial oligomer and monomer; −/+, mostly monomer; −, no oligomer detected; *, oligomer migrated more slowly than wild-type caveolin-1. Column 5 (DRM): association with TX100-resistant membranes after extraction of raft-containing mixed proteoliposomes. +, similar to wild-type caveolin-1 (~70% insoluble); +/−, about equal amounts soluble and insoluble; −, poor DRM association (~30% insoluble). ND, not determined.

![Figure 9](image-url)  
**Figure 9.** Persistence of caveolin-1 mutant proteins in the Golgi apparatus after cycloheximide treatment. The localization of the indicated proteins (A, D, G, J, and M) and GM130 (B, E, H, J, and N) in transfected FRT cells after cycloheximide treatment for 3 h (except cells expressing 118A5, treated for 5 h) is shown. C, F, I, L, and O; merged anti-caveolin-1 (green) and anti-GM130 (red) images.
Caveolin-1 Mutant Proteins

Caveolin-1 mutant proteins that lacked residues 66–70 accumulated in the ER, supporting a previously established role for that domain in ER exit (Machleidt et al., 2000). By contrast, a surprising number of caveolin-1 mutant proteins accumulated in the Golgi apparatus. Previous workers have used a mutagenesis approach in an attempt to identify specific Golgi targeting signals in caveolin-1 (Luetterforst et al., 1999), caveolin-2 (Brezza et al., 2002), or Golgi exit signals in caveolin-1 (Machleidt et al., 2000). Our data suggest that most of these alterations caused Golgi accumulation indirectly, by affecting protein conformation. Golgi accumulation of these proteins probably resulted from inhibition of Golgi exit, rather than exposure of retention signals, as we showed that signal-mediated Golgi retention would require at least three separate signals.

However, ΔN1 accumulated in the Golgi apparatus, even though its oligomerization and raft affinity suggested that its conformation might not be grossly disturbed. Thus, the first half of the N-terminal domain may be especially important in Golgi exit. Consistent with this possibility, caveolin-1 and caveolin-2 (which accumulates in the Golgi apparatus in the absence of caveolin-1) show little sequence similarity in this region.

Golgi Transport of Caveolin-1 Is Unusually Difficult
Cargo proteins are generally transported rapidly and synchronously through the Golgi apparatus. By contrast, Golgi passage of caveolin-1 seems to be unusually difficult. One function of the optimized conformation of caveolin-1 may be to partially overcome this difficulty. Disruption of this optimized conformation by mutation would then lead to Golgi accumulation. One model explaining how this might occur is described next.

Munro and Bretscher proposed a model to explain how newly synthesized plasma membrane proteins in transit through the Golgi apparatus are sorted from Golgi resident proteins (Bretscher and Munro, 1993; Munro, 1995). Transmembrane spans of plasma membrane proteins are longer than those of Golgi proteins. Munro and Bretscher proposed that microdomains of different thickness coexist in the Golgi apparatus. Cholesterol increases bilayer thickness, and they proposed that thick domains contain more cholesterol than thin domains. They also proposed that Golgi enzymes and cargo proteins destined for the plasma membrane partition into the appropriate domains according to transmembrane domain length.

According to the cisternal maturation model of Golgi dynamics, cargo proteins move through the Golgi apparatus passively, as individual cisternae progress through the stack (Pelham and Rothman, 2000; Martínez-Menárguez et al., 2001; Mironov et al., 2001). This model predicts that Golgi resident proteins, as well as any cargo proteins—like caveolin-1—that move through the Golgi more slowly than the rate of cisternal flow, must transiently leave the cisternae, probably in retrograde-directed recycling vesicles. To accommodate these ideas, the model of Munro and Bretscher might predict that recycling vesicles are thinner than bulk cisternae. Thus, long transmembrane spans of plasma membrane proteins would keep these proteins in forward-directed cisternae and prevent incorporation into retrograde-directed carriers.

