Expression of Caveolin-3 in Skeletal, Cardiac, and Smooth Muscle Cells

Caveolin, a 21–24-kDa integral membrane protein, is a principal component of the caveola membrane. Recently, we and others have identified a family of caveolin-related proteins; caveolin has been termed caveolin-1. Caveolin-3 is most closely related to caveolin-1, but caveolin-3 mRNA is expressed only in muscle tissue types. Here, we examine (i) the expression of caveolin-3 protein in muscle tissue types and (ii) its localization within skeletal muscle fibers by immunofluorescence microscopy and subcellular fractionation. For this purpose, we generated a novel monoclonal antibody (mAb) probe that recognizes the unique N-terminal region of caveolin-3, but not other members of the caveolin gene family. A survey of tissues and muscle cell types by Western blot analysis reveals that the caveolin-3 protein is selectively expressed only in heart and skeletal muscle tissues, cardiac myocytes, and smooth muscle cells. Immunolocalization of caveolin-3 in skeletal muscle fibers demonstrates that caveolin-3 is localized to the sarcolemma (muscle cell plasma membrane) and coincides with the distribution of another muscle-specific plasma membrane marker protein, dystrophin. In addition, caveolin-3 protein expression is dramatically induced during the differentiation of C2C12 skeletal myoblasts in culture. Using differentiated C2C12 skeletal myoblasts as a model system, we observe that caveolin-3 co-fractionates with cytoplasmic signaling molecules (G-proteins and Src-like kinases) and members of the dystrophin complex (dystrophin, α-sarcoglycan, and β-dystroglycan), but is clearly separated from the bulk of cellular proteins. Caveolin-3 co-immunoprecipitates with antibodies directed against dystrophin, suggesting that they are physically associated as a discrete complex. These results are consistent with previous immunoelectron microscopic studies demonstrating that dystrophin is localized to plasma membrane caveolae in smooth muscle cells.

Caveolae are small bulb-shaped invaginations located at or near the cell surface (1–5). They represent a microdomain or subcomponent of the plasma membrane (3–5). Recent biochemical and morphological studies have implicated caveolae in a subset of transmembrane signaling events, including G-protein-coupled signaling (reviewed in Refs. 4 and 5). Caveolin, a 21–24-kDa integral membrane protein, is an important structural and regulatory component of caveolae membranes (6–11). Using both Triton-based methods (12–17) and detergent-free methods (18, 19), caveolin co-purifies with a number of lipid-modified cytoplasmic signaling molecules, including G-proteins (α and βγ subunits), protein kinase Ca, Src-family tyrosine kinases, and Ras proteins. Based on these observations, we have proposed the “caveola signaling hypothesis” which states that compartmentalization of certain cytoplasmic signaling molecules within caveolae membranes could allow rapid and efficient coupling of activated receptors to more than one effector system (4, 12).

Caveolin may act as a scaffolding protein within caveolae membranes (20). Caveolin forms high molecular mass homooligomers (14–16 monomers per oligomer) (20–22), and these caveolin homooligomers have the capacity to bind cholesterol (22, 23) and self-associate into larger structures that resemble caveolae (20). Within these structures, individual caveolin homo-oligomers (~4–6 nm) apparently pack side by side to form 25–50-nm structures that are the same size as caveolae (20). This would provide a “caveolin platform or scaffold” for the recruitment of caveolin-interacting proteins, such as G sub-units and Ras proteins, to caveolin-rich areas of the plasma membrane (20). In accordance with this idea, recombinant caveolin is sufficient to recruit nonlipid modified forms of G sub-units and Ras onto membranes both in vitro and in vivo (19, 22). These structural properties of caveolin are also consistent with a role for caveolin in orchestrating the formation of caveolae microdomains.

Additional evidence suggests that expression of caveolin is sufficient to form caveola. Caveolin mRNA and protein expression levels are highest in cell types that contain numerous caveolae, i.e. adipocytes, endothelial cells, smooth muscle cells, and fibroblasts (9, 13). Caveolin expression levels directly correlate with the morphological appearance of caveolae: (i) caveolin and caveolae are both induced ~10–25-fold...
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during the differentiation of 3T3-L1 fibroblasts to the adipocyte form (24–26), and (ii) caveolin levels are dramatically reduced and caveolae are morphologically absent in NIH 3T3 cells transformed by various activated oncogenes (v-akt, activated ras, and others) (27). Furthermore, recombinant expression of caveolin in caveolin-negative cell lines results in the correct targeting of caveolin to caveola-enriched membrane fractions (28) and drives the de novo formation of caveolae (29). These results indicate that caveolin represents an important structural protein for directing the formation of caveolae membranes.

