Phenotypic behavior of caveolin-3 R26Q, a mutant associated with hyperCKemia, distal myopathy, and rippling muscle disease

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Sotgia, Federica, Scott E. Woodman, Gloria Bonuccelli, Franco Capozza, Carlo Minetti, Philipp E. Scherer, and Michael P. Lisanti. Phenotypic behavior of caveolin-3 R26Q, a mutant associated with hyperCKemia, distal myopathy, and rippling muscle disease. Am J Physiol Cell Physiol 285:C1150–C1160, 2003. First published July 2, 2003; 10.1152/ajpcell.00166.2003.—Four different phenotypes have been associated with CAV3 mutations: limb girdle muscular dystrophy-1C (LGMD-1C), rippling muscle disease (RMD), and distal myopathy (DM), as well as idiopathic and familial hyperCKemia (HCK). Detailed molecular characterization of two caveolin-3 mutations (P104L and ΔTFT), associated with LGMD-1C, shows them to impart a dominant-negative effect on wild-type caveolin-3, rendering it dysfunctional through sequestration in the Golgi complex. Interestingly, substitution of glutamine for arginine at amino acid position 26 (R26Q) of caveolin-3 is associated not only with RMD but also with DM and HCK. However, the phenotypic behavior of the caveolin-3 R26Q mutation has never been evaluated in cultured cells. Thus we characterized the cellular and molecular properties of the R26Q mutant protein to better understand how this mutation can manifest as such distinct disease phenotypes. Here, we show that the caveolin-3 R26Q mutant protein is mostly retained at the level of the Golgi complex. The caveolin-3 R26Q mutant formed oligomers of a much larger size than wild-type caveolin-3 and was excluded from caveoleae-enriched membranes. However, caveolin-3 R26Q did not behave in a dominant-negative fashion when coexpressed with wild-type caveolin-3. Thus the R26Q mutation behaves differently from other caveolin-3 mutations (P104L and ΔTFT) that have been previously characterized. These data provide a possible explanation for the scope of the various disease phenotypes associated with the caveolin-3 R26Q mutation. We propose a haploinsufficiency model in which reduced levels of wild-type caveolin-3, although not much larger than that of a single allele, caveolin-3 R26Q mutant protein, are sufficient for normal muscle cell function.

Thoracic cavity; muscle cell caveolae; caveolin-3; muscular dystrophy

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teins (44). A direct interaction between β-dystroglycan and caveolin-3 has been recently demonstrated (46). However, under certain conditions caveolin-3 can be physically separated from the dystrophin complex (5). This indicates that although caveolin-3 is dystrophin-associated, it is not absolutely required for the biogenesis of the dystrophin complex (5). In addition, studies have demonstrated a transient association of caveolin-3 with transverse tubules (T tubules) during differentiation of mouse skeletal fibers (29).

Mutations in the caveolin-3 gene have been associated with a variety of muscle diseases, including limb-girdle muscular dystrophy (LGMD), hyperCKemia (HCK), distal myopathy (DM), and rippling muscle disease (RMD). Mutations in the caveolin-3 gene were first reported to be associated with the autosomal dominant form of LGMD type 1C (LGMD-1C). Two heterozygous mutations were identified: 1) a microdeletion (ΔTFT/63–65) in the caveolin scaffolding domain, and 2) a missense mutation (P104L) in the transmembrane domain. Both mutations cause a severe deficiency (~95%) of caveolin-3 protein expression in muscle fibers (24). The main clinical features of the patients include calf hypertrophy, mild to moderate proximal muscle weakness, and myopathic changes in the muscle biopsies. Interestingly, both mutations involve 2 of the 12 residues conserved among all the members of the caveolin protein family. However, other pathogenic mutations (A46T, A46V, T64P) have since been found to be associated with LGMD-1C that do not involve any of the 12 conserved residues.

