A caveolin-3 mutant that causes limb girdle muscular dystrophy type 1C disrupts Src localization and activity and induces apoptosis in skeletal myotubes

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Summary

Caveolins are membrane proteins that are the major coat proteins of caveolae, specialized lipid rafts in the plasma membrane that serve as scaffolding sites for many signaling complexes. Among the many signaling molecules associated with caveolins are the Src tyrosine kinases, whose activation regulates numerous cellular functions including the balance between cell survival and cell death. Several mutations in the muscle-specific caveolin, caveolin-3, lead to a form of autosomal dominant muscular dystrophy referred to as limb girdle muscular dystrophy type 1C (LGMD-1C). One of these mutations (here termed the 'TFT mutation') results in a deletion of a tripeptide (ΔTFT(63-65)) that affects the scaffolding and oligomerization domains of caveolin-3. This mutation causes a 90-95% loss of caveolin-3 protein levels and reduced formation of caveolae in skeletal muscle fibers. However, the effects of this mutation on the specific biochemical processes and cellular functions associated with caveolae have not been elucidated. We demonstrate that the TFT caveolin-3 mutation in post-mitotic skeletal myotubes causes severely reduced localization of caveolin-3 to the plasma membrane and to lipid rafts, and significantly inhibits caveolar function. The TFT mutation reduced the binding of Src to caveolin-3, diminished targeting of Src to lipid rafts, and caused abnormal perinuclear accumulation of Src. Along with these alterations of Src localization and targeting, there was elevated Src activation in myotubes expressing the TFT mutation and an increased incidence of apoptosis in those cells compared with control myotubes. The results of this study demonstrate that caveolin-3 mutations associated with LGMD-1C disrupt normal cellular signal transduction pathways associated with caveolae and cause apoptosis in muscle cells, all of which may reflect pathogenetic pathways that lead to muscle degeneration in these disorders.

Key words: Caveolin-3, Caveolae, Limb girdle muscular dystrophy, Lipid rafts, Signal transduction, Src kinase

Introduction

Lipid rafts are microdomains within cellular membranes, including the plasma membrane, characterized by a high cholesterol and sphingolipid content and densely packed, highly organized, acylated fatty acids (Brown and London, 2000). Caveolae are specialized lipid rafts that appear as 50-100 nm invaginations in the plasma membrane of most cell types (Kurzchalia and Parton, 1999; Schlegel and Lisanti, 2001). Caveolae are involved in many processes, including cholesterol trafficking within the cell, endocytosis of certain molecules (e.g. albumin) and regulation of many signal transduction pathways (Schlegel and Lisanti, 2001). Caveolae have a characteristic protein coat, a major component of which is caveolin, and studies of caveolin-null mice have demonstrated that caveolin targeting to the plasma membrane is required for normal caveolar formation (Galbiati et al., 2001; Park et al., 2002). To date, three major caveolin isoforms have been identified. Caveolin-1 and caveolin-2 are expressed in many cell types; caveolin-3 is a muscle-specific isoform (Tang et al., 1996; Way and Parton, 1996). In mature skeletal muscle, caveolin-3 is predominantly localized at the plasma membrane (Razani et al., 2000). In cultured post-mitotic muscle cells (myotubes), this protein is also found in foci throughout the cytosol (Carozzi et al., 2002). Caveolin-3 is clearly involved in the formation of the T-tubule system (Carozzi et al., 2000; Parton et al., 1997; Galbiati et al., 2001; Lee et al., 2002), but other distinctive roles it plays in normal muscle cell function are only beginning to be investigated.

Caveolins and caveolae have well-established roles in mediating signal transduction processes at the plasma membrane. Caveolin proteins are considered primarily to have a scaffolding role mediated by a 'caveolin scaffolding domain' which specifically binds many signaling molecules (Li et al., 1996; Venema et al., 1997; Song et al., 1996). Growth factor receptors, signaling molecules (e.g. Src, Ras, NOS, PKCα, GPI-linked proteins and G-protein α-subunits), and their downstream effector molecules localize to caveolae (Shaul and Anderson, 1998). Based on these observations, it has been suggested that caveolae act as platforms for the accumulation of signaling molecules and provide a physical location for the initiation of downstream signaling events (Zajchowski and Robbins, 2002). Furthermore, it was

recently demonstrated that the expression of caveolin proteins is necessary for normal trafficking and subcellular localization of certain caveolae-associated signaling molecules (Sotgia et al., 2002).

Src tyrosine kinase is one of the major signaling molecules known to accumulate within caveolae and to bind directly to the caveolin scaffolding domain (Li et al., 1996). In many cell types, there is abundant evidence for the role of Src kinases in regulating the balance of signals that determine whether cells will survive or will undergo apoptosis in response to various stressful stimuli (Thomas and Brugge, 1997). Both activation and inhibition of Src kinases have been shown to promote apoptosis (Thomas and Brugge, 1997), demonstrating that the consequences of alterations of Src signaling depend on the cellular environment. Src kinases regulate acetylcholine receptor clustering during neuromuscular junction formation (Smith et al., 2001). In addition, Src kinases have a role in the intracellular signaling cascade involved in activation and phosphorylation of the vitamin D receptor in myotubes (Buitrago et al., 2001; Morelli et al., 2000), and there is evidence that the Src kinase, Lyn, is involved in insulin receptor signaling in skeletal muscle (Muller and Frick, 1999). Taken together, these studies indicate that Src kinases have a major role in regulating muscle cell survival and function and that these signaling pathways are likely to be regulated, at least in part, by the association of Src with caveolae and caveolins.

Several disease-causing mutations in the gene encoding caveolin-3 have been identified. A microdeletion (referred to here as the TFT mutation) resulting in the loss of three amino acid residues (ΔTFT(63-65)) within the scaffolding domain was first identified in association with human limb girdle muscular dystrophy (LGMD), which was then termed LGMD-1C (Minetti et al., 1998). Immunohistochemical analysis of biopsies from patients suffering from this disease reveal caveolin-3 in the cytosol and at the plasma membrane, but western blot analysis demonstrates a 90-95% reduction in the levels of the protein (Minetti et al., 1998; Minetti et al., 2002). Scanning electron microscopic analysis of caveolae formation at the cell surface demonstrated that caveolae are present but in severely reduced numbers (Minetti et al., 2002). Expression of this TFT mutation along with wild-type caveolin-3 in NIH3T3 fibroblasts shows that the mutation causes retention of caveolin-3 protein in the Golgi complex (Galbiati et al., 1999b) and causes caveolin-3 to form high molecular mass oligomers that are unstable and highly susceptible to proteolysis (Galbiati et al., 2000). However, no studies to date have examined the function of the TFT mutation in post-mitotic skeletal myotubes.