However, there are certain cell lines which morphologically contain caveolae, but fail to express caveolin (12). This finding has suggested that other caveolin-related proteins may exist that are immunologically distinct from caveolin. In support of this notion, two novel caveolin-related proteins have recently been identified and cloned. These proteins, termed caveolin-2 and caveolin-3, are the products of separate caveolin genes (26, 30, 31). Thus, caveolin (referred caveolin-1) is the first member of a multigene family (26).

Caveolin-3 is most closely related to caveolin-1 based on protein sequence homology; caveolin-1 and caveolin-3 are ~65% identical and ~85% similar. (See Tang et al. (30) for an alignment.) However, caveolin-3 mRNA is expressed predominantly in muscle tissue types (skeletal muscle, diaphragm, and heart) (30). Identification of a muscle-specific member of the caveolin gene family may have implications for understanding the role of caveolin in different muscle cell types (smooth, cardiac, and skeletal), as previous morphological studies have demonstrated that caveolae are abundant in these cells. This appears relevant to the pathogenesis of Duchenne’s muscular dystrophy. More specifically, dystrophin has been localized to plasma membrane caveolae in smooth muscle cells using immunoelectron microscopy techniques (32), and skeletal muscle caveolae undergo characteristic changes in their size and distribution in patients with Duchenne’s muscular dystrophy, but not in other forms of neuromuscularly based muscular dystrophies examined (33). This indicates that muscle cell caveolae may play an important role in muscle membrane biology.

Here, we (i) characterize the protein expression of caveolin-3 in skeletal, cardiac, and smooth muscle cells and (ii) report its co-localization, co-fractionation, and co-immunoprecipitation with dystrophin, a known muscle-specific caveolar marker protein.

**EXPERIMENTAL PROCEDURES**

Materials—The cDNAs for caveolin-1, caveolin-2, and caveolin-3 were as described previously (12, 26, 30). Antibodies and their sources were as follows: anti-caveolin-1 IgG (mAb 12297; gift of Dr. J. O. R. Glenney, Transduction Labs); anti-myc epitope IgG (mAb 9E10; Santa Cruz Biotech); anti-dystrophin (rabbit polyclonal; gift of Drs. Louis Kunkel and Elizabeth McNally, Children’s Hospital, Boston, MA); anti-β-dystroglycan (mAb 43DAG18DS; Novocastra Laboratories); anti-α-sarcoglycan (Adhalin; mAb Ad1/20A6; Novocastra Laboratories); anti-G41 (Dupont NEN); anti-G2 (Transduction Labs); anti-Src (Oncogene Sciences); anti-Lyn (Santa Cruz Biotech); anti-glutathione S-transferase (Santa Cruz Biotech). A rabbit polyclonal antibody directed against the C-terminal 44 amino acids of caveolin-1 (residues 135–178) was as characterized previously (28). This antibody specifically recognizes both α- and β-isoforms of caveolin-1, but does not recognize caveolin-2 or caveolin-3. A variety of other reagents were purchased commercially: fetal bovine serum (JRH Biosciences); prestained protein markers (Life Technologies, Inc.); Slow-fade anti-fade reagent (Molecular Probes, Eugene, OR).

Hybridoma Production—A monoclonal antibody to caveolin-3 was generated by multiple immunizations of Balb/c female mice with the synthetic peptide TEEHTDLEARIKIDCHICELD. This peptide corresponds to amino acids 3–24 of the rat caveolin-3 protein sequence. Mice showing the highest titer of anti-caveolin-3 immunoreactivity were used to create fusions with myeloma cells using standard protocols (34). Positive hybridomas were cloned twice by limiting dilution and injected into mice to produce ascites. The ascites were purified by immunoaffinity chromatography on protein A-Sepharose. These antibodies were produced in collaboration with Drs. Roberto Campos-Gonzalez and J. R. Glenney, Jr. (Transduction Laboratories, Lexington, KY).

Transient Expression of Caveolin Genes in COS-7 Cells—Constructs encoding C-terminally myc-epitope tagged forms of caveolin-1, caveolin-2, or caveolin-3, were as described previously (26, 28, 30). These constructs (~5–10 μg) were transiently transfected into COS-7 cells by the DEAE-dextran method (35). Forty-eight hours post-transfection, cells were scraped into lysis buffer (20 mM Tris, pH 8.0, 150 mM NaCl, 1% Triton X-100). Recombinant expression was analyzed by SDS-PAGE (35% acrylamide) followed by Western blotting. Epitope-tagged forms of caveolin-1, caveolin-2, and caveolin-3 were detected using the monoclonal antibody, 9E10, that recognizes the myc-epitope (EQKLISEEDLN).

Tissue Western—Approximately 200 mg of various mouse tissues were lysed in immunoprecipitation buffer and homogenized on ice with a Polytron tissue grinder, as described elsewhere (25). Equal amounts (100 μg protein) were loaded on an SDS-PAGE gel (12% acrylamide). After transfer to nitrocellulose, the blot was probed with antibodies directed against caveolin-1 and caveolin-3.