Subsequently, a novel sporadic mutation in the caveolin-3 gene was found in two unrelated children with persistent elevated levels of serum creatine kinase (hyperCKemia) without muscle weakness or other symptoms of myopathy (2). Sequence analysis of the caveolin-3 gene identified a heterozygous mutation leading to a substitution of a glutamine for an arginine at amino acid position 26 (R26Q). Immunohistochemistry and Western blot analysis showed that the expression level of caveolin-3 was only partially reduced at the muscle cell surface. Interestingly, this mutation also involves 1 of the 12 conserved residues in the caveolin protein family.

This same caveolin-3 R26Q mutation was later reported in one patient with sporadic DM in which muscle atrophy was restricted to the small muscles of the hands and feet (48). Immunohistochemistry and Western blot analysis showed that the expression level of caveolin-3 was reduced (48).

Recently, heterozygous mutations in the caveolin-3 gene have been identified as the molecular basis of RMD, in both autosomal dominant families and a sporadic case (1, 49). RMD is a rare disorder characterized by involuntary, mechanically induced contractions of skeletal muscle. Direct sequencing of the caveolin-3 gene revealed four different missense mutations: P104L, A46T, A46V, and R26Q. Curiously, these mutations are the same as the ones described in patients with LGMD-1C, HCK, and DM.

These findings clearly reveal the clinical heterogeneity associated with mutations within the caveolin-3 gene. To better understand the relationship between caveolin-3 mutations and their disease phenotypes, we previously expressed the P104L and ΔTFT caveolin-3 mutant proteins in NIH 3T3 cells (10). We found that these mutants cause the formation of unstable high-molecular-mass aggregates of caveolin-3 that are not targeted to the plasma membrane. Moreover, these caveolin-3 mutants (P104L and ΔTFT) behaved in a dominant-negative fashion, causing the intracellular retention of wild-type caveolin-3 at the level of the Golgi complex. These results provide a clear molecular explanation for the autosomal dominant nature of the disease observed in these kindreds.

In this report, we characterize the phenotypic behavior of the caveolin-3 R26Q mutant in detail. Interestingly, this mutant protein forms unstable high molecular mass aggregates but does not behave in a dominant-negative fashion. The cellular profile of the caveolin-3 R26Q mutation is more akin to haploinsufficiency, where one normally functioning allele and the resulting gene product are insufficient for normal cellular function. Thus our data provide a possible explanation for the scope of the various disease phenotypes associated with the caveolin-3 R26Q mutation.

MATERIALS AND METHODS

Materials. Antibodies and their sources were as follows: anti-Cav-3 IgG (mouse MAb 26; gift of Dr. Roberto Campos-Gonzalez, BD Transduction Laboratories) (44); anti-Cab-45 IgG (rabbit PAb) (36); anti-Myc IgG (rabbit PAb; Santa Cruz Biotechnology); anti-GFP IgG (rabbit PAb; Santa Cruz Biotechnology); [9,10-3H(N)]palmitic acid was from American Radiolabeled Chemicals, and EXPRES250S35S protein-labeling mix (containing L-[35S]methionine) was from NEN Life Sciences. The proteasome inhibitor MG-132 was from Calbiochem. All other biochemicals used were of the highest purity available and were obtained from regular commercial sources.

Construction of the nontagged caveolin-3 mutant. The cDNA encoding wild-type caveolin-3 (Cav-3 WT) (47) was subcloned into the pCAGGS expression vector (10). The Cav-3 R26Q mutant was generated by the replacement of arginine at amino acid position 26 with glutamine. The mutant was generated by PCR amplification, using appropriate internal primers, and subcloned into the pcAGGS expression vector. The accuracy of intended base substitution and the absence of undesired mutations were verified by DNA sequencing.

Cell culture and transfection. NIH/3T3 cells were grown in DMEM supplemented with glutamine, antibiotics (penicillin and streptomycin), and 10% donor bovine calf serum (7, 15). Cos-7 and L6 cells were grown in DMEM supplemented with glutamine, antibodies (penicillin and streptomycin), and 10% fetal calf serum (40). Cells (~30–50% confluent) were transiently transfected using a lipid-based method (Qiagen) and analyzed 48 h posttransfection.

