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Phenotypic Behavior of Caveolin-3 Mutations That Cause Autosomal Dominant Limb Girdle Muscular Dystrophy (LGMD-1C)

RETENTION OF LGMD-1C CAVEOLIN-3 MUTANTS WITHIN THE GOLGI COMPLEX*

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Caveolin-3, a muscle-specific caveolin-related protein, is the principal structural protein of caveolae membrane domains in striated muscle cell types (cardiac and skeletal). Autosomal dominant limb girdle muscular dystrophy (LGMD-1C) in humans is due to mutations within the caveolin-3 gene: (i) a 9-base pair microdeletion that removes three amino acids within the caveolin scaffolding domain (ΔTFT) or (ii) a missense mutation within the membrane spanning domain (P → L). The molecular mechanisms by which these two mutations cause muscular dystrophy remain unknown.

Here, we investigate the phenotypic behavior of these caveolin-3 mutations using heterologous expression. Wild type caveolin-3 or caveolin-3 mutants were transiently expressed in NIH 3T3 cells. LGMD-1C mutants of caveolin-3 (ΔTFT or P → L) were primarily retained at the level of a perinuclear compartment that we identified as the Golgi complex in double-labeling experiments, while wild type caveolin-3 was efficiently targeted to the plasma membrane. In accordance with these observations, caveolin-3 mutants formed oligomers of a much larger size than wild type caveolin-3 and were excluded from caveolae-enriched membrane fractions as seen by sucrose density gradient centrifugation. In addition, these caveolin-3 mutants were expressed at significantly lower levels and had a dramatically shortened half-life of ~45–60 min. However, caveolin-3 mutants were palmitoylated to the same extent as wild type caveolin-3, indicating that targeting to the plasma membrane is not required for palmitoylation of caveolin-3. In conclusion, we show that LGMD-1C mutations lead to formation of unstable high molecular mass aggregates of caveolin-3 that are retained within the Golgi complex and are not targeted to the plasma membrane. Consistent with its autosomal dominant form of genetic transmission, we demonstrate that LGMD-1C mutants of caveolin-3 behave in a dominant-negative fashion, causing the retention of wild type caveolin-3 at the level of the Golgi. These data provide a molecular explanation for why caveolin-3 levels are down-regulated in patients with this form of limb girdle muscular dystrophy (LGMD-1C).

Caveolae are 50–100-nm vesicular invaginations of the plasma membrane (1). It has been proposed that caveolae participate in vesicular trafficking events and signal transduction processes (2–4). Caveolin, a 21–24-kDa integral membrane protein, is a principal component of caveolae membranes *in vivo* (5–9). Caveolin is only the first member of a new gene family; as a consequence, caveolin has been retermed caveolin-1 (10).

The mammalian caveolin gene family consists of caveolins 1, 2, and 3 (4, 10–12). Caveolins 1 and 2 are co-expressed and form a hetero-oligomeric complex (13) in many cell types, with particularly high levels in adipocytes, whereas expression of caveolin-3 is muscle-specific and found in both cardiac and skeletal muscle, as well as smooth muscle cells (14).

It has been proposed that caveolin family members function as scaffolding proteins (15) to organize and concentrate specific lipids (cholesterol and glycosphingolipids; Refs. 16–18) and lipid-modified signaling molecules (Src-like kinases, H-Ras, eNOS, and G-proteins; Refs. 16 and 19–23) within caveolae membranes. These caveolin family members all share three characteristic properties: (i) detergent insolubility at low temperatures; (ii) self-oligomerization; and (iii) incorporation into low density Triton-insoluble membrane fractions that are enriched in caveolae membranes (24).

The specialized lipid composition of caveolae is thought to convey resistance of this membrane domain to detergent solubilization by Triton X-100 (at low temperatures) (25–31). This property appears to be unique to caveolae membranes. For example, when intact cells were fixed in paraformaldehyde and extracted with Triton X-100 and then examined by electron microscopy, the insoluble membranes that remained were found to be caveolae (32).

The expression of caveolin-3 is induced during the differentiation of skeletal myoblasts and caveolin-3 is localized to the muscle cell plasma membrane (sarcolemma), where it forms a complex with dystrophin and its associated glycoproteins (14). However, under certain conditions caveolin-3 can be physically separated from the dystrophin complex (33). This indicates that although caveolin-3 is dystrophin-associated, it is not absolutely required for the biogenesis of the dystrophin complex (33).

In collaboration with Minetti and colleagues (34), we have recently identified an autosomal dominant form of limb girdle muscular dystrophy (LGMD-1C)[1] in two Italian families that is due to a deficiency in caveolin-3 expression. Analysis of their genomic DNA reveals two distinct mutations in *CAV3*: (i) a

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[1] The abbreviations used are: LGMD, limb girdle muscular dystrophy; PBS, phosphate-buffered saline; PAGE, polyacrylamide gel electrophoresis; Mes, 4-morpholino-ethanesulfonic acid; GFP, green fluorescent protein; WT, wild type.

9-base pair microdeletion that removes the sequence TFT from the caveolin scaffolding domain; and (ii) a missense mutation that changes a proline to a leucine (P → L) in the transmembrane domain (34). Both mutations lead to a loss of ~85–90% of caveolin-3 protein expression.

The identity of the *CAV3* lesions in these LGMD families is instructive. Of the 12 amino acid residues that are invariant in all three human caveolins as well as the two *Caenorhabditis elegans* homologs, two of these invariant residues are affected by the mutations identified in these two Italian families. One of the invariant prolines is changed to leucine in family A, while one of the invariant phenylalanines is deleted in family B (34). In addition, alanine scanning mutagenesis of a peptide encoding the caveolin scaffolding domain reveals that the FTV(T/S) sequence in caveolins 1 and 3 is important for the correct recognition of certain caveolin-binding signaling molecules *in vitro* (35, 36), and the FT residues in this sequence are deleted in family B. This finding provides genetic evidence that this region of the caveolin scaffolding domain is critical *in vivo*.

One hypothesis is that down-regulation of the caveolin-3 protein in patients with LGMD-1C may reflect the targeting of misfolded caveolin-3 oligomers to a degradative pathway. Here, we evaluate the phenotypic behavior of these caveolin-3 mutations using heterologous expression in NIH 3T3 cells.

EXPERIMENTAL PROCEDURES

Materials—Antibodies and their sources were as follows: anti-caveolin-3 IgG (mouse mAb 26; Ref. 14), gift of Dr. Roberto Campos-Gonzalez, Transduction Laboratories; anti-Cab45 IgG (rabbit pAb; Ref. 37), gift of Dr. Philipp E. Scherer, Albert Einstein College of Medicine. [9,10-^{3}H]palmitic acid was from American Radiolabeled Chemicals, Inc. EXPRE^{35}S^{35}S-protein labeling mix (containing L-[^{35}S]methionine) was from NEN Life Science Products. All other biochemicals used were of the highest purity available and were obtained from regular commercial sources.

Construction of Untagged LGMD-1C Caveolin-3 Mutants—The cDNA encoding wild type caveolin-3 (Cav-3/WT; Ref. 12) was cloned into the pCAGGS expression vector (gift of Dr. Armin Rehm, Ploegh Laboratory, Harvard Medical School). The following two caveolin-3 mutants were generated: (i) replacement of proline at amino acid position 104 with leucine (Cav-3/P→L) and (ii) deletion of amino acids threonine-phenylalanine-threonine (amino acids 63–65) (Cav-3/ΔTFT). Both caveolin-3 mutants were generated by polymerase chain reaction amplification using appropriate internal primers and subcloned into the pCAGGS expression vector. The correctness of intended base substitutions and the absence of unwanted mutations was verified by DNA sequencing.