Since caveolar function is integrally linked to signal transduction processes, the aim of this study was to examine the effects of the TFT caveolin-3 mutation in skeletal myotubes on caveolar function and associated signal transduction processes. In particular, we focused on those processes that may be involved in the regulation of myotube survival, since cell death is a key feature of the pathology of muscular dystrophies (Tews, 2002) and since the disruption of cell signaling pathways are increasingly being found to contribute to the pathogenesis of muscular dystrophies (Rando, 2001). Our results demonstrate a dramatic impairment of caveolar function and an associated disruption of the localization and function of Src tyrosine kinase. In addition, myotubes expressing the mutant form of caveolin-3 had an increased incidence of apoptotic cell death. These results demonstrate mechanisms of cellular dysfunction and pathological changes that are mediated by caveolin-3 mutations associated with LGMD-1C and relate them to potential pathogenetic mechanisms in this disorder.

Materials and Methods

Antibodies and other reagents

The anti-caveolin-3 antibody was obtained from Transduction Laboratories (Palo Alto, CA), and the anti-caveolin-1 antibody was from Santa Cruz Biotechnology (Santa Cruz, CA). Anti-skeletal muscle myosin (slow) was obtained from Sigma Chemicals (St Louis, MO). Anti-c-Src was obtained from Santa Cruz Biotechnology. Texas Red-conjugated albumin and anti-Texas Red antibody were obtained from Molecular Probes (Eugene, OR). Secondary antibodies for western blotting were obtained from Amersham Biosciences (Piscataway, NJ), and secondary antibodies for immunofluorescence were obtained from Molecular Probes.

Cell culture and transfection

Primary myoblasts were derived from neonatal C57Bl/6 mice (Rando et al., 1998; Disatnik and Rando, 1999) and maintained in growth medium (GM) consisting of Ham's F10 nutrient mixture (Mediatech, Herndon, VA) supplemented with 20% fetal bovine serum (Omega Scientific, Tarzana, CA), penicillin/streptomycin (Mediatech) and basic fibroblast growth factor (2.5 ng/ml; Atlanta Biologicals, Norcross, GA) (Franco et al., 1999; Zhou et al., 2001). Myoblasts were induced to undergo differentiation and fusion to form multinucleated myotubes in differentiation medium (DM) consisting of Dulbecco's modified Eagle's medium supplemented with 2% horse serum (Invitrogen Life Technology, Carlsbad, CA) and penicillin/streptomycin (Franco et al., 1999). All experiments were conducted on myotubes 3 days after the induction of differentiation unless otherwise specified. Myoblasts and myotubes were grown on plastic tissue culture plates coated with a mixture of mouse laminin (5 μg/ml) (Invitrogen) and calf skin collagen (0.01%) (Sigma), or on glass chamber slides coated with mouse fibronectin (5 μg/ml) (Invitrogen).

The wild-type and the ΔTFT(63-65) mutant caveolin-3 cDNAs were kindly provided by Dr Michael Lisanti (Albert Einstein Medical Center). These cDNAs were subcloned into the pLXSN retroviral plasmid and driven by the LTR promoter. The plasmid also encodes the neomycin-resistance gene (*neo*) under the control of the SV40 promoter. PT67 cells were transfected with the retroviral constructs and the supernatants were used to stably transfect proliferating primary myoblasts. As controls, myoblasts were transfected with the virus encoding *neo* alone. Transfected myoblasts were selected in the presence of G418 (100 μg/ml) in GM. In all of the figures, cells expressing the TFT mutation are referred to as 'TFT', those expressing wild-type caveolin-3 are referred to as 'WT', and those expressing the control vector are referred to as 'Control'.

Isolation of caveolae-enriched membrane fractions

Six 100 mm culture dishes were used for each sucrose gradient and fractionations were performed as described by Galbiati et al. (Galbiati et al., 1999b). Cells were washed twice in ice-cold phosphate-buffered saline (PBS) and scraped into a final volume of 2 ml morpholino-ethanesulfonic acid (MES)-buffered saline (1 M MES, 0.15 M NaCl) containing 1% Triton X-100. Lysates were passed through a Dounce homogenizer (10 strokes), placed in the bottom of a 12 ml ultracentrifuge tube, and an equal volume of 80% sucrose (in double-

distilled water) was added to a final concentration of 40% sucrose. Discontinuous sucrose gradients were created by overlaying the lysate with 4 ml of 30% and 4 ml of 5% sucrose. Samples were centrifuged at 200,000 ***g*** for 16 hours at 4°C in an SW41Ti swinging bucket rotor in an L-7 ultracentrifuge (Beckman Coulter Incorporated, Fullerton, CA). A buoyant layer formed at the 5%-30% sucrose interface that is known to contain lipid rafts, including the bulk of cellular caveolae (Galbiati et al., 1999b; Meacci et al., 2000). Gradients were separated into twelve 1 ml fractions from the top to the bottom of the gradient. The lipid raft fraction coincided with fractions 4-5. A 40 μl sample of each fraction was mixed with 10 μl of 5× Laemmli sample buffer and western blotting was performed as outlined below.

Albumin uptake

Myotubes were treated with Texas Red-conjugated albumin (Molecular Probes) at 20 μg/ml for 30 minutes. For immunofluorescence analysis of Texas Red uptake, cells were washed twice in ice-cold PBS, fixed in paraformaldehyde (4% w/v in PBS) for 10 minutes at room temperature, washed thoroughly, and mounted with Vectashield (Vector Laboratories, Burlingame, CA). Quantitative analysis of albumin uptake was determined by western blotting (see below) using an antibody directed against Texas Red (Molecular Probes).

Western blotting

Western blotting was performed as previously described (Disatnik et al., 2002). Cells were washed twice with ice-cold PBS, scraped into lysis buffer (150 mM NaCl, 50 mM Tris-HCl, 1% v/v NP-40, 0.5% w/v DOC, 1 mM EDTA, 1 mM EGTA) containing protease and phosphatase inhibitors. Samples were centrifuged to remove nuclei and insoluble debris and the protein content was determined by the Bio-Rad protein assay (Bio-Rad, Hercules, CA). The required amount of protein was mixed with sample buffer, denatured by boiling for 5 minutes, separated by SDS-PAGE, and transferred to a 0.45 μm nitrocellulose membrane (Osmonics Laboratory Products, Minnetonka, MN). Membranes were blocked in non-fat milk (5% w/v in PBS containing 0.5% Tween-20) and incubated in the primary antibody (diluted 1:500-1:2500 in 5% milk) overnight. Primary antibody binding was detected with either horseradish peroxidase-conjugated anti-mouse immunoglobulins, or horseradish peroxidase-conjugated anti-rabbit immunoglobulins diluted 1:5000 in PBS containing 1% BSA and 0.05% Tween 20 for 1 hour at room temperature with gentle agitation. The complex was detected by standard enhanced chemiluminescence.