Immunofluorescence—Mouse skeletal muscle (psos) was excised and incubated for 30 min in 0.1% Triton X-100 in EAGTA-Ringer’s solution (100 mM NaCl, 2 mM KCl, 2 mM MgCl2, 6 mM potassium phosphate, 1 mM EGTA, 0.1% glucose). The tissue was further incubated in this solution with the addition of 1 mM ATP. The muscle samples were then fixed in 4% paraformaldehyde/PBS, rinsed in PBS, and cryoprotected in 0.6 M sucrose, PBS prior to freezing them in an isopentane–liquid nitrogen bath. Frozen sections (5 μm thick) were extracted in ice-cold acetone for 2 min and rinsed in PBS prior to antibody staining. To prevent nonspecific binding, the sections were preincubated in 3% BSA in PBS for 1 h at 37°C. Mouse monoclonal anti-caveolin-3 and rabbit polyclonal dystrophin antibodies were then applied to the sections and incubated in a similar fashion. Sections were then washed with three changes of PBS, 0.1% Triton X-100. Binding of the primary antibodies was detected by incubating the sections for 1 h at 37°C, with fluorescein-conjugated goat anti-mouse IgG and lissamine rhodamine-conjugated donkey anti-rabbit IgG. Sections were finally washed as above and mounted in 1:1 normal glycerol in 90% glycerol. All samples were examined and photographed using a Zeiss Axioskop microscope with 40× and 63× objectives.

Cell Culture—C2C12–3 cells (36) were derived from a single colony of C2C12 cells (37) cultured at clonal density and display a more stable phenotype than the parental cell line. C2C12–3 myoblasts were cultured as described elsewhere (36). Briefly, proliferating C2C12–3 cells were cultured in high mitogen medium (Dulbecco’s modified Eagle’s medium containing 15% fetal bovine serum and 1% chicken embryo extract) and induced to differentiate at confluence in low mitogen medium (Dulbecco’s modified Eagle’s medium containing 3% horse serum). Overt differentiation was indicated by the assembly of multineucleated syncytia, which commenced 36–48 h after the cells were switched to low mitogen media.

Cell Fractionation—Differentiated C2C12 cells grown to confluence in 150-mm dishes were prepared to caveolin-enriched membrane fractions, essentially as we described previously for other cell lines (12, 13, 25, 28, 38, 39). However, two specific modifications were introduced. First, we used the purification of the caveolin-rich domains without the use of detergent (19). Triton X-100 was replaced with sodium carbonate buffer and a sonication step was introduced to more finely disrupt cellular membranes (19). After two washes with ice-cold PBS, differentiated C2C12 cells (two confluent 150-mm dishes) were scraped into 2 ml of 500 mM sodium carbonate, pH 11.0. Homogenization was carried out sequentially in the following order using (i) a Polytron homogenizer (10 strokes); (ii) a Polytron tissue grinder (three 10-s bursts; Kinematica GmbH, Brinkmann Instruments, Westbury, NY); and (iii) a sonicator (three 20-s bursts; Branson Sonifier 250, Branson Ultrasonic Corp., Danbury, CT). The homogenate was then adjusted to 15% sucrose by addition of 2 ml of 90% sucrose prepared in MBS (25 mM Mes, pH 6.5, 0.15 M NaCl) and placed at the bottom of an ultracentrifuge tube. A ~35% discontinuous sucrose gradient was formed above (4 ml of 5% sucrose/4 ml of 35% sucrose); both in MBS containing 250 mM sodium carbonate) and centrifuged at 39,000 rpm for 16 h in an
RESULTS

Characterization of a mAb Probe Specific for Caveolin-3—Caveolin-1, -2, and -3 are distinct gene products with different molecular masses, all in the range of ~18–24 kDa. C-terminally myc-tagged forms of caveolin-1, caveolin-2, and caveolin-3 were transiently expressed in COS-7 cells. Lysates were generated and used to determine the specificity of caveolin antibody probes by immunoblotting. As a control for equal loading, immunoblotting was first performed with mAb 9E10 that recognizes the myc-epitope; this antibody reveals all three myc-tagged caveolin gene products (left panel). Note that mAb 2297 recognizes only caveolin-1 (middle panel), while a mAb generated against an N-terminal peptide that is unique to caveolin-3 recognizes only caveolin-3 (right panel).

Immunoblotting of Gradient Fractions—From the top of each gradient, 1-ml gradient fractions were collected to yield a total of 13 fractions. As shown previously, caveolin-1 migrates mainly in fractions 5 and 6 of these sucrose density gradients (13, 19, 25, 28, 38, 39). Gradient fractions were separated by SDS-PAGE (15% acrylamide) and transferred to nitrocellulose