Triton insolubility. Transiently transfected NIH/3T3 cells were washed twice with ice-cold PBS, and a buffer containing 25 mM MES, pH 6.5, 0.15 M NaCl, 1% Triton X-100, and protease inhibitors was added on cells (45). After 30 min of incubation at 4°C without agitation, the soluble fraction was collected. The insoluble fraction was extracted using 1% SDS.
Equal volumes of the soluble and insoluble fractions were resolved by SDS-PAGE (12.5% acrylamide) and analyzed by caveolin-3 immunoblotting.

**Velocity gradient centrifugation.** Samples were lysed in MES-buffered saline (MBS; 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 SW60 rotor (Beckman). Gradient fractions were collected starting from the top of the gradient and subjected to immunoblot analysis. Molecular mass standards for velocity gradient centrifugation were as described previously (34, 37, 39, 47).

**Preparation of caveolae-enriched membrane fractions.** Transiently transfected NIH/3T3 cells were scraped into 0.7 ml of MBS containing 1% (vol/vol) Triton X-100 (4, 17, 19, 21, 31, 33, 35, 38, 40, 41, 43). Homogenization was carried out with 10 strokes of a tight-fitting Dounce homogenizer. The homogenate was adjusted to 40% sucrose by the addition of 0.7 ml of 80% sucrose prepared in MBS and was placed at the bottom of an ultracentrifuge tube. A 5–30% linear sucrose gradient was formed above the homogenate and centrifuged at 44,000 rpm for 16–20 h in a SW60 rotor (Beckman Instruments). A light-scattering band confined to the 15–20% sucrose region was observed that contained wild-type Cav-3 but excluded most other cellular proteins. From the top of each gradient, 0.375-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.

**Immunoblotting.** Samples were separated by SDS-PAGE under reducing conditions and transferred to nitrocellulose membranes (Schleicher and Schuell). The protein bands were visualized with Ponceau S (Sigma). Membranes were blocked with 5% low-fat dried milk in TBST (20 mM Tris/HCl, 150 mM NaCl, 0.5% Tween 20). Blots were then incubated at room temperature (RT) for 1 h with either Cav-3 antibody or green fluorescent protein (GFP) antibody, washed in TBST, and incubated with a secondary antibody conjugated with horseradish peroxidase (Transduction Laboratories). Bound IgG were detected using a chemiluminescent substrate (Pierce).

**Immunofluorescence microscopy.** Transiently transfected NIH/3T3 cells grown on glass coverslips were washed three times with PBS and fixed for 30 min at RT with 2% paraformaldehyde in PBS. After fixation, cells were permeabilized with 0.1% Triton X-100, 0.2% BSA for 10 min. Cells were then treated with 25 mM NH4Cl in PBS for 10 min at RT to quench free aldehyde groups. Cells were rinsed with PBS and incubated with the primary antibody for 1 h at RT: anti-Cav-3 IgG (MAb) and anti-Myc (PAb) were diluted 1:1,000 in PBS with 0.1% Triton X-100, 0.2% BSA. After three washes with PBS (10 min each), cells were incubated with the secondary antibody for 1 h at RT: rhodamine-conjugated antirabbit antibody (5 μg/ml) and fluorescein-conjugated antimouse antibody (5 μg/ml) (Jackson ImmunoResearch). Finally, cells were washed three times with PBS (10 min each wash), and slides were mounted with slow-Fade aldehyde free (Invitrogen).