Immunofluorescence

Myoblasts or myotubes grown on fibronectin-coated glass chamber slides were washed twice with cold PBS, fixed in paraformaldehyde (4% w/v in PBS) for 30 minutes, washed thoroughly in PBS, and permeabilized for 10 minutes in 0.1% Triton X-100 in PBS containing 1% BSA. Immunostaining was performed as previously described (Disatnik et al., 2000; Langenbach and Rando, 2002). Cells were then incubated overnight with anti-mouse caveolin-3 (Transduction Laboratories) or anti-mouse caveolin-1 (Santa Cruz Biotechnology) antibodies diluted 1:500 in 0.1% Triton X-100 in PBS containing 1% BSA at 4°C. Cells were washed thoroughly in three changes of PBS, incubated with the secondary antibody (anti-mouse Cy-3) diluted 1:500 in permeabilization solution for 2 hours at room temperature, washed three times in PBS, mounted with Vectashield (Vector Laboratories, Burlingame, CA) and coverslipped.

Isolation of nuclear proteins

Cells were washed twice in ice-cold PBS and scraped into hypotonic solution A [10 mM Hepes (pH 7.9), 10 mM KCl, 1.5 mM $MgCl_2$, 0.1 mM EGTA, 0.1 mM EDTA] containing protease and phosphatase inhibitors, and incubated on ice for 15 minutes. Cells were lysed by adding Triton X-100 to a final concentration of 1%, mixed thoroughly and centrifuged at 16,000 ***g*** for 30 seconds. The pellet was washed with hypotonic solution A, resuspended in hypotonic solution B [20 mM Hepes (pH 7.9), 25% glycerol, 0.4 M NaCl, 1 mM EDTA, 1 mM EGTA] containing protease and phosphatase inhibitors, homogenized with 20 strokes of a Dounce homogenizer, and centrifuged at 16,000 ***g*** for 15 minutes. The supernatant containing the bulk of solubilized nuclear proteins was removed to a fresh tube and the protein content was analyzed using the Bio-Rad protein assay. Expression of nuclear proteins was then analyzed by western blotting as described above.

TUNEL staining

Cells were washed twice in ice-cold PBS and fixed in 4% paraformaldehyde in PBS for 5 minutes followed by 5 minutes in ice-cold absolute methanol. Cells were permeabilized in 0.1% Triton X-100 in 0.1% sodium citrate for 5 minutes. TUNEL labeling was performed using the In Situ Cell Death Detection Kit (Roche Applied Science, Indianapolis, IN) as previously described (Langenbach and Rando, 2002). The percentage of myotubes undergoing apoptosis was calculated by counting the number of myotubes containing at least one apoptotic nucleus as a percentage of the total number of myotubes in a given field at 40× magnification. For each culture, at least five non-overlapping fields were counted, and for each cell line at least four cultures were analyzed. Data from all experiments were pooled and one-way analysis of variance was used to determine if differences between cell populations were statistically significant at the 0.05% confidence level using Minitab 5.0 software.

Immunoprecipitation

Cells were prepared as outlined above for western blotting. Immunoprecipitation was performed as previously described (Disatnik et al., 2002). The anti-Src antibody was added at a dilution of 1:100 and incubated with gentle agitation overnight at 4°C. Protein G-conjugated agarose beads (Santa Cruz Biotechnology) were added at a dilution of 1:2 and mixed gently for 2 hours at 4°C. Samples were then centrifuged at 200 ***g*** for 2 minutes, the supernatant was removed, the pellet was washed with cold lysis buffer and the samples were again centrifuged at 200 ***g*** for 2 minutes. The wash procedure was repeated twice. After the final wash, the pellet was resuspended in 30 μl 5× Laemmli sample buffer, denatured at 100°C for 5 minutes, and resolved by SDS-PAGE as described above.

Statistical analysis

The results presented are from at least four separate experiments. Data are presented as means±s.d. One-way analysis of variance was used for comparisons, and $P<0.05$ was considered statistically significant.

Results

Structurally, caveolin-3 is most closely related to caveolin-1 (85% similar, 65% identical). Caveolin-3 contains the characteristic 'caveolin signature sequence' (Fig. 1), which is conserved across all mammalian caveolin isoforms, in addition to a hydrophobic sequence that forms the putative intramembrane domain (Tang et al., 1996). Importantly, caveolin-3 contains a 20 amino acid sequence (55-74) that corresponds to a similar domain in caveolin-1 that is required for caveolin binding to signaling proteins including G-proteins, Src and H-Ras (Okamoto et al., 1998). The TFT caveolin-3 mutant that causes LGMD-1C affects this caveolin scaffolding domain (Fig. 1, bracket).

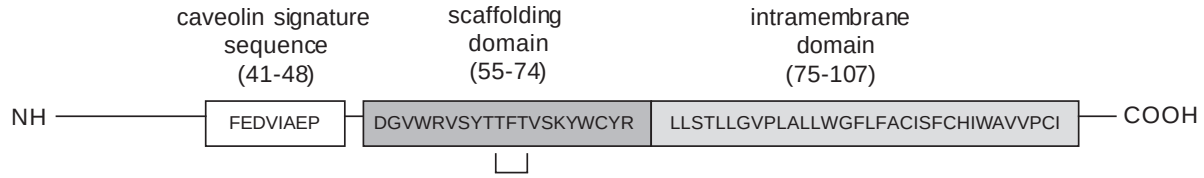

Fig. 1. Schematic of caveolin-3 protein. This protein has a caveolin signature sequence (conserved in all caveolin proteins), a scaffolding domain known to bind various signaling proteins and a hydrophobic intramembrane domain. The bracket within the scaffolding domain indicates the site of the TFT mutation known to cause LGMD-1C.

Expression of caveolin-3 in lipid rafts in myotubes expressing the TFT caveolin-3 mutant

We first characterized the localization of caveolin-3 protein in myoblasts and myotubes expressing the TFT mutation. Myoblasts were stably transfected with the pLXSN retrovirus encoding TFT mutant caveolin-3 cDNA. Since myoblasts do not express endogenous caveolin-3, myoblasts were transfected with wild-type caveolin-3 cDNA in order to compare the localization of mutant and wild-type protein (Fig. 2A). In myoblasts, wild-type caveolin-3 protein was localized predominantly at the plasma membrane, with some cytosolic and perinuclear expression, while mutant caveolin-3 protein was expressed exclusively in the perinuclear region (Fig. 2A). Myoblasts were then induced to undergo terminal differentiation and the localization of caveolin-3 was analyzed in multinucleated myotubes. When caveolin-3 was examined, it was found predominantly at the plasma membrane with some expression in the perinuclear and cytosolic compartments in control myotubes (Fig. 2A). In contrast, in myotubes expressing the TFT mutation, caveolin-3 was localized primarily within a perinuclear region consistent with the distribution of the Golgi complex, although a small amount of caveolin-3 was also observed at the plasma membrane (Fig. 2A). The expression of mutant caveolin-3 did not interfere with the process of myogenic differentiation since the formation of multinucleated myotubes was indistinguishable from that in control cells (Fig. 2A). Furthermore, the expression of a muscle-specific differentiation marker, skeletal muscle slow myosin, followed the same time course in cells expressing mutant caveolin-3 as in control cells transfected with the empty vector (Fig. 2B). Similar levels of myosin expression were observed during the differentiation of myoblasts transfected with wild-type caveolin-3 (data not shown). In all subsequent experiments, no differences were noted between cells transfected with wild-type caveolin-3 or with vector alone, indicating that the expression of caveolin-3 in myoblasts did not noticeably alter cellular properties related to growth or differentiation.