Cell Culture and Transient Transfection—NIH 3T3 cells were grown in Dulbecco's modified Eagle's medium supplemented with glutamine, antibiotics (penicillin and streptomycin), and 10% donor bovine calf serum (38, 39). COS-7 cells were grown in Dulbecco's modified Eagle's medium supplemented with glutamine, antibiotics (penicillin and streptomycin), and 10% fetal calf serum (40). Cells (~30–50% confluent) were transfected using a modified calcium phosphate precipitation method and analyzed 48 h post-transfection.

Triton Insolubility—Transfected NIH 3T3 cells were washed twice with PBS and lysed 30 min at 4 °C in a buffer containing 10 mM Tris, pH 8.0, 0.15 M NaCl, 5 mM EDTA, and 1% Triton X-100 (24). Samples were centrifuged at 14,000 rpm for 10 min at 4 °C. Pellet (insoluble fraction) and supernatant (soluble fraction) were resolved by SDS-PAGE (12.5% acrylamide) and analyzed by immunoblotting.

Velocity Gradient Centrifugation—Samples were dissociated in Mes-buffered saline (25 mM Mes, pH 6.5, 0.15 M NaCl) containing 60 mM octyl glucoside. Solubilized material was loaded atop a 5–40% linear sucrose gradient and centrifuged at 50,000 rpm (34,000 × *g*) for 10 h in a SW 60 rotor (Beckman) (10, 12, 13, 15). Gradient fractions were collected from above and subjected to immunoblot analysis. Molecular mass standards for velocity gradient centrifugation were as described previously (10, 12, 13, 15).

Preparation of Caveolae-enriched Membrane Fractions—Transfected NIH 3T3 cells were scraped into 2 ml of Mes-buffered saline containing 1% (v/v) Triton X-100 (19, 25, 27, 28, 31, 40–45). Homogenization was carried out with 10 strokes of a loose fitting Dounce homogenizer. The homogenate was adjusted to 40% sucrose by the addition of 2 ml of 80% sucrose prepared in Mes-buffered saline and placed at the bottom of an ultracentrifuge tube. A 5–30% linear sucrose gradient was formed above the homogenate and centrifuged at 39,000 rpm for 16–20 h in a SW41 rotor (Beckman Instruments). A light scattering band confined to the 15–20% sucrose region was observed that contained endogenous caveolin-1, but excluded most of other cellular proteins. From the top of each gradient, 1-ml gradient fractions were collected to yield a total of 12 fractions. An equal amount of protein from each gradient fraction was separated by SDS-PAGE and subjected to immunoblot analysis.

Immunoblot Analysis—Cellular proteins were resolved by SDS-PAGE (12.5% acrylamide) and transferred to nitrocellulose membranes. Blots were incubated for 2 h in TBST (10 mM Tris-HCl, pH 8.0, 150 mM NaCl, 0.2% Tween 20) containing 2% powdered skim milk and 1% bovine serum albumin. After three washes with TBST, membranes were incubated for 2 h with the primary antibody (~1000-fold diluted in TBST) and for 1 h with horseradish peroxidase-conjugated goat anti-rabbit/mouse IgG (~5000-fold diluted). Proteins were detected using an ECL detection kit (Amersham Pharmacia Biotech).

Immunofluorescence Microscopy—Transfected NIH 3T3 cells grown on glass coverslips were washed three times with PBS and fixed for 30 min at room temperature with 2% paraformaldehyde in PBS. Fixed cells were rinsed with PBS and permeabilized with 0.1% Triton X-100, 0.2% bovine serum albumin for 10 min. Then cells were treated with 25 mM NH_4Cl in PBS for 10 min at room temperature to quench free aldehyde groups. Cells were rinsed with PBS and incubated with the primary antibody for 1 h at room temperature: anti-caveolin-3 IgG (mouse mAb 26) and anti-Cab45 IgG (rabbit pAb) diluted 1:1000 in PBS with 0.1% Triton X-100, 0.2% bovine serum albumin. After three washes with PBS (10 min each), cells were incubated with the secondary antibody for 1 h at room temperature: lissamine rhodamine B sulfonyl chloride-conjugated goat anti-rabbit antibody (5 μg/ml) and fluorescein isothiocyanate-conjugated goat anti-mouse antibody (5 μg/ml). Finally, cells were washed three times with PBS (10 min each wash), slides were mounted with slow-Fade anti-fade reagent (Molecular Probes, Inc., Eugene, OR) and observed under a Bio-Rad MR 600 confocal microscope.

Metabolic Labeling and Immunoprecipitation—Forty-eight hours post-transfection, COS-7 cells were labeled for 4 h with [9,10-^{3}H]palmitic acid (150 μCi/ml) in Dulbecco's modified Eagle's medium supplemented with 5% dialyzed fetal bovine serum, 5 mM sodium pyruvate, antibiotics, and L-glutamine. Cells were washed twice with PBS and lysed 30 min at 4 °C in a buffer containing 10 mM Tris, pH 8.0, 0.15 M NaCl, 5 mM EDTA, 1% Triton X-100, 60 mM octyl glucoside. Samples were precleared for 1 h at 4 °C using protein A-Sepharose (20 μl, 50% slurry) and subjected to overnight immunoprecipitation at 4 °C using anti-caveolin-3 antibody (10 μl, mAb 26) and protein A-Sepharose (30 μl, 50% slurry). After three washes with the immunoprecipitation buffer, samples were separated by SDS-PAGE (12.5% acrylamide), and the gel was treated with 1 M sodium salicylate for 30 min to maximize sensitivity. The gel was dried at 70 °C for 1 h and exposed on Kodak X-OMAT film at −70 °C.

Measurement of the Turnover Rate of Caveolin-3 Mutants—In order to determine the half-life of transiently expressed caveolin-3/WT, caveolin-3/P → L, and caveolin-3/ΔTFT, COS-7 cells were labeled 24 h post-transfection for 1 h with 50 μCi/ml EXPRE^{35}S^{35}S-protein labeling mix (L-[^{35}S]methionine) in methionine-free culture medium (46). Cells were collected at the end of the labeling period (time 0) and 1, 7, 24, and 48 h after replacement of the culture medium with fresh normal growth medium and processed for immunoprecipitation as described above.

Co-expression of Tagged Wild Type and Mutant Forms of Caveolin-3—To examine whether LGMD-1C mutants of caveolin-3 behave in a dominant negative fashion, we co-transfected NIH 3T3 cells with C-terminally Myc-tagged WT caveolin-3 and three different N-terminally GFP-tagged forms of caveolin-3 (WT, P → L, or DTFT). Briefly, the GFP-tagged caveolin-3 fusions were created using the pEGFP-C1 vector (CLONTECH, Inc.). The C-terminally Myc-tagged WT caveolin-3 cDNA was as we described previously (12), except it was subcloned into the pCAGGS expression vector. Myc-tagged caveolin-3 was visualized using pAb A-14 (Santa Cruz Biotechnology, Inc., Santa Cruz, CA) that is directed against the Myc epitope (EQKLISEEDLN). GFP-tagged caveolin-3 was detected as we previously described for GFP-tagged caveolin-1 (47).