**Metabolic labeling and immunoprecipitation.** Forty-eight hours posttransfection, Cos-7 cells were labeled for 4 h with [9,10-3H]palmitic acid (150 μCi/ml) in DMEM supplemented with 5% dialyzed fetal bovine serum, 5 mM sodium pyruvate, antibiotics, and 1-glutamine. Cells were washed twice with PBS and lysed for 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, and 60 mM octyl glucoside. Samples were precleared for 1 h at 4°C using protein A-Sepharose (20 μl, 50% slurry) and subjected to 5 h of immunoprecipitation at 4°C using anti-Cav-3 antibody (10 μl, MAb 26) and protein A-Sepharose (30 μl, 50% slurry). After three washes with immunoprecipitation buffer, samples were separated by SDS-PAGE (12% acrylamide), and the gel was treated with 1 M sodium saliclylate for 30 min to maximize sensitivity. The gel was dried at 70°C for 2 h and exposed on Kodak X-OMAT film at −70°C.

**Measurement of the turnover rate of Cav-3 mutant.** To determine the half-life of transiently expressed Cav-3 WT and the Cav-3 R26Q mutant, we labeled Cos-7 cells 24 h posttransfection for 1 h with 50 μCi/ml EXPRE35S35S protein-labeling mix ([35S]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 were processed for immunoprecipitation as described in Metabolic labeling and immunoprecipitation.

**Proteasomal inhibitor treatment.** Twenty hours posttransfection, Cos-7 cells were treated for 16 h with either vehicle alone (DMSO) or MG-132 (10 μM). Proteasome inhibitors were dissolved in DMSO.

**Coexpression of tagged WT and mutant forms of Cav-3.** To examine whether the Cav-3 R26Q mutant behaves in a dominant-negative fashion, we cotransfected NIH/3T3 cells with COOH-terminally Myc-tagged Cav-3 WT and two different COOH-terminally GFP-tagged forms of Cav-3 (WT, R26Q). Briefly, the GFP-tagged Cav-3 fusions were subcloned into pEGFP-C1 vector (Clontech) using SacI and BamHI sites. The COOH-terminally Myc-tagged WT Cav-3 cDNA was as we described previously (12), except that it was subcloned into the pCAGGS expression vector. Myc-tagged caveolin-3 was visualized using a Myc MAb. For Western blot analysis, GFP-tagged Cav-3 was detected using a GFP PAb.

**RESULTS**

**Expression of Cav-3 WT and the Cav-3 R26Q mutant in NIH/3T3 cells.** To understand the pathogenic mechanisms underlying those muscle diseases caused by a Cav-3 R26Q mutation, we expressed and analyzed the phenotypic behavior of the Cav-3 R26Q mutant in a heterologous expression system. We introduced the same amino acid substitution detected in patients into a heterologous expression system. We introduced the same amino acid substitution detected in patients into a heterologous expression system.

**NIIH/3T3 cells were transiently transfected either with Cav-3 WT or the Cav-3 R26Q mutant.** Cell lysates were subjected to Western blot analysis using a specific MAb raised against Cav-3. Figure 1B shows that the Cav-3 R26Q mutant is expressed at significantly lower levels compared with Cav-3 WT. In addition, the mutant migrates with a characteristic upward mobility shift, which could be due to conformational changes. However, the lower expression levels of the Cav-3 R26Q mutant did not impede our study of the phenotypic behavior of the mutant.
Caveolin-3 R26Q is detergent soluble and forms abnormally high-molecular-mass oligomers, which are excluded from caveolar membrane microdomains. To evaluate the biochemical properties of the Cav-3 R26Q mutant, we first assessed its detergent solubility pattern. Caveolin family proteins are clustered in cell membrane microdomains, named lipid rafts, which are characterized by a peculiar aggregation of cholesterol and sphingolipids. Such a lipid composition provides these membrane microdomains with special structural and biochemical properties. The close lateral association of the lipid-saturated acyl chain creates a relatively ordered environment and impedes the free movement of proteins. As a consequence of such a “liquid-ordered” status, these membrane microdomains have been found to be resistant to solubilization by certain nonionic detergents, such as Triton X-100, at low temperatures. Because caveolins reside in such a lipid microenvironment, they share the property of being Triton X-100 insoluble (20, 35, 39, 47). As a consequence, the Cav-3 R26Q mutant was expressed at lower levels compared with the WT. In addition, the Cav-3 R26Q mutant showed an upward shift in migration.