While endogenous caveolin-3 is absent in myoblasts and is upregulated upon differentiation and fusion, caveolin-1 is conversely expressed in myoblasts and downregulated during differentiation and fusion (Lee et al., 2002). Caveolin-3 exhibits highest sequence homology to the caveolin-1 isoform. Like caveolin-1, caveolin-3 expression at the plasma membrane is absolutely required for caveolae formation, while caveolin-2 does not target

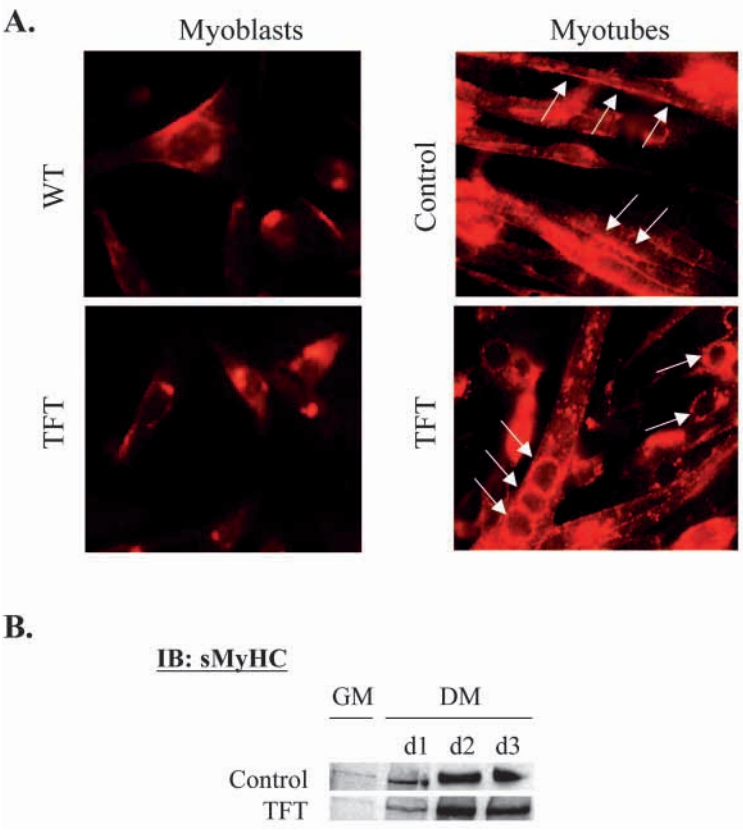

Fig. 2. Localization of wild-type and mutant caveolin-3 protein in myoblasts and myotubes. (A) Myoblasts were stably transfected with wild-type caveolin-3 (WT) or with the TFT mutant caveolin-3 (TFT). Caveolin-3 localization was assessed immunocytochemically in myoblasts or in myotubes. In myoblasts, the TFT mutant protein was exclusively perinuclear, whereas wild-type caveolin-3 was also membrane-associated and cytosolic. In control myotubes (derived from myoblasts transfected with vector alone), endogenous caveolin-3 was strongly expressed at the plasma membrane (arrows) with very limited intracellular and perinuclear expression. In contrast, in myotubes expressing the TFT mutation, caveolin-3 was most abundantly expressed in the perinuclear region (arrows), with only small amounts of protein present at the plasma membrane. (B) To test for any effects of the TFT mutation on the biochemical differentiation of myoblasts, cultures were analyzed by western blotting for the expression of the differentiation-specific protein, skeletal muscle slow myosin heavy chain (sMyHC). Control myoblasts (vector alone) and myoblasts expressing the TFT mutation were analyzed before differentiation with the cells maintained in GM, and on days 1, 2 and 3 of differentiation after the cells have been switched to DM. No differences in the induction of myosin expression were observed between the cell populations.

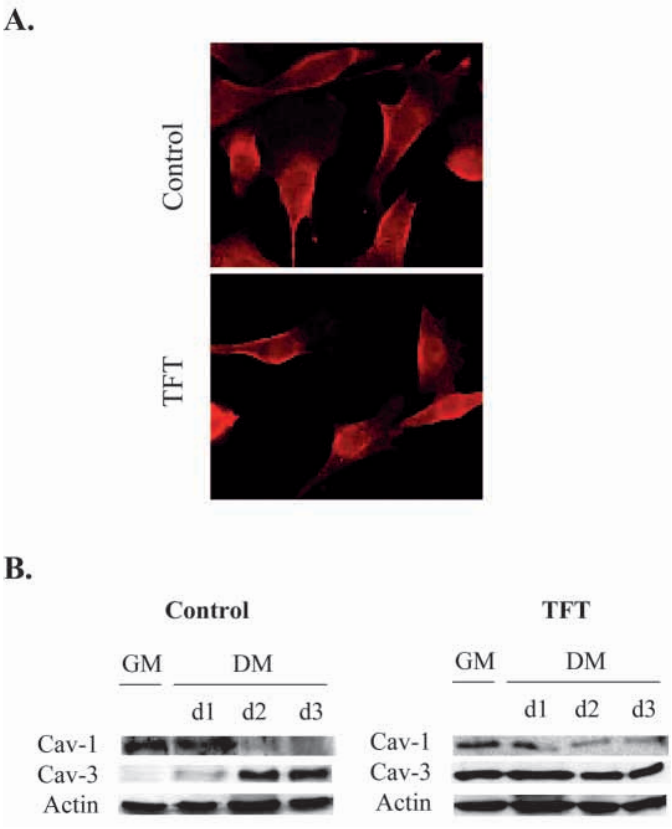

Fig. 3. Normal localization and expression patterns of caveolin-1 in the presence of the TFT mutant caveolin-3. (A) Myoblasts stably expressing the TFT mutation or vector alone (Control) were analyzed for localization of caveolin-1. Caveolin-1 localized almost exclusively to the plasma membrane and no differences between cell populations were observed. (B) The level of caveolin-1 and caveolin-3 expression was assessed by western blot analysis in myoblasts maintained in GM or undergoing differentiation in DM. Expression was analyzed 1, 2 and 3 days after the initiation of differentiation. In control cells and in cells expressing the TFT mutation, caveolin-1 was highly expressed in myoblasts and declined during myogenic differentiation. Expression of caveolin-3 increased with differentiation in control cells but was expressed at high levels throughout differentiation in cells constitutively expressing the TFT mutation. Actin was used as the loading control in all experiments.

to the plasma membrane in the absence of caveolin-1 (Schlegel and Lisanti, 2001). Therefore, we wanted to determine whether expression of the TFT mutation would alter the normal regulation of caveolin-1. In particular, we wanted to test whether there was a compensatory upregulation of caveolin-1 in muscle cells with impaired caveolin-3 expression and localization. In control myoblasts transfected with vector alone, caveolin-1 was almost exclusively localized to the plasma membrane and this was not affected by expression of the TFT mutation (Fig. 3A). Transfection of myoblasts with wild-type caveolin-3 also had no effect on caveolin-1 localization (data not shown). Furthermore, western blotting demonstrated that caveolin-1 expression was appropriately downregulated upon differentiation and fusion in both cell populations (Fig. 3B). These results demonstrate that caveolin-1 expression is not upregulated to compensate for the effects of the TFT mutation on caveolin-3 localization.