RESULTS

Expression of Wild Type and LGMD-1C Mutants of Caveolin-3

In order to begin to understand the pathogenesis of limb girdle muscular dystrophy 1C (LGMD-1C), we generated the

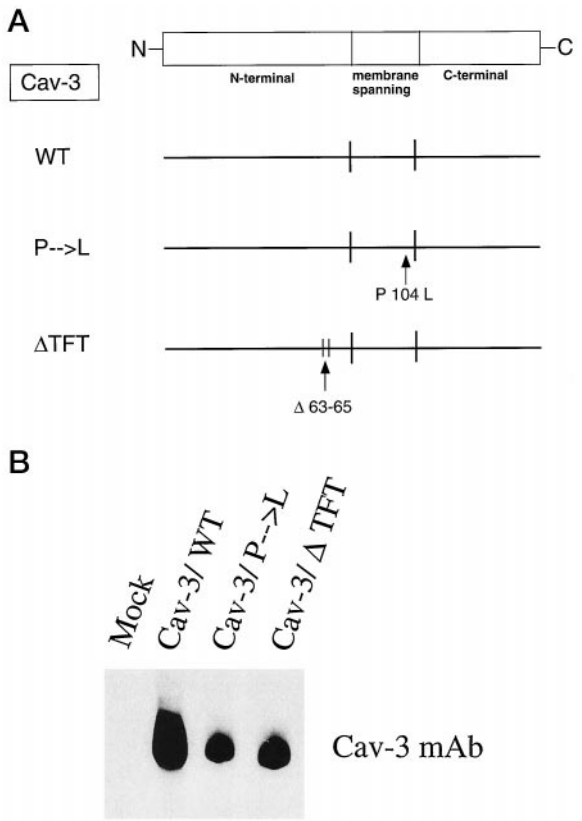

FIG. 1. **Construction and expression of LGMD-1C mutants of caveolin-3.** *A*, schematic diagram. The two LGMD-1C mutations in caveolin-3 are as indicated. Note that the ΔTFT mutation occurs within the scaffolding domain, and the P → L mutation occurs within the membrane-spanning domain. *B*, Western blot analysis. Wild type caveolin-3 and caveolin-3 mutants (ΔTFT or P → L) were transiently expressed in COS-7 cells, and their expression was assessed by immunoblotting with a specific caveolin-3 monoclonal antibody probe (mAb 26). Note that both mutant forms were expressed at significantly lower levels than achieved with wild type caveolin-3. Quantitation revealed that these LGMD-1C mutants were expressed at ~15–20% the level of expression observed for wild type caveolin-3.

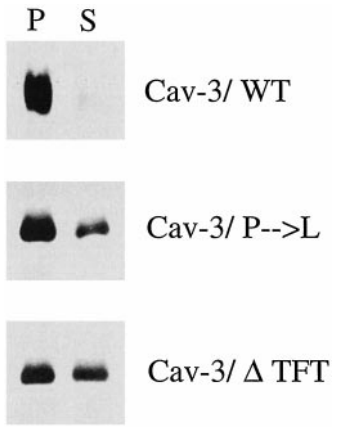

FIG. 2. **Detergent solubility of LGMD-1C caveolin-3 mutants.** NIH 3T3 cells were transiently transfected with wild type and mutant forms of caveolin-3. After extraction in a buffer containing 1% Triton X-100, samples were separated into supernatant (*S*, soluble) and pellet (*P*, insoluble) fractions, resolved by SDS-PAGE, and analyzed by immunoblotting. Note that wild type caveolin-3 is >95% Triton-insoluble, while both LGMD-1C mutants of caveolin-3 are significantly more Triton-soluble.

same two mutations in wild type caveolin-3 that are seen in patients with the symptomatic disease. These two mutations, as well as wild type caveolin-3, are illustrated schematically in Fig. 1*A*.

We next transiently transfected these caveolin-3 mutants (ΔTFT or P → L) into COS-7 cells and assessed their expression relative to wild type by Western blot analysis with a specific caveolin-3 monoclonal antibody probe (mAb 26) (Fig. 1*B*). Our results indicate that both wild type and mutant forms of caveolin-3 are well expressed, but both mutant forms are expressed at significantly lower levels than achieved with wild type caveolin-3. Quantitation revealed that these LGMD-1C mutants were expressed at ~15–20% the level of expression observed for wild type caveolin-3. However, this did not impede our analysis of the phenotypic behavior of these mutants in cultured cells.

Detergent Insolubility, Oligomeric State, and Caveolar Targeting of LGMD-1C Mutants of Caveolin-3

Triton Insolubility—Endogenous caveolins 1, 2, and 3 are insoluble in nonionic detergents such as Triton X-100 at low temperatures (10, 12, 25, 26); however, they can be efficiently solubilized by the mild detergent, octyl glucoside (25, 26). It is thought that octyl glucoside solubilization occurs through the displacement of endogenous lipid components (such as glycosphingolipids and cholesterol) that are concentrated within caveolae membranes and interact directly with the caveolins (25–31).

Fig. 2 shows that in transiently transfected NIH 3T3 cells, wild type caveolin-3 is >95% Triton-insoluble, while both LGMD-1C mutants of caveolin-3 are significantly more Triton-soluble. More specifically, Cav-3/P → L was ~25–30% Triton-soluble, while Cav-3/ΔTFT was ~40–50% Triton-soluble. These results clearly indicate that these mutations interfere with the ability of caveolin-3 to achieve optimal Triton insolubility. These results are perhaps surprising, since the mutation within the caveolin scaffolding domain (ΔTFT) more dramatically affects Triton insolubility than the mutation within the membrane-spanning domain (P → L).

Oligomerization—Caveolin-1 forms ~350-kDa homo-oligomers containing ~14–16 caveolin monomers per oligomer (15, 48). These homo-oligomers are thought to function as building blocks in the construction of caveolae membranes. Similarly, caveolin-3 forms homo-oligomers of the same size as caveolin-1 (12). In contrast, caveolin-2 exists as a homodimeric complex or as a high molecular mass hetero-oligomer with caveolin-1 (10, 13).

Thus, we next investigated the oligomeric state of LGMD-1C caveolin-3 mutants. For this purpose, we employed an established velocity gradient system developed previously to study the oligomeric state of caveolins 1, 2, and 3 (10, 12, 15). Fig. 3 shows that wild type caveolin-3 behaved as a high molecular mass complex, migrating between the 200- and 443-kDa molecular mass standards (peak fractions 7 and 8). In contrast, both LGMD-1C caveolin-3 mutants migrated predominantly as high molecular mass oligomers of >443 kDa, forming high molecular mass aggregates. More specifically, virtually all of the P → L mutant migrated in fractions 11 and 12, while ~60% of the ΔTFT mutant was confined to fractions 11 and 12 and ~40% migrated to fractions 7 and 8. Our results dramatically show that these LGMD-1C mutations adversely affect the oligomerization process.

Incorporation into Low Density Triton-insoluble Membrane Fractions That Are Enriched in Caveolae Membranes—To separate membranes enriched in caveolae from the bulk of cellular membranes and cytosolic proteins, an established equilibrium sucrose density gradient system was utilized (19, 25, 27, 28, 31,

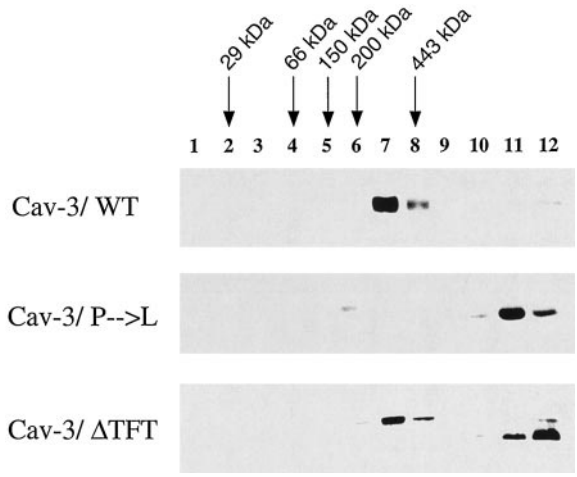

FIG. 3. **Velocity gradient centrifugation analysis of the oligomeric state of LGMD-1C mutants of caveolin-3.** NIH 3T3 cells were transiently transfected with wild type and mutant forms of caveolin-3. After extraction in a buffer containing 60 mM octyl glucoside, the solubilized material was loaded atop a 5–40% linear sucrose gradient and centrifuged. Gradient fractions were collected from above and subjected to immunoblot analysis. Note that wild type caveolin-3 behaved as a high molecular mass complex, migrating between the 200- and 443-kDa molecular mass standards (peak fractions 7 and 8). In contrast, both LGMD-1C caveolin-3 mutants migrated predominantly as high molecular mass oligomers of ≫443 kDa, forming high molecular mass aggregates. Molecular mass standards for velocity gradient centrifugation are as indicated.