Caveolin-3 is known to self-associate and to form oligomeric complexes, each composed of ~14–16 monomers. The interaction of these oligomers with each other is thought to act as the driving force in the formation of caveolae invaginations (34, 39, 47). In an attempt to evaluate the oligomerization status of the Cav-3 mutant, we subjected lysates from NIH/3T3 cells expressing either Cav-3 WT or the Cav-3 R26Q mutant to velocity gradient ultracentrifugation. Figure 3 shows that Cav-3 WT forms oligomeric complexes of 350–440 kDa (fractions 5 and 6). On the contrary, Cav-3 R26Q mutant formed an abnormally large oligomeric complex that migrated at the bottom of the gradient (fraction 12).
that Cav-3 WT forms complexes of ~350–400 kDa. On the contrary, the Cav-3 R26Q mutant behaves as a very high-molecular-mass aggregate, which resides at the bottom of the gradient.

Because the Cav-3 R26Q mutant has abnormal oligomerization properties, we hypothesized that the Cav-3 R26Q mutant could not be properly targeted to caveolar membrane microdomains. To test this hypothesis, we performed sucrose gradient ultracentrifugation on NIH/3T3 cells transiently transfected either with Cav-3 WT or the Cav-3 R26Q mutant. This procedure allows the separation of caveolin-containing membranes from other cytosolic and membranous cellular components, by taking advantage of the low buoyant density and detergent insolubility of caveolae membranes (4, 17, 19, 21, 31, 33, 35, 38, 40, 41, 43). Figure 4 shows that Cav-3 WT is enriched in fractions 4 and 5, which are of caveolar origin. However, the Cav-3 R26Q mutant is mainly excluded from these fractions. Taken together, these results suggest that the biochemical behavior of the Cav-3 R26Q mutant is greatly compromised.

**Cav-3 R26Q is retained in a perinuclear compartment.** We next evaluated the subcellular distribution of the Cav-3 R26Q mutant. NIH/3T3 cells transiently transfected either with Cav-3 WT or Cav-3 R26Q were subjected to immunofluorescence analysis with a specific MAb directed against Cav-3. Figure 5A shows that Cav-3 WT is mainly targeted to the plasma membrane and is distributed in a very fine

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**Fig. 4. Cav-3 R26Q is mainly excluded from caveolar membrane microdomains.** Caveolar microdomains were separated from other membranous and cytosolic cellular components by sucrose gradient fractionation. NIH/3T3 cells were transiently transfected with cDNAs encoding either Cav-3 WT or Cav-3 R26Q. Thirty-six hours after transfection, cells were lysed in a buffer containing 1% Triton X-100, loaded at the bottom of a discontinuous sucrose gradient, and subjected to overnight ultracentrifugation. Twelve fractions were collected, and an equal amount of protein (5 μg) from each fraction was subjected to Western blot analysis. The distribution of Cav-3 was visualized using a specific MAb. Cav-3 WT was targeted to fractions 4 and 5, which are of caveolar origin. Conversely, Cav-3 R26Q was mostly excluded from the caveolar membrane microdomains.

**Fig. 5. Cav-3 R26Q localizes to a perinuclear intracellular compartment.** A: NIH/3T3 cells were transiently transfected with cDNAs encoding either Cav-3 WT or Cav-3 R26Q. Thirty-six hours after transfection, cells were formaldehyde-fixed and immunostained with a MAb against Cav-3. Cav-3 WT (top) is targeted to the plasma membrane (arrows). In contrast, Cav-3 R26Q (bottom) localizes in an intracellular perinuclear compartment (arrowhead), not the membrane (arrows). N, nucleus. B: Cos-7 cells were transiently transfected with the cDNA encoding Cav-3 R26Q. Thirty-six hours after transfection, cells were formaldehyde-fixed and doubly immunostained with a mouse MAb directed against Cav-3 (47) and a rabbit polyclonal antibody (PAb) directed against Cab-45, a Golgi marker protein (36). The perinuclear distribution of the Cav-3 R26Q mutant and Cab-45 significantly coincides (arrowheads).
punctate pattern. On the contrary, the Cav-3 R26Q mutant is retained intracellularly, in a perinuclear compartment.