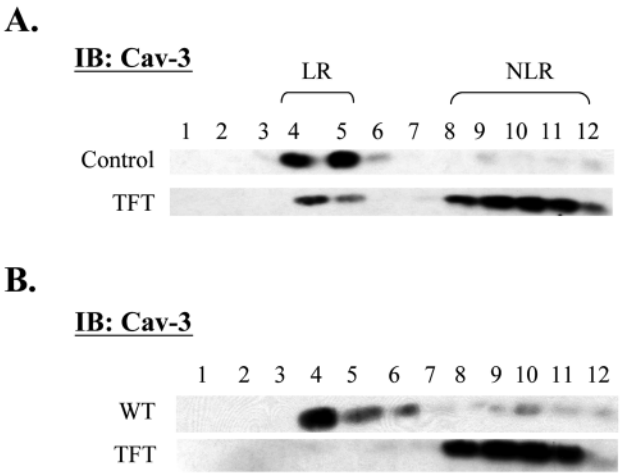

Fig. 4. Diminished incorporation of caveolin-3 into lipid rafts in myotubes expressing the TFT mutant caveolin-3. (A) Extracts of myotubes expressing vector alone (Control) or the TFT mutation were subjected to sucrose density gradient fractionation. 50 μl samples from each fraction (1-12) were analyzed by western blotting for expression of caveolin-3. In control myotubes, caveolin-3 was detected exclusively in the lipid raft (LR) fraction. In myotubes expressing the mutant caveolin-3, most of the caveolin-3 (endogenous plus mutant) was instead found in the non-lipid raft (NLR) fraction with only a small proportion present in the LR fraction. (B) Extracts of myoblasts expressing wild-type caveolin-3 or the TFT mutation were subjected to sucrose density gradient fractionation and analyzed for caveolin-3 expression as in A. Wild-type caveolin-3 preferentially localized to the LR fractions, while mutant protein was completely excluded from lipid rafts.

While it has previously been demonstrated that the TFT mutant caveolin-3 protein is excluded from lipid rafts in fibroblasts (Galbiati et al., 1999b), the effects of this mutation on incorporation of caveolin-3 into lipid rafts in muscle cells expressing endogenous caveolin-3 have not been examined. We were interested to determine the effect of the TFT mutation on the targeting of caveolin-3 to lipid rafts in these cells since some caveolin-3 is still targeted to the plasma membrane (and perhaps lipid rafts there) in the presence of the mutant protein (see Fig. 2A), and since it is well established that lipid rafts exist within the Golgi (Gkantiragas et al., 2001) where most caveolin-3 appears to be retained in cells expressing the mutation. We prepared lysates from myotubes and separated the lipid raft fraction using sucrose density gradients. In all samples, a buoyant layer containing lipid rafts formed at the 5%-30% sucrose interface, corresponding to fractions 4 and 5 of the gradient (Galbiati et al., 1999b). This fraction was referred to as the lipid raft fraction, while fractions 9-12, containing almost all remaining cellular protein, was referred to as the non-lipid raft fraction. Western blotting clearly demonstrated that endogenous caveolin-3 localized exclusively to the lipid raft fraction in control myotubes (Fig. 4A). However, in myotubes expressing the TFT mutation, there was an obvious dual localization, with the bulk of caveolin-3 (endogenous wild-type plus exogenous mutant) localizing to the non-lipid raft fraction while a small proportion remained in the lipid raft fraction (Fig. 4A). Thus, expression of the TFT mutation not only prevented localization of caveolin-3 to the plasma membrane but also markedly inhibited its incorporation

into lipid raft regions of membrane throughout the trafficking process.

To determine if mutant caveolin-3 protein is incorporated into the lipid raft fraction in the absence of endogenous caveolin-3, the TFT mutation was expressed in myoblasts and the lipid rafts were resolved on sucrose gradients. Western blot analysis showed that mutant caveolin-3 protein was localized exclusively to the non-lipid raft fraction (Fig. 4B). In contrast, myoblasts transfected with wild-type caveolin-3 showed that almost all caveolin-3 localized to the lipid raft fraction (Fig. 4B). We observed similar results in NIH3T3 fibroblasts transfected with wild-type or mutant caveolin-3 (data not shown), consistent with previous studies (Galbiati et al., 1999b). These results demonstrate that despite the presence of lipid rafts within the Golgi (Gkantiragas et al., 2001), the TFT mutant form is excluded from this compartment and markedly reduces the targeting of endogenous caveolin-3 there as well.

The TFT mutation causes diminished caveolar function at the plasma membrane

Since there is marked depletion of caveolin-3 from the lipid raft fraction in myotubes expressing the TFT mutation, we next examined the extent of loss of plasma membrane caveolar function in those cells. This was performed by assessing the ability of myotubes to endocytose albumin, a molecule known to be taken up primarily by caveolae (Schubert et al., 2001). Texas Red-conjugated albumin was added to the cell culture medium and myotubes were analyzed for the uptake of this marker. In control myotubes, Texas Red was readily taken up and distributed in punctate foci throughout the cytosol (Fig. 5A). In myotubes expressing the TFT mutation, by contrast, Texas Red uptake was extremely limited and discrete foci were only rarely detected within the cytoplasm (Fig. 5A). Western blot analysis of Texas Red levels in total cell lysates confirmed that albumin uptake was inhibited (Fig. 5B). Therefore, the decreased targeting of caveolin-3 to the plasma membrane and incorporation into lipid rafts in myotubes expressing the TFT mutation results in significantly impaired caveolar function.