40–45). In this fractionation scheme, immunoblotting with anti-caveolin IgG can be used to track the position of caveolae-derived membranes within these bottom-loaded sucrose gradients. Using this procedure, caveolin-1 is purified ~2000-fold relative to total cell lysates as ~4–6 μg of caveolin-rich domains (containing ~90–95% of total cellular caveolin-1) are obtained from 10 mg of total cellular proteins (19, 43). We and others have shown that these caveolae-enriched fractions exclude >99.95% of total cellular proteins and also markers for noncaveolar plasma membrane, Golgi, lysosomes, mitochondria, and endoplasmic reticulum (25, 27, 28).

Fig. 4 illustrates that in this fractionation scheme wild type caveolin-3 is correctly targeted to these low density Triton-insoluble membranes (fractions 4 and 5) that are enriched in caveolae membranes. In contrast, both LGMD-1C caveolin-3 mutants (P → L and ΔTFT) were quantitatively excluded from these caveolae-enriched fractions. These results indicate that the LGMD-1C mutations clearly prevent the incorporation of caveolin-3 into caveolae membranes.

Immunolocalization of LGMD-1C Mutants of Caveolin-3 to a Perinuclear Intracellular Compartment

As the LGMD-1C mutants of caveolin-3 were excluded from caveolae membranes, we next determined their subcellular localization by immunofluorescence using confocal microscopy. Fig. 5 shows the localization of these mutants. The distribution of wild type caveolin-3 is shown for comparison. Both LGMD-1C mutants were primarily retained at the level of a perinuclear compartment and did not reach the plasma membrane. In contrast, wild type caveolin-3 was efficiently targeted to the plasma membrane under these conditions. We identified this perinuclear compartment as the Golgi complex (Fig. 6) by performing double-labeling experiments with antibodies directed against the resident Golgi marker protein, Cab45, that is endogenously expressed (37).

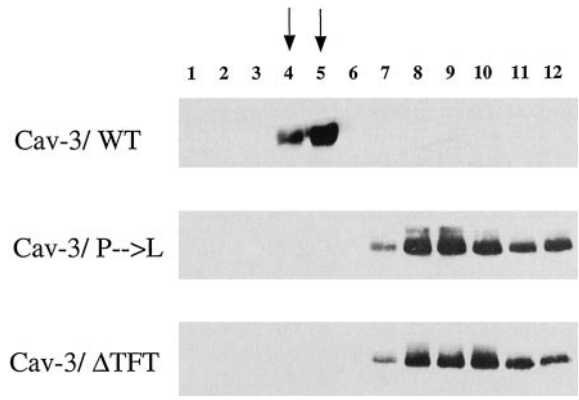

FIG. 4. **LGMD-1C mutants of caveolin-3 are not incorporated into caveolae-enriched membrane fractions.** NIH 3T3 cells were transiently transfected with wild type and mutant forms of caveolin-3. To separate membranes enriched in caveolins from the bulk of cellular membranes and cytosolic proteins, an established equilibrium sucrose density gradient system was utilized (see "Experimental Procedures"). In this fractionation scheme, immunoblotting with anti-caveolin IgG can be used to track the position of caveolae-derived membranes within these bottom-loaded sucrose gradients. Note that wild type caveolin-3 is correctly targeted to these low density Triton-insoluble membranes (fractions 4 and 5; indicated by *arrowheads*) that are enriched in caveolae. In contrast, both LGMD-1C caveolin-3 mutants are quantitatively excluded from these caveolae-enriched fractions.

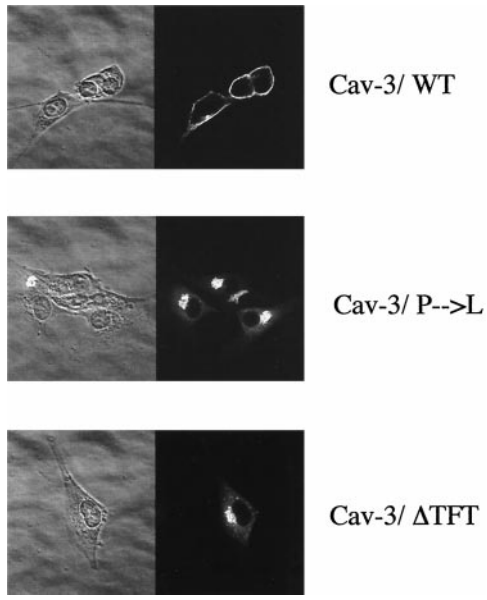

FIG. 5. **Immunolocalization of LGMD-1C mutants of caveolin-3.** Transfected NIH 3T3 cells grown on glass coverslips were immunolabeled with antibodies directed against caveolin-3 and observed under a Bio-Rad MR 600 confocal microscope. Note that both LGMD-1C mutants were primarily retained intracellularly in a perinuclear compartment and did not reach the plasma membrane. In contrast, wild type caveolin-3 was efficiently targeted to the plasma membrane.

LGMD-1C Mutants of Caveolin-3 Have a Much Shorter Half-life but Still Undergo Palmitoylation to Normal Levels

Given that the LGMD-1C mutants of caveolin-3 are not expressed to the same level as the wild type protein (Fig. 1*B*) and they are retained in an intracellular compartment (Figs. 5 and 6), these results indirectly suggest that such mutations may affect the stability of the caveolin-3 protein product. Thus, we performed a series of pulse-chase experiments designed to evaluate the turnover rate of caveolin-3 in transiently trans-

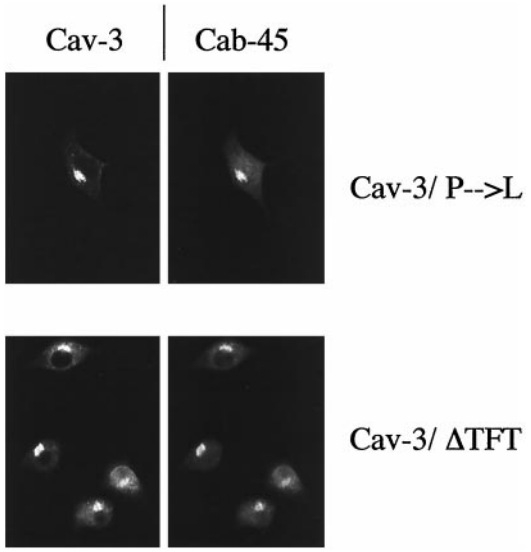

FIG. 6. **LGMD-1C mutants of caveolin-3 co-localize with a resident Golgi marker protein, Cab45.** NIH 3T3 cells were transiently transfected with mutant forms of caveolin-3 and subjected to double labeling with antibodies directed against caveolin-3 and a Golgi marker protein, Cab45. Note that the distribution of caveolin-3 mutants and Cab45 precisely coincides, identifying this perinuclear compartment as the Golgi complex.