To better define the perinuclear compartment where the Cav-3 R26Q mutant resides, we next performed double labeling with a Golgi marker protein, known as Cab-45 (36). Cos-7 cells were transiently transfected with the Cav-3 R26Q cDNA and subjected to immunostaining with anti-Cav-3 IgG (mouse MAb) and anti-Cab-45 IgG (rabbit PAb). Figure 5B shows that the distribution of the Cav-3 R26Q mutant clearly coincides with that of Cab-45, thus identifying the perinuclear compartment as the Golgi complex.

Cav-3 R26Q has a short half-life but is palmitoylated. Because the Cav-3 R26Q mutant is expressed at much lower levels than the wild type, we investigated the idea that the turnover rate of the Cav-3 R26Q mutant was affected. To assess the validity of this hypothesis, we performed pulse-chase experiments on Cos-7 cells transiently transfected with Cav-3 WT or Cav-3 R26Q. Figure 6A indicates that Cav-3 WT has a half-life of ~7 h. However, the Cav-3 R26Q mutant shows a faster turnover rate and appears to have a much shorter half-life of ~1 h.

We next explored the possibility that the lower protein expression of the Cav-3 R26Q mutant was due to...
protein degradation, occurring through the proteasomal pathway. As a consequence, we analyzed the effect of proteasome inhibitor treatment on Cos-7 cells transiently transfected with the Cav-3 R26Q cDNA. The expression level of Cav-3 was detected using a specific Cav-3 MAb probe. Figure 6B shows that treatment for 16 h with the proteasomal inhibitor MG-132 (10 μM) is sufficient to rescue the expression level of Cav-3 R26Q. However, no effect was observed when cells were treated for the same time with vehicle alone.

Because Cav-3 is normally palmitoylated (6, 10), we next evaluated whether the Cav-3 R26Q mutant would undergo palmitoylation. Cos-7 cells expressing either Cav-3 WT or Cav-3 R26Q were metabolically labeled with [3H]palmitic acid. Subsequently, cell lysates were immunoprecipitated using a Cav-3 MAb. After extensive washing, samples were separated by SDS-PGE, and gels were exposed for autoradiography (Fig. 7, top). To assess the relative expression level, we subjected a portion of the cell lysates, before immunoprecipitation, to Western blot analysis using a Cav-3 antibody (Fig. 7, bottom). The Cav-3 R26Q mutant appears to undergo normal palmitoylation, if we equalize for the relatively lower expression level of the mutant compared with the wild type.

Cav-3 R26Q does not impart a dominant-negative effect on Cav-3 WT. Mutations in the Cav-3 gene have been associated with an autosomal dominant form of LGMD-1C. These mutations include the substitution of a proline for a leucine at position 104 (P104L) and a three-amino acid in-frame deletion (ΔTFT) in the Cav-3 scaffolding domain (24). Previous studies have revealed the pathogenic mechanism of these LGMD-1C Cav-3 mutants and showed that they act in a dominant-negative fashion (10).

As such, we next evaluated whether the Cav-3 R26Q mutant behaved in a similar dominant-negative fashion. We used a cotransfection approach. We reasoned that, if the Cav-3 R26Q mutant showed dominant-negative behavior, we could detect changes in the expression levels and subcellular distribution of Cav-3 WT when coexpressed with the mutant. To distinguish Cav-3 WT from the Cav-3 R26Q mutant, we placed epitope tags (Myc and GFP) at the COOH terminus of each construct. We first checked whether the tagged constructs maintained the expected subcellular distri-