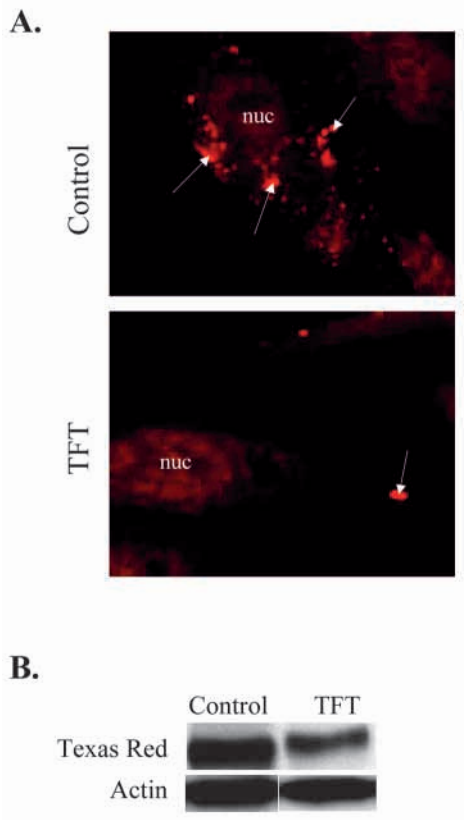

Fig. 5. Reduced albumin uptake in myotubes expressing the TFT mutant caveolin-3. (A) Control myotubes and myotubes expressing the TFT mutation were incubated with Texas Red-conjugated albumin (20 μg/ml) for 30 minutes and then analyzed by fluorescence microscopy for cellular uptake of Texas Red. In control myotubes, Texas Red was observed in foci throughout the cytosol (arrows) and surrounding the nucleus (nuc). In contrast, only occasional Texas Red-positive foci were observed in the cytosol of myotubes expressing the caveolin-3 mutant. (B) Control myotubes and myotubes expressing the TFT mutation were incubated with Texas Red-conjugated albumin as described above, and total cell lysates were analyzed by western blotting using an antibody against Texas Red. The levels of Texas Red were substantially lower in myotubes expressing mutant caveolin-3 compared with control myotubes. Actin was used as the loading control.

Disruption of interactions between Src and caveolin-3 by the caveolin-3 TFT mutation

Caveolin mutations cause diseases and disrupt normal caveolar function, and caveolae are central regulators of signal transduction pathways (Schlegel and Lisanti, 2001). Thus, it is important to determine the specific alterations in signaling pathways caused by caveolin-3 mutations. Members of the Src family of tyrosine kinases are associated with caveolae and bind the caveolin scaffolding domain, which inhibits their activities (Li et al., 1996). Since the Src kinases are involved in many signaling pathways associated with cell survival and cell death (Thomas and Brugge, 1997), we were interested to determine to what extent the normal association of Src with caveolin-3, and Src function, are altered by the expression of the TFT mutation.

We first examined the binding between Src and caveolin-3. After immunoprecipitating an equal amount of cellular Src from control myotubes and myotubes expressing the TFT mutation, the amount of caveolin-3 that was bound to Src was analyzed by western blotting. Although total cellular Src remained unchanged, there was a diminished association between Src and caveolin-3 in myotubes expressing the TFT mutation compared with control myotubes (Fig. 6A). The converse experiment, immunoprecipitating caveolin-3 and doing western blot analysis on associated Src, suggested the same result (data not shown) but was confounded by the fact that it was not possible to immunoprecipitate equal amounts of caveolin-3 from control myotubes compared with those overexpressing the mutant form of the protein.

The TFT mutation limits localization of Src to lipid rafts

Caveolae are considered to be important in regulating the subcellular localization of Src and other signaling molecules, an association that is mediated by caveolin proteins (Zajchowski and Robbins, 2002). Since the data presented above clearly demonstrate disrupted interactions between

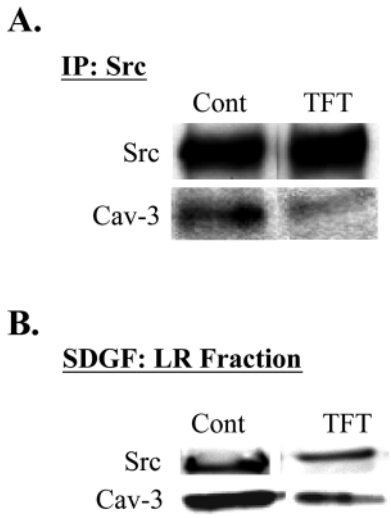

Fig. 6. Effects of the TFT caveolin-3 mutation on caveolin-3 binding to Src and targeting of Src to lipid rafts. (A) Equal amounts of protein from control myotubes and myotubes expressing the TFT mutation were used for immunoprecipitation of Src. The total immunoprecipitated samples were then analyzed by western blotting for levels of Src and caveolin-3. Even though the total levels of Src were similar in both cell populations, there was a decrease in associated caveolin-3 in myotubes expressing the TFT mutation compared to control myotubes. (B) In order to test for the effects of the TFT mutation on the targeting of Src to lipid rafts, extracts from control myotubes or myotubes expressing the TFT mutation were subjected to sucrose density gradient fractionation (SDGF) as in Fig. 4. For ease of analysis, fractions 4 and 5 were pooled from each gradient and referred to as the lipid raft (LR) fraction. An equal amount of protein (10 μg) from the LR fraction of each cell population was analyzed by western blotting for levels of Src and caveolin-3. Both Src and caveolin-3 levels were reduced in LR fractions from myotubes expressing the mutant caveolin-3.

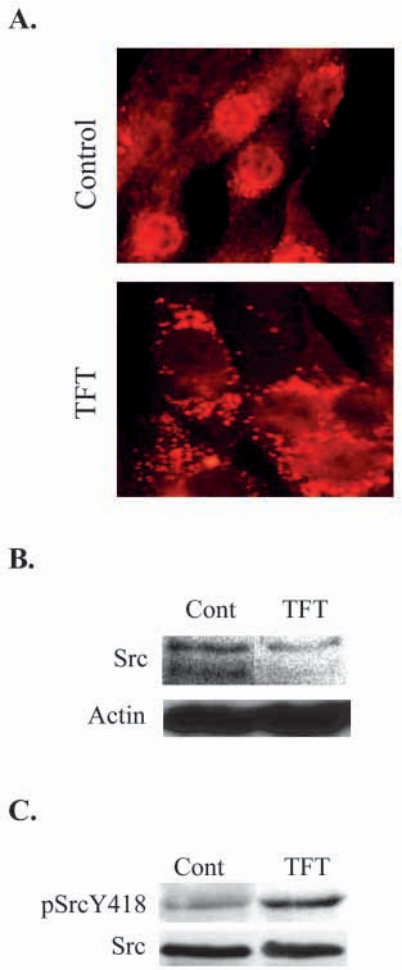

Fig. 7. Failed targeting of Src to the nucleus and altered Src activation in myotubes expressing the TFT mutant caveolin-3. (A) Control myotubes and myotubes expressing the TFT mutation were analyzed immunocytochemically for the cellular distribution of Src. While Src was present diffusely in the membrane and cytoplasm and concentrated in nuclei of control myotubes, it was not targeted to these cellular compartments but was instead retained in a perinuclear distribution in myotubes expressing the mutant caveolin-3. (B) The nuclear fraction from control myotubes and myotubes expressing the TFT mutation were analyzed by western blotting for expression of Src. There was clearly diminished Src targeting to the nuclei of mutant myotubes. Actin was used as the loading control. (C) Equal amounts of protein from total cell lysates of control myotubes and myotubes expressing the TFT mutation were analyzed by western blotting for phosphorylation of Src at tyrosine 418 (pSrcY418) and total Src. Hyperphosphorylation of Src at this residue was observed in myotubes expressing the TFT mutation compared with control myotubes, while total cellular Src did not differ between the two cell populations.

caveolin-3 and Src, we next examined the expression of Src in lipid rafts in control myotubes and myotubes expressing the TFT mutation. For ease of analysis, fractions 4 and 5 from each sucrose gradient were pooled (see Fig. 4A), and an equivalent quantity of protein from each myotube culture was analyzed by western blotting for expression of Src and caveolin-3. The levels of caveolin-3 in lipid rafts were significantly reduced in myotubes expressing the TFT mutation compared with the controls (Fig. 6B) as was shown in Fig. 4A. In these myotubes, there was reduced targeting of Src to the lipid raft fraction compared with control myotubes (Fig. 6B). Thus, the expression of the TFT mutation appeared to alter the targeting of Src to lipid rafts.