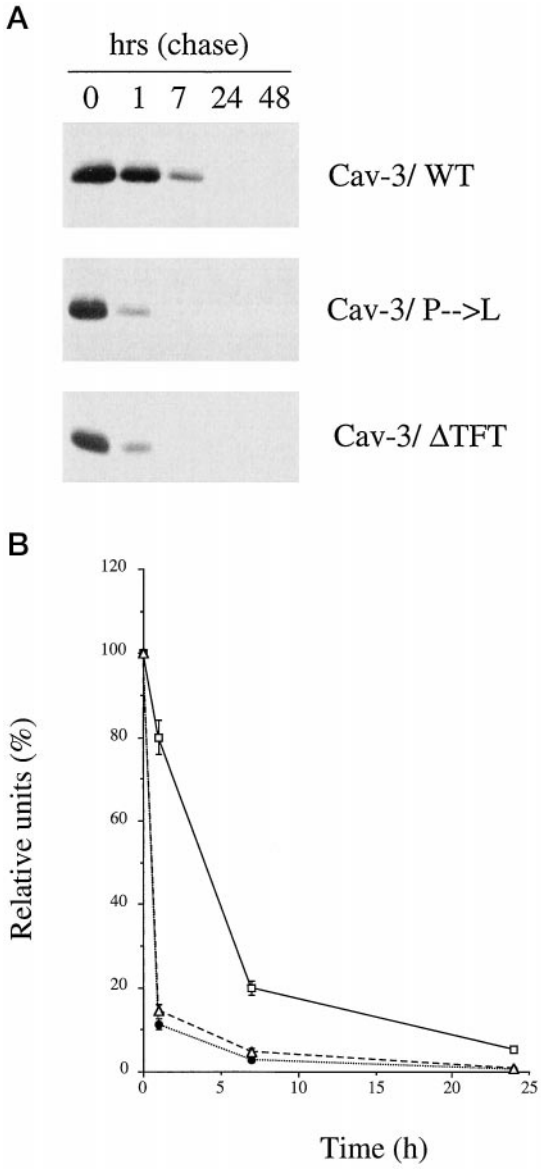

FIG. 7. **LGMD-1C mutants of caveolin-3 are unstable.** To follow the turnover of caveolin-3 (wild type and LGMD-1C mutants), COS-7 cells were labeled 24 h post-transfection for 1 h with 50 μCi/ml of [^{35}S]methionine in methionine-free culture medium. Cells were collected at the end of the labeling period (0) and at 1, 7, 24, and 48 h of chase and subjected to immunoprecipitation with antibodies directed against caveolin-3. *A*, after immunoprecipitation, samples were separated by SDS-PAGE and visualized by autoradiography. *B*, graphical representation of *A* (derived from several independent experiments). Note that wild type caveolin-3 has a half-life of ~5.25 h. In striking contrast, both LGMD-1 mutants have a half-life of ~45–60 min. □, Cav-3 WT; ●, Cav-3 P → L; △, Cav-3 ΔTFT.

fected cells. Fig. 7 shows that wild type caveolin-3 has a half-life of ~5.25 h. In striking contrast, both LGMD-1C mutants have a half-life of ~ 45–60 min. As such, the half-life of these mutant forms of caveolin-3 is shortened by ~5–7-fold. This dramatic reduction in half-life is predicted to reduce the steady state levels of these LGMD-1C mutant to ~15–20% of wild type levels (⅕th–⅐th), as we observe experimentally by Western blot analysis (Fig. 1). These results may explain why both mutations lead to a loss of ~85–90% of caveolin-3 protein expression in patients with LGMD-1C (34).

Since caveolin-1 is palmitoylated on three cysteine residues within its C-terminal region (49) and these cysteines are absolutely conserved between caveolins 1 and 3 (12), we next evaluated the palmitoylation state of caveolin-3. As predicted, wild type caveolin-3 was heavily palmitoylated (Fig. 8*A*). Similarly, both LGMD-1C mutants of caveolin-3 were also palmitoylated. As a control for expression levels, we also assessed the relative amount of wild type and mutant caveolin-3 in lysates derived from these experiments (Fig. 8*B*).

If we normalize for the fact that these caveolin-3 mutants are not expressed as well as the wild type (Fig. 8*C*), we find that both mutants undergo palmitoylation to a similar extent as wild type caveolin-3. Since the LGMD-1C mutants of caveolin-3 are retained at the level of the Golgi, these results indicate that palmitoylation of caveolin-3 can take place prior to its transport to the cell surface.

LGMD-1C Mutants of Caveolin-3 Have a Dominant Negative Phenotype, Causing the Retention of Wild Type Caveolin-3 at the Level of the Golgi

To examine whether LGMD-1C mutants of caveolin-3 behave in a dominant negative fashion, we next co-transfected NIH 3T3 cells with C-terminally Myc-tagged WT caveolin-3 and three different N-terminally GFP-tagged forms of caveolin-3 (WT, P → L, or ΔTFT).

Fig. 9*A* shows that in cells co-expressing wild type caveolin-3 (Myc) and LGMD-1C mutant forms of caveolin-3 (GFP), both are co-localized to the Golgi complex (*middle* and *lower panels*). In contrast, in cells co-expressing wild type tagged forms of caveolin-3 (Myc and GFP), both tagged forms are co-localized to the plasma membrane (*upper panel*). These results clearly indicate that both the LGMD-1C mutants of caveolin-3 behave in a dominant negative fashion, causing the retention of wild type caveolin-3 at the level of the Golgi.

In addition, co-expression of wild type caveolin-3 and caveolin-3 mutants (ΔTFT or P → L) was assessed by immunoblotting with antibodies directed against the Myc epitope and GFP. In cells co-expressing wild type tagged forms of caveolin-3 (Myc and GFP), both wild type tagged forms were well expressed (Fig. 9*B*, *lane 1*). However, in cells co-expressing wild type caveolin-3 (Myc) and LGMD-1C mutant forms of caveolin-3 (GFP) (Fig. 9*B*, *lanes 2* and *3*), both wild type and LGMD-1C