Fig. 9. Cav-3 R26Q does not behave in a dominant-negative fashion: immunofluorescence analysis. A: NIH/3T3 cells were transiently cotransfected with cDNAs encoding Cav-3 WT-Myc and Cav-3 WT-GFP. Thirty-six hours after transfection, cells were formaldehyde-fixed and immunostained with a PAb against Myc (to detect Cav-3 WT-Myc). Cav-3 WT-GFP was visualized using autofluorescence. In cells coexpressing both tagged forms of Cav-3 WT, the wild-type protein was properly localized to the cell surface. B: NIH/3T3 cells were transiently cotransfected with cDNAs encoding Cav-3 WT-Myc and Cav-3 R26Q-GFP. Thirty-six hours after transfection, cells were formaldehyde-fixed and immunostained with a PAb against Myc (to detect Cav-3 WT-Myc). Cav-3 R26Q-GFP was visualized using autofluorescence. In cells coexpressing Cav-3 WT and Cav-3 mutant, the WT form was mainly targeted to the cell surface (arrows), whereas the mutant was retained intracellularly (arrowhead). These results suggest that the Cav-3 R26Q mutant does not behave in a dominant-negative fashion, because it did not interfere with the localization pattern of Cav-3 WT.
bution. Figure 8 shows that the tagged Cav-3 WT constructs (both Myc and GFP tagged) are properly targeted to the cell surface, whereas the Cav-3 R26Q GFP mutant is retained in an intracellular perinuclear compartment.

Next, NIH/3T3 cells were transiently cotransfected with either 1) Cav-3 WT-Myc and Cav-3 WT-GFP or 2) Cav-3 WT-Myc and Cav-3 R26Q-GFP. Cells were then subjected to immunofluorescence analysis. Figure 9A shows that when coexpressed in a single cell, Cav-3 WT-Myc (as detected with the Myc antibody) and Cav-3 WT-GFP (as detected by autofluorescence) were both able to reach the cell membrane. Interestingly, as shown in Fig. 9B, coexpression of Cav-3 WT-Myc (as detected with the Myc antibody) and Cav-3 R26Q-GFP (as detected by autofluorescence) did not impede the targeting of Cav-3 WT to the plasma membrane. As expected, the Cav-3 R26Q mutant was localized in an intracellular compartment. These results suggest that the expression of the Cav-3 R26Q mutant did not affect the subcellular distribution of Cav-3 WT.

We next evaluated whether the Cav-3 R26Q mutant affected the expression level of Cav-3 WT. Figure 10 shows that Cav-3 WT-Myc does not change its expression level (as detected with the Myc antibody) when coexpressed with either Cav-3 WT-GFP or Cav-3 R26Q-GFP. Taken as a whole, these results clearly indicate that the Cav-3 R26Q mutant does not affect the expression level or the localization pattern of Cav-3 WT. From these findings, we can conclude that the Cav-3 R26Q mutant does not behave in a dominant-negative fashion.

Cav-3 mutants (R26Q, P104L, and ΔTFT) are all retained intracellularly in L6 skeletal muscle cell line. Because the expression of Cav-3 is normally restricted to muscle cell types, we also evaluated the subcellular localization of the Cav-3 R26Q mutant in the context of a skeletal muscle cell line, namely, L6 myoblasts. For comparison, we also assessed the distribution of two other Cav-3 mutants, namely, Cav-3 P104L and Cav-3 ΔTFT. L6 cells were transiently transfected with the Cav-3 cDNAs (WT or mutants) and immunostained with a specific MAab probe directed against Cav-3.
Figure 11 shows that Cav-3 WT is properly targeted to the plasma membrane in L6 cells, as expected. On the contrary, Cav-3 R26Q, Cav-3 P104L, and Cav-3 ΔTFT were all retained in an intracellular perinuclear Golgi compartment in L6 myoblasts.