Despite the reduced targeting of Src to lipid rafts in cells expressing the TFT mutation, these cells still expressed a significant fraction of total cellular Src in that subcellular compartment (Fig. 6B). Given that lipid rafts are present in several membranous structures throughout the cell, we examined Src subcellular localization in more detail using immunofluorescence analysis. In control myotubes, Src was diffusely distributed to the membrane and cytoplasm and was also highly concentrated in nuclei (Fig. 7A). In contrast, in myotubes expressing the TFT mutation, Src accumulated almost entirely in the perinuclear region typical of distribution within the Golgi (Fig. 7A). Western blotting was used to confirm the reduced targeting of Src to nuclei in myotubes expressing the TFT mutation (Fig. 7B). Thus, expression of a dominant-negative caveolin-3 mutant resulted in an inhibition of normal Src trafficking and localization indicating that normal caveolin function is necessary for normal Src distribution and activity (and see below).

As stated previously, both increases and decreases of

activation of the Src signaling pathway can lead to apoptosis, depending on the cellular context (Thomas and Brugge, 1997). Since it is well-established that Src binding to the caveolin scaffolding domain inhibits Src tyrosine kinase activity (Li et al., 1996), we next examined whether the decreased binding of Src to caveolin-3 observed in myotubes expressing the TFT mutation led to an enhancement of Src activation. Activated Src was analyzed using an antibody specific for Src phosphorylated at tyrosine 418 (pSrcY418), since autophosphorylation at this site is known to be an absolute requirement for initiation of kinase activity (Thomas and Brugge, 1997). Western blotting clearly demonstrated that pSrcY418 expression was elevated in myotubes expressing the TFT mutation compared with control myotubes, while total cellular Src expression did not change (Fig. 7C). These results are consistent with the known inhibitory effect of binding of Src to the caveolin scaffolding domain and demonstrate hyperphosphorylation of Src when binding to caveolin-3 is disrupted in skeletal myotubes.

The TFT mutation causes an increased incidence of apoptosis

Since Src signaling is known to be involved in the regulation of cell survival (Thomas and Brugge, 1997), we examined whether there is an increase in cell death in myotubes expressing the TFT mutation in which Src localization and activity were disrupted. Muscular dystrophies, such as those caused by caveolin-3 mutations, are associated with both apoptotic and necrotic cell death (Tews, 2002). Myotube cultures were examined for evidence of apoptotic cell death at day 1, day 3 and day 6 of differentiation using a TUNEL assay. While there were no significant differences between the populations after 1 day of differentiation, there was a significant increase in TUNEL-positive nuclei in myotubes expressing the TFT mutation 3 and 6 days after the initiation of differentiation compared with control cells (Fig. 8). Over the same time period, there was no significant increase in apoptosis in control cultures compared with that on the first day of differentiation. This was true when the control cells were transfected with vector alone or with wild-type caveolin-3. Thus, the initiation of apoptotic signaling was dependent both on the expression of the mutant form of caveolin-3 and on the differention state of the cells.

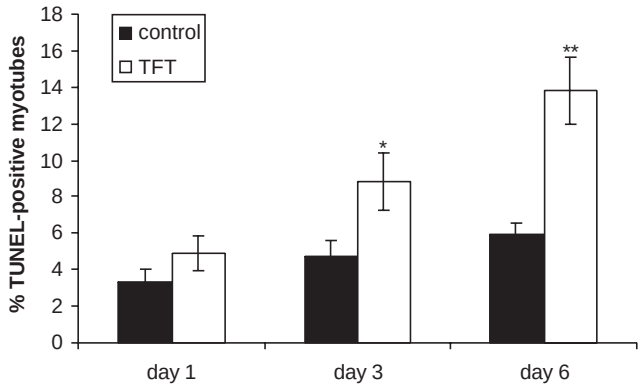

Fig. 8. The TFT mutant caveolin-3 causes an increased incidence of apoptosis in myotubes. Control myotubes and myotubes expressing the TFT mutation were stained at day 1, day 3 and day 6 of differentiation using the TUNEL assay. The percentages of myotubes containing TUNEL-positive nuclei were determined. At least four non-overlapping fields were counted for each culture, and four separate cultures for each cell population were analyzed. At day 3 and day 6 of differentiation, there were significantly more apoptotic TFT-expressing myotubes than apoptotic control myotubes. Data are presented as mean±s.d. At each time point, one-way analysis of variance was used to compare the data for each mutant to the control data (*P<0.05, **P<0.005).

Discussion

Behavior of caveolin-3 TFT mutation in primary myotubes

The results of this study demonstrate that expression of the TFT mutation causes an impairment of caveolar function, altered localization and activation of Src tyrosine kinase, and apoptotic death of differentiated muscle cells. In myotubes expressing the TFT mutation, the bulk of caveolin-3 protein localizes to the Golgi complex and there is diminished targeting of caveolin-3 to lipid rafts. This loss of targeting of caveolin-3 to the plasma membrane and incorporation into lipid rafts significantly affects caveolar function as demonstrated by poor uptake of fluorophore-conjugated albumin, a molecule known to be endocytosed by caveolae (Schubert et al., 2001). These results are consistent with previous studies that demonstrated a requirement for incorporation of caveolin protein into lipid rafts for the formation of caveolae at the plasma membrane (Galbiati et al., 2001; Park et al., 2002) and that a reduction of the number of functional caveolae at the cell surface causes a deficiency in albumin uptake (Schubert et al., 2001).

The mechanisms by which the TFT mutation causes reduced targeting of caveolin-3 protein to the plasma membrane and diminished incorporation into lipid rafts are unclear. It has been demonstrated that the TFT mutation causes misfolding of the caveolin-3 protein (Galbiati et al., 2000) and conformational changes that affect oligomerization and subcellular targeting (Jagannadham et al., 2002). Our results demonstrate that this mutant form of the caveolin-3 protein expressed in the absence of endogenous caveolin-3 was completely excluded from lipid rafts, consistent with studies in non-muscle cells (Galbiati et al., 1999b). It is known that the TFT mutant protein forms unstable, high molecular mass oligomers (Galbiati et al., 1999b), so one possibility is that the mutant proteins oligomerize with endogenous caveolin-3 to have the dominant effect that characterizes the inheritance pattern of the associated form of muscular dystrophy.