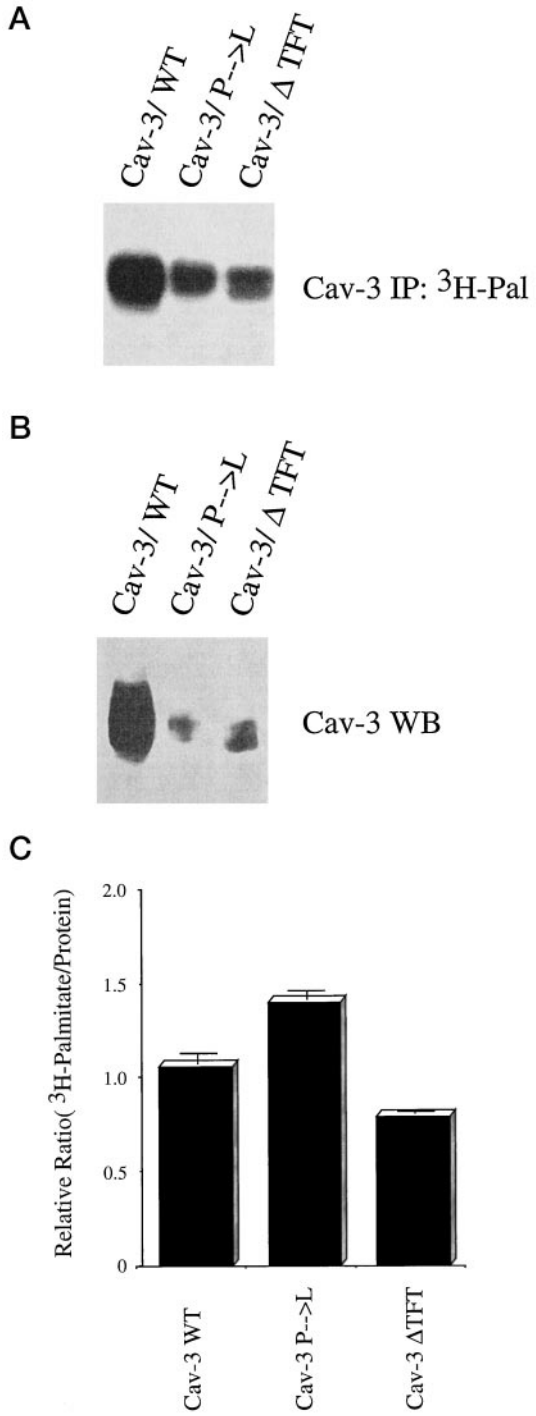

FIG. 8. **Wild type and mutant forms of caveolin-3 both undergo palmitoylation to the same extent.** *A*, metabolic labeling and immunoprecipitation. COS-7 cells were transiently transfected with wild type and mutant forms of caveolin-3. Forty-eight hours post-transfection, cells were labeled for 4 h with [9,10-^{3}H]palmitic acid (150 μCi/ml). After immunoprecipitation with caveolin-3-specific antibodies, samples were separated by SDS-PAGE and visualized by autoradiography. *B*, Western blot analysis. As a control for relative expression levels, lysates were also subjected to immunoblotting with antibodies directed against caveolin-3. Note that both caveolin-3 mutants undergo palmitoylation to the same extent as wild type caveolin-3 if we normalize for the fact that these caveolin-3 mutants are not expressed as well as the wild type. *C*, graphical representation of *A* and *B* (derived from several independent experiments). After quantitation, a relative ratio (palmitate incorporation/protein) was calculated and normalized to 1 for wild type caveolin-3. Note that the P → L mutant showed a ratio of ~1.4, while the ΔTFT mutant yielded a ratio of ~0.8. These results indicate that both LGMD-1C mutants of caveolin-3 are palmitoylated to a similar level as wild type caveolin-3.

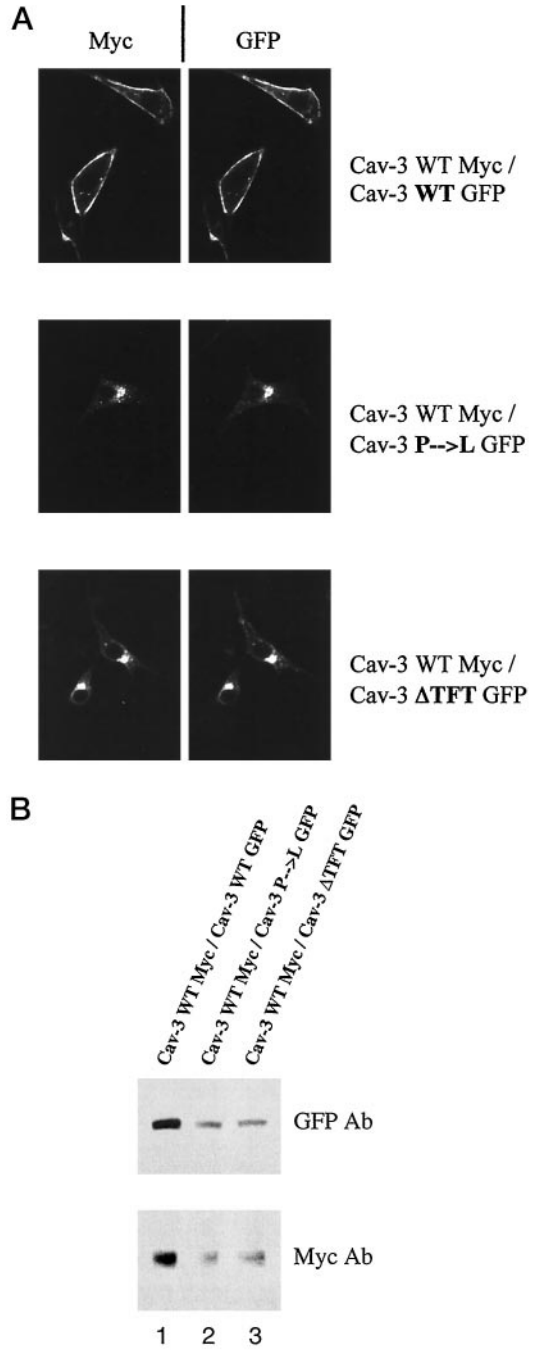

FIG. 9. **Co-expression of wild type and LGMD-1C mutant forms of caveolin-3 in a single cell.** To examine whether LGMD-1C mutants of caveolin-3 behave in a dominant negative fashion, we co-transfected NIH 3T3 cells with C-terminally Myc-tagged WT caveolin-3 and three different N-terminally GFP-tagged forms of caveolin-3 (WT, P → L, or ΔTFT). *A*, immunolocalization. Note that in cells co-expressing wild type tagged forms of caveolin-3 (Myc and GFP), both tagged forms are co-localized to the plasma membrane (*upper panel*). However, in cells co-expressing wild type caveolin-3 (Myc) and LGMD-1C mutant forms of caveolin-3 (GFP), both are co-localized to the Golgi complex (*middle* and *lower panels*). *B*, Western blot analysis. The expression of wild type caveolin-3 and caveolin-3 mutants (ΔTFT or P → L) was assessed by immunoblotting with antibodies directed against the Myc epitope and GFP. Note that in cells co-expressing wild type tagged forms of caveolin-3 (Myc and GFP), both wild type tagged forms are well expressed (*lane 1*). However, in cells co-expressing wild type caveolin-3 (Myc) and LGMD-1C mutant forms of caveolin-3 (GFP) (*lanes 2* and *3*), both wild type and LGMD-1C mutants are expressed at dramatically lower levels (~15–20% the level of expression seen in *lane 1*). Taken together, these results indicate that the LGMD-1C mutants of caveolin-3 behave in a dominant negative fashion, causing the retention of wild type caveolin-3 at the level of the Golgi.