Caveolin-1 lacks defined transmembrane spans. It may not be able to sense the thickness of cholesterol-rich domains and may not partition as strongly into these domains as other proteins. Instead, at each sorting step, some caveolin-1 may be incorporated into retrograde-directed vesicles. The resulting cycle would slow the net rate of forward transport. However, caveolin-1 is transported through the Golgi apparatus faster than most of the mutants we examined and thus may partition better into thick domains than they do. Caveolin-1 may recognize some other property of these domains, rather than their thickness. This property may be shared with membrane rafts. This property might be the cholesterol-induced increase in acyl-chain packing found in rafts (Xu and London, 2000), or some other feature of the higher cholesterol concentration. In any case, the same structural features that target caveolin-1 to rafts also may increase its affinity for cholesterol-rich Golgi domains. Mutants with reduced raft affinity may have lower affinity for these domains, enhancing their incorporation into retrograde vesicles, and thus their Golgi accumulation.
This model also could explain slow Golgi transport of some other proteins. Like caveolins, peripheral membrane proteins that are transported to the plasma membrane via the secretory pathway cannot be sorted in the Golgi based on transmembrane span length. Palmitoylation of several of these proteins is required for efficient Golgi exit (Choy et al., 1999; Kanaani et al., 2002; Navarro-Lérida et al., 2002; Taki-moto et al., 2002; Takida and Wedegaertner, 2003). Palmi-toylation can target proteins to rafts (Brown and London, 2000) and thus also might sort these proteins into cholester-ol-rich Golgi domains in the Golgi. Palmitoylation also en-hances Golgi transport of some transmembrane proteins (van’t Hof and Crystal, 2002; Kalinina and Fricker, 2003), possibly through the same mechanism.

Three caveolin-1 mutants, 71A75, 76A80, and ΔN1, did not fit this model. They had apparently normal raft affinity but accumulated in the Golgi apparatus. It is possible that the sensitivity of our DRM assay is not high enough to detect small changes in raft affinity that are important in vivo.

It is also possible that Golgi accumulation of caveolin-1 mutants is related to quality control, a process of recognition and degradation of misfolded proteins. Many of the mutant proteins that we examined were degraded rapidly, as re-portedly previously (Machleidt et al., 2000). Golgi accumula-tion of misfolded caveolin-1 mutant proteins might occur as part of a post-ER quality control system.

Caveolin-1 Oligomerization

Blue native gel analysis gave some new insights into caveo-lin-1 oligomerization. We saw remarkably few intermediate sized oligomers. We occasionally saw a band that migrated slightly more slowly than the monomer, when examining mutants that were otherwise entirely monomeric. Some mu-tants were present in both monomeric and oligomeric forms, suggesting that oligomerization was inefficient or oligomers unstable. However, we never saw any other intermediate-sized forms of these proteins. This suggested that oligomer-ization of caveolin-1 is highly cooperative.

A few mutant proteins oligomerized, but did not quite comigrate with wild-type caveolin-1 on blue native gels. These complexes may contain different numbers of mono-mers, or may have a different shape or ability to bind dye. These mutants also may bind differentially to other proteins, although caveolin-1 oligomers are thought to contain no additional proteins except caveolin-2 (Monier et al., 1995; Scheiffele et al., 1998). Oligomers of Y148A5 and H135A5 migrated slightly faster than wild-type caveolin-1. These mutant proteins oligomerized as efficiently as wild-type caveolin-1 and showed normal cellular localization and DRM association. This suggested that their abnormal migra-tion on blue native gels reflected only minor conformational changes.

Two classes of mutant proteins formed oligomers that migrated unusually slowly. P132L was the only member of the first class. The other class included six Ala-scan mutants in and near the CSD (Figure 7), and also a W85A point mutant (our unpublished data). As all these oligomers were similarly affected, changes throughout this region seemed to cause the same structural alteration. P132L oligomers mi-grated more slowly than those of the CSD mutants. Oli-gomers of both classes migrated as distinct bands, suggest-ing that they did not aggregate nonspecifically. We saw sig-nificant amounts of monomeric forms of all these mutant proteins, suggesting that oligomerization was inefficient or unstable. All these mutant proteins accumulated in the Golgi apparatus. Thus, structural changes that reduced oligomer mobility seemed to affect caveolin-1 conformation more se-verely than those that increased migration.

The correlation between in vitro DRM association and oligomerization suggested that wild-type caveolin-1 and oli-gomerization-competent mutant proteins were probably in-corporated into liposomes as oligomers. Blue native gel analy-sis showed that the wild-type protein remained oligomeric after reconstitution (our unpublished data), and we expect that this was true of the mutant proteins as well.

Further work will clearly be required to fully understand how so many different mutations can alter the conformation of caveolin-1 and how these changes can have such pro-found effects on Golgi exit, oligomerization, and raft affinity of the protein.

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