We observed no effects of the caveolin-3 mutation on the expression and localization of caveolin-1 in myoblasts or on the downregulation of caveolin-1 expression during myoblast differentiation and fusion (Fig. 3). This indicates that caveolin-1 expression is not upregulated to compensate for the failure of caveolin-3 to reach normal expression levels and appropriate localization at the plasma membrane. Furthermore, we have not observed any effects on the process of differentiation of myoblasts expressing the TFT mutation. Galbiati and colleagues found that an antisense-mediated down-regulation of caveolin-3 did not affect the biochemical differentiation of cells of the C2C12 muscle cell line but did inhibit myoblast

fusion (Galbiati et al., 1999a). These results are intriguing, but it is clear that myoblast fusion proceeds normally in the absence of caveolin-3 since muscle development is normal in the caveolin-3-deficient mouse (Hagiwara et al., 2000) and in patients with functional deficiency of caveolin-3 by virtue of the expression of the TFT dominant mutation (Minetti et al., 1998). Our findings of normal myogenic differentiation in vitro in the presence of the caveolin-3 mutant protein were obtained using myoblasts derived from freshly dissociated primary cultures. Thus the dissociation of biochemical and morphological differentiation in caveolin-3-deficient C2C12 myoblasts found by Galbiati et al. might be due to unique properties of that cell line or the clones selected (Galbiati et al., 1999a) and are not likely to reflect a general effect of caveolin-3 deficiency on muscle differentiation.

LGMD-1C mutation causes altered localization and function of Src

Insertion of caveolin proteins into cholesterol- and sphingolipid-rich lipid rafts within cellular membranes results in the formation of caveolae. These microdomains are extremely rich in signaling molecules and there is evidence that they are important in regulating the subcellular localization, trafficking, and activity of these molecules (Zajchowski and Robbins, 2002). The results of the present study demonstrate a striking effect of the TFT mutation on Src localization and activation. We observed a remarkably similar perinuclear accumulation of Src in myotubes expressing the TFT mutation to what Sotgia and colleagues (Sotgia et al., 2002) found when they examined the localization of Src and GPI-anchored proteins in fibroblasts derived from caveolin-1-null mice. Even though Src normally interacts directly with the caveolin scaffolding domain (Li et al., 1996), either the absence of caveolin proteins or the expression of a dominant negative mutant of caveolin-3 results in a similar mis-localization of Src. The pattern of expression of mis-localized Src in myotubes expressing the TFT mutation closely corresponds to the localization of caveolin-3 in these cells, suggesting that Src is also retained within the Golgi. Together, these data suggest that normal caveolin expression is required for normal trafficking and subcellular localization of signaling molecules and that it is a functional deficiency in cells expressing the mutations, rather than a direct binding and retention, that results in Src localization in perinuclear distribution.

The TFT mutation caused significantly increased Src phosphorylation. It is probable these changes are the direct result of decreased binding of Src to caveolin-3 in the presence of the mutant. The specific mutation not only affects the caveolin scaffolding domain, but it also overlaps with a sequence of four amino acids that is crucial to binding many signaling molecules, including Src (Couet et al., 1997). Furthermore, the mutant protein interacts directly with normal caveolin-3, causing retention of caveolin-3 to the Golgi where it forms unstable, high molecular mass aggregates (Galbiati, Volonte et al., 1999b). This would obviously preclude normal caveolin activity (such as binding and inhibition of Src activation) of the wild-type protein and explains the dominant phenotype of LGMD-1C. That some function of the mutant protein is conserved, despite its instability and susceptibility to proteasomal degradation (Galbiati et al., 2000), is indicated by the fact that the TFT mutant protein undergoes normal palmitoylation when expressed in NIH3T3 fibroblasts (Galbiati et al., 1999b). Palmitoylation is one of several post-translational modifications of caveolin that are required for its binding to Src (Song et al., 1997; Lee et al., 2001).

Effects of the LGMD-1C mutation on Src binding to caveolin-3 and targeting to lipid rafts

Caveolin-3 acts as the major scaffolding protein for Src within lipid rafts in caveolae where this interaction regulates Src activation (Li et al., 1996). In myotubes expressing the TFT mutation, Src accumulation within lipid rafts is significantly reduced, and this correlates directly with the decreased co-immunoprecipitation of Src and caveolin-3 in these cells, indicative of a decreased binding. We have observed that, in resting myotubes, activated Src is almost undetectable in lipid rafts, and that caveolin-3 fails to co-immunoprecipitate with activated Src (data not shown). There is increasing evidence that the interaction and regulation of caveolin proteins with signaling molecules is extremely complex and overlaps with the role of caveolins in cholesterol trafficking (Incardona and Eaton, 2000; Ikonen and Parton, 2000). Cholesterol content in caveolae and its binding to caveolins are likely to be involved in the lipid modifications required for signaling proteins to bind to the caveolin scaffolding domain. Therefore, the possibility that altered intracellular cholesterol content and localization are involved in the disruption of Src-caveolin dynamics cannot be excluded. Taken together, these results further support a crucial role for caveolins not only in regulating Src activity, but also in controlling its subcellular localization. While the downstream effects of abnormal Src hyperactivation in myotubes are currently unclear, this is likely to have a strong influence on the outcome of intracellular signals transduced by Src activation.

LGMD-1C mutation causes an increased susceptibility to apoptosis

Apoptosis is involved in the progression of several myopathies and muscular dystrophies (Tews, 2002). While this has not been examined in human LGMD-1C resulting from the TFT mutation, a recent study reported the upregulation of certain apoptosis-related genes in transgenic mice expressing a different caveolin-3 mutation that also causes LGMD-1C (Sunada et al., 2002). Our results clearly show that myotubes expressing the TFT mutation have a two- to threefold increased incidence of apoptosis compared with normal myotubes. Interestingly, we also observed increased activation of Src in myotubes expressing the TFT mutation, as demonstrated by analysis of phosphorylation of Src at tyrosine-418, an autophosphorylation site required for Src kinase activity (Thomas and Brugge, 1997). This increase in Src activity in cells expressing the mutation is predicted since the binding to caveolin-3 is reduced (Fig. 6A) and the binding of Src to caveolins inhibits its activity (Li et al., 1996). Although the regulation of cell survival and cell death by the activation of Src is complex and depends upon the specific cellular context, it is clear that Src activation can promote apoptotic cell death as seen in myotubes expressing the TFT mutation (Thomas and

Brugge, 1997). However, in addition to Src, numerous other cell survival signaling pathways are associated with caveolae and are likely to be disrupted by the expression of caveolin-3 mutants. There is a growing body of literature demonstrating that alterations of normal signal transduction pathways play critical roles in the pathogenesis of various muscular dystrophies (Rando, 2001), but there is no evidence that a single pathway predominates or that there is a final common pathway for cell death in the dystrophies. Our data demonstrate that expression of a mutant caveolin-3 protein associated with LGMD-1C leads to the disruption of Src localization and activity in muscle cells. That same mutation induces apoptotic myotube death. Further studies will be necessary to examine the interactions among the various signal transduction pathways associated with caveolae to determine the relative contributions of these pathways to the pathogenetic mechanisms of muscular dystrophies associated with caveolin-3 mutations.

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