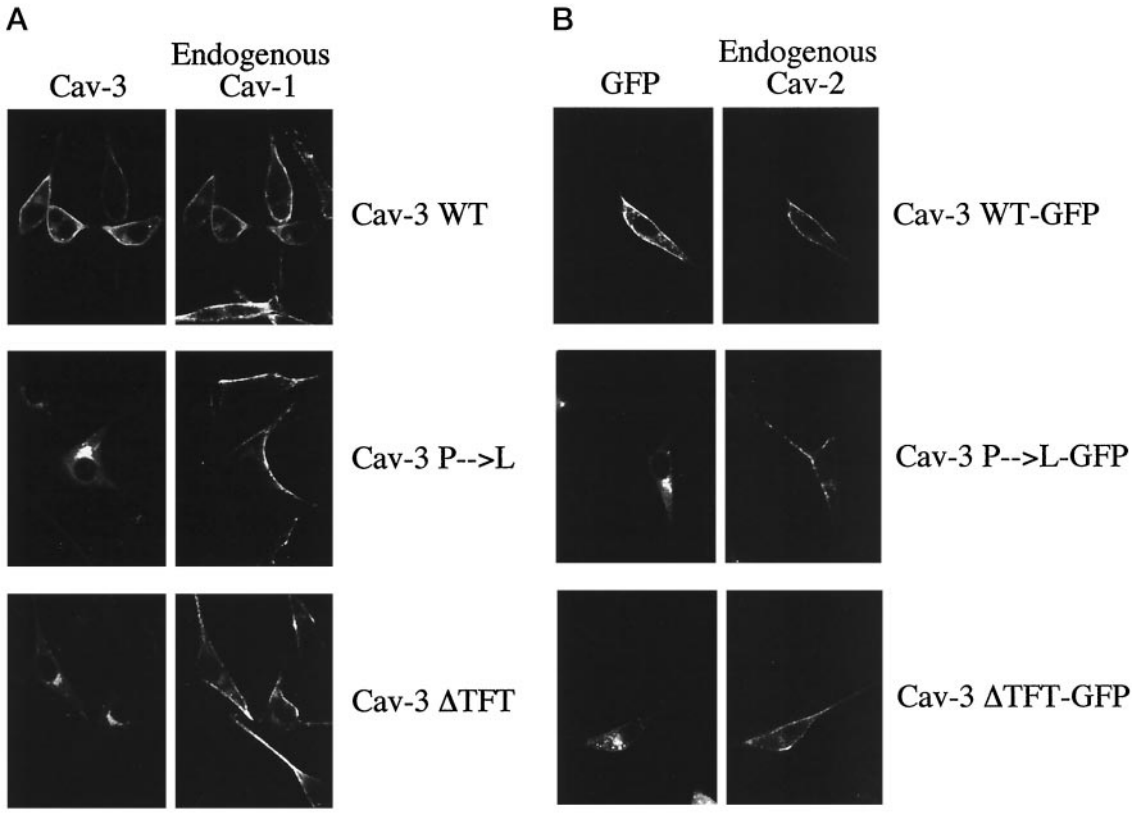

FIG. 10. **Transient expression of the LGMD-1C mutants of caveolin-3 does not affect the localization of caveolins 1 and 2.** *A*, localization of caveolin-1. NIH 3T3 cells were transiently transfected with untagged wild type caveolin-3 or untagged LGMD-1C mutants of caveolin-3. Cells were doubly immunostained with antibodies directed against caveolin-3 (mouse mAb 26) and caveolin-1 (rabbit pAb N-20). Note that the LGMD-1C mutants of caveolin-3 do not affect the localization of caveolin-1 when transiently co-expressed within the same cells. *B*, localization of caveolin-2. NIH 3T3 cells were transiently transfected with GFP-tagged wild type caveolin-3 or GFP-tagged LGMD-1C mutants of caveolin-3. Cells were immunostained with an antibody directed against caveolin-2 (mouse mAb 65). GFP-tagged caveolin-3 was detected as we previously described for GFP-tagged caveolin-1 (47). Note that the LGMD-1C mutants of caveolin-3 do not affect the localization of caveolin-2 when transiently co-expressed within the same cells. In addition, Western blot analysis revealed that transient expression of wild type caveolin-3 or the LGMD-1C mutants of caveolin-3 did not affect the expression levels of endogenous caveolins 1 and 2 (data not shown).

mutants are expressed at dramatically lower levels (~15–20% the level of expression seen in *lane 1*). These results directly demonstrate that co-expression of wild type caveolin-3 with LGMD-1C mutant forms can dramatically reduce the steady-state expression levels of wild type caveolin-3 by ~80–85%.

These observations are consistent with the previous genetic observation that the transmission of LGMD-1C occurs in an autosomal dominant fashion; *i.e.* only one mutant copy of caveolin-3 is sufficient to cause the symptomatic disease and reduce caveolin-3 levels by ~85–90% in patients (34).

LGMD-1C Mutants of Caveolin-3 Do Not Affect the Distribution or the Expression of Endogenous Caveolins 1 and 2

LGMD-1C mutants of caveolin-3 may also affect the expression, properties, or distribution of caveolins 1 and 2 that are endogenously expressed in NIH 3T3 cells. For example, LGMD-1C mutants of caveolin-3 may form mixed hetero-oligomers with caveolins 1 and 2, leading to their retention in the Golgi and degradation. However, this is unlikely, since we have recently shown that wild type caveolin-3 does not form mixed oligomers with caveolin-1 or -2 (50).

To examine whether LGMD-1C mutants of caveolin-3 behave in a dominant negative fashion with regard to the localization of caveolins 1 and 2, we next transiently transfected NIH 3T3 cells with wild type caveolin-3 or the LGMD-1C mutant forms of caveolin-3. To monitor the effects of LGMD-1C mutants on endogenous caveolins 1 and 2, we employed mono-specific antibody probes that recognize only caveolin-1 or caveolin-2, as we have generated and described previously (13, 40).

Fig. 10 shows that in NIH 3T3 cells transiently expressing either wild type or mutant forms of caveolin-3, the distribution of caveolins 1 and 2 was not affected; *i.e.* they were efficiently targeted to the plasma membrane. However, in these same cells, the LGMD-1C mutants of caveolin-3 were retained at the level of the Golgi complex. These results clearly indicate that the LGMD-1C mutants do not affect the distribution of caveolins 1 and 2.

To assess whether LGMD-1C mutants of caveolin-3 can affect the steady state expression levels of caveolins 1 and 2, we next derived NIH 3T3 cells stably expressing either wild type caveolin-3 or LGMD-1C mutants of caveolin-3. Interestingly, the expression levels of endogenous caveolins 1 and 2 were not affected by the expression of LGMD-1C mutants of caveolin-3 (Fig. 11*A*). Consistent with these observations, LGMD-1C mutants of caveolin-3 also did not affect the localization of caveolin-1 (Fig. 11*B*).

Taken together, these results clearly demonstrate that LGMD-1C mutants of caveolin-3 selectively cause the retention of wild type caveolin-3, but not caveolins 1 and 2, at the level of the Golgi complex, dramatically reducing its steady-state expression levels.

DISCUSSION

LGMD-1C is an autosomal dominant form of limb girdle muscular dystrophy that is genetically caused by mutations