**DISCUSSION**

LGMD-1C results from heterozygous mutations within the coding region of the caveolin-3 gene (14, 24). In these patients, the levels of the caveolin-3 protein are markedly reduced. The pathogenic mutations include a microdeletion (ΔTFT) in the caveolin scaffolding domain, a point mutation in the transmembrane domain (P104L), and a point mutation in the NH2-terminal region (A46T) (14, 24). In addition, a novel sporadic point mutation (T64P) in the caveolin scaffolding domain has been described that also results in LGMD-1C (22).

We recently investigated the cellular behavior of the ΔTFT and P104L caveolin-3 mutations by heterologous expression in NIH/3T3 cells. Our data indicated that these LGMD-1C mutations cause the formation of unstable high-molecular-mass aggregates of mutant caveolin-3 that are retained within the Golgi complex and are not targeted to the plasma membrane (10). In addition, these two mutants cause the retention of Cav-3 WT at the level of the Golgi complex, thus behaving in a dominant-negative fashion. These results provide a molecular explanation for the dramatic loss of membrane-associated caveolin-3 in patients with LGMD-1C due to these mutations.

We also identified a novel heterozygous mutation in the coding region of the caveolin-3 gene in both familial and sporadic cases of HCK (2). This mutation consists of a substitution of a glutamine for an arginine at amino acid position 26. Since its identification, several different disease phenotypes have been associated with the Cav-3 R26Q mutation, including RMD, DM, and HCK. However, the phenotypic behavior of the Cav-3 R26Q mutation has never been evaluated. The mechanism by which the Cav-3 R26Q mutation results in these varied disease phenotypes remains unclear.

Here, we have shown that the Cav-3 R26Q mutation 1) decreases the steady-state expression levels of caveolin-3, 2) increases the Triton solubility and oligomeric state of caveolin-3, 3) causes caveolin-3 to be partially excluded from lipid rafts/caveolae-enriched membrane domains, 4) leads to intracellular retention of caveolin-3 in a perinuclear Golgi compartment, and 5) dramatically shortens the half-life of caveolin-3. However, this mutation does not prevent the palmitylation of the caveolin-3 protein product. Interestingly, the Cav-3 R26Q mutation does not behave in a dominant-negative fashion, because it does not affect the expression or the cellular distribution of the WT Cav-3 protein, in contrast with the results obtained for the previously tested LGMD-1C mutations (ΔTFT or P104L) (10). These data provide a likely explanation for the observed differences in detectable levels of the caveolin-3 protein in human muscle tissue biopsies taken from patients with ΔTFT/P104L mutations [−90−95% reduction in caveolin-3 levels (24)] vs. the R26Q mutation [−60−80% reduction in caveolin-3 levels (2)]. These differences in the levels and functionality of the remaining WT Cav-3 protein may explain the varied clinical presentations as well.

For example, the higher levels of caveolin-3 that were detected in muscle fibers from HCK patients with the Cav-3 R26Q mutation may still be sufficient to form caveolae and maintain relatively normal muscle function with asymptomatic release of creatine kinase. In support of this hypothesis, we recently identified a novel Cav-3 P28L mutation that results only in familial HCK (23). In patient biopsies, the Cav-3 P28L mutation leads to a similar reduction of caveolin-3 membrane association as the caveolin-3 R26Q mutant. That the Cav-3 R26Q mutation can also result in RMD and DM in other populations is most likely due to modifying genetic background effects, allowing for a more fulminant effect of the Cav-3 R26Q mutation. Another possibility, of course, is that in addition to reduced WT Cav-3 levels, the Cav-3 R26Q mutant may itself negatively affect muscle cell function by destabilizing the muscle cell plasma membrane directly or through its interaction with other proteins. Ultimately, further investigation of the genetic backgrounds of the populations in which the Cav-3 R26Q mutation is expressed will yield a better understanding of the determinants of the different resultant phenotypes.

Recently, the Cav-3 R26Q mutation was found to be associated with a patient that was identified as having a form of LGMD (22). This finding further illustrates the broad phenotypic spectrum associated with the Cav-3 R26Q mutation.

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**DISCLOSURES**

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