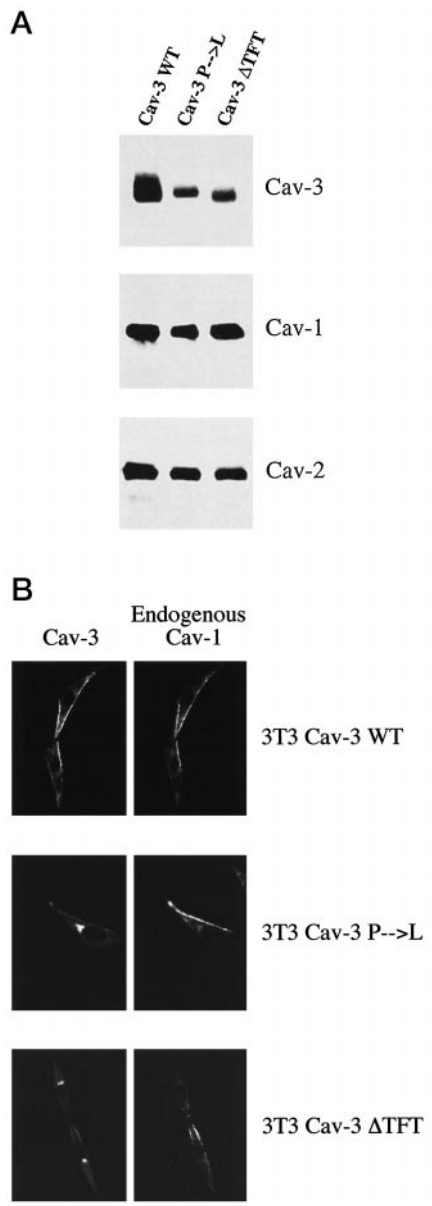

FIG. 11. **Stable expression of the LGMD-1C mutants of caveolin-3 does not affect the expression of endogenous caveolins.** NIH 3T3 cells stably expressing either wild type caveolin-3 or the LGMD-1C mutants of caveolin-3 (all untagged) were derived. *A*, Western blot analysis. The expression of caveolins 1, 2, and 3 was assessed by immunoblotting with a panel of isoform-specific caveolin monoclonal antibody probes (Cav-1, mAb 2297; Cav-2, mAb 65; and Cav-3, mAb 26). Note that the expression levels of endogenous caveolins 1 and 2 are not affected by the expression of the LGMD-1C mutants of caveolin-3. *B*, immunolocalization. Cells were doubly immunostained with antibodies directed against caveolin-3 (mouse mAb 26) and caveolin-1 (rabbit pAb N-20). Note that in all cases, endogenous caveolin-1 is targeted to the plasma membrane. In contrast, both LGMD-1C mutants of caveolin-3 are retained at the level of the Golgi complex. Thus, the LGMD-1C mutants of caveolin-3 do not affect the expression or the localization of caveolin-1 when stably co-expressed within the same cell.

within the coding regions of the caveolin-3 gene. In collaboration with Minetti and colleagues (34), we have recently identified two different families in Italy with this autosomal dominant form of limb girdle muscular dystrophy that is due to a deficiency in caveolin-3 expression. In these patients, by quantitative immunofluorescence and Western blot analysis, the levels of the caveolin-3 protein are reduced by ~85–90%.

Analysis of their genomic DNA reveals two distinct mutations: (i) a 9-base pair microdeletion that removes the sequence TFT from the caveolin scaffolding domain and (ii) a missense mutation that changes a proline to a leucine (P → L) in the transmembrane domain (34). Since these mutations are heterozygous and show an autosomal dominant form of transmission, we postulated that these mutations must cause the formation of a dominant-negative form of caveolin-3 (34). One hypothesis is that this may lead to the degradation of wild type caveolin-3, since caveolins 1, 2, and 3 are known to form both hetero- and homo-oligomers (10, 12, 13, 15, 51). However, the mechanisms that underlie the pathogenesis of this form of muscular dystrophy remain unknown.

Here, we show that these LGMD-1C mutations (i) decrease the steady-state expression levels of caveolin-3, (ii) increase the Triton solubility and oligomeric state of caveolin-3, (iii) cause caveolin-3 to be excluded from caveolae-enriched membrane domains, (iv) lead to the intracellular retention of caveolin-3 in a perinuclear compartment that we identify as the Golgi complex, and (v) dramatically shorten the half-life of caveolin-3 by ~5–7-fold. However, these mutations do not prevent the palmitoylation of the caveolin-3 protein product. These results are summarized in Table I. Taken together, these data indicate that LGMD-1C mutations cause the formation of unstable high molecular mass aggregates of caveolin-3 that are retained within the Golgi complex and are not targeted to the plasma membrane. In addition, these results provide a molecular explanation for why caveolin-3 levels are dramatically downregulated in patients with LGMD-1C.

Interestingly, comparison of the known protein sequences of mammalian caveolins 1, 2, and 3 with *C. elegans* caveolins 1 and 2, reveals that only 12 amino acid residues are invariant between worms and humans (51). These include two charged residues (Arg and Asp), five aromatic residues (three Phe, Trp, and Tyr), two prolines, two serines, and a glycine. Of these 12 invariant residues, two are affected by the LGMD-1C mutations identified in caveolin-3. One of the invariant prolines is changed to leucine in family A (P → L), while one of the invariant phenylalanines is deleted in family B (ΔTFT) (34). Our current results with caveolin-3 suggest that mutation of these evolutionarily conserved residues may have dire consequences for the structure or functioning of caveolin-3 and possibly the entire caveolin gene family. Consistent with its autosomal dominant form of genetic transmission, we demonstrate here that LGMD-1C mutants of caveolin-3 behave in a dominant negative fashion, causing the retention of wild type caveolin-3 at the level of the Golgi.

Two other mutations within the coding sequence of the caveolin-3 gene have been described that are associated with a proximal form of muscular dystrophy (52). One of these mutations was homozygous, suggesting a possible autosomal recessive inheritance (52). Interestingly, these two additional mutations map to the caveolin-3 scaffolding domain. Thus, three out of the four mutations identified are clustered within the caveolin scaffolding domain, a 20-amino acid membrane-proximal region of caveolin-3.

Given that these LGMD-1C mutations cause caveolin-3 to form abnormal high molecular mass oligomers that are retained intracellularly and have a much higher turnover rate, it is likely that these mutations cause misfolding of the caveolin-3 protein and lead to its rapid degradation ($t_{1/2}$ ~1 h). In support of this notion, protein misfolding secondary to mutations or environmental factors is now thought to be a common mechanism that underlies the pathogenesis of a number of human diseases, including cystic fibrosis, Prion disease, and Alzheimer's disease (53–55). This interpretation is consistent with the finding that three out of four thus far identified caveolin-3

TABLE I
Summary of the properties of the LGMD-1C mutants (P → L or ΔTFT) of caveolin-3, as compared with WT caveolin-3

Cav-3	Expression	Triton solubility	Oligomer formation	Caveolar targeting	Cellular distribution	Turnover rate ($t_{1/2}$)	Lipid modification
		%	*kDa*				
WT	Normal	<5	~200–443	+	Plasma membrane	~5.25 h	+
P → L	Decreased	~25–30	≫443	−	Golgi	~45–60 min	+
ΔTFT	Decreased	~40–50	≫443	−	Golgi	~45–60 min	+

mutations cluster within the caveolin scaffolding domain, which is involved both in the self-assembly of caveolins into high molecular mass homo-oligomers and in the hetero-oligomeric interaction of caveolins with signaling molecules (4).

Wild type caveolin-3 has been shown to interact with a variety of signaling molecules and to inhibit their signaling activity both *in vitro* and *in vivo* (14, 35, 36, 56). Thus, these LGMD-1C mutants of caveolin-3 may cause the retention of certain signaling molecules at the level of the Golgi. One prediction is that retention of signaling molecules at the level of the Golgi by mutant forms of caveolin-3 will occur and, therefore, these mutants will be better inhibitors of signal transduction, although they may be expressed at lower levels. Alternatively, these mutants may no longer recognize signaling molecules and, therefore, will fail to act as inhibitors of signal transduction.

Future studies will address the effects of expressing LGMD-1C mutants of caveolin-3 in skeletal muscle cells in culture. Since C2C12 cells normally up-regulate endogenous caveolin-3 expression during myoblast differentiation and fusion (12, 14), these cells are a good model system to assess the effects of co-expressing wild type and mutant forms of caveolin-3. The added benefit of using C2C12 cells is that this would allow us to examine the behavior of the LGMD-1C mutants in the context of skeletal muscle cell differentiation. However, we feel that it is more prudent to first establish the behavior of LGMD-1C mutants of caveolin-3 in the simpler context of a heterologous expression system, before moving to the slightly more complicated C2C12 differentiation system. We predict that expression of the LGMD-1C mutants of caveolin-3 in C2C12 cells will induce the extensive down-regulation/degradation of wild type caveolin-3, as seen in LGMD-1C patients (34) and NIH 3T3 cells (this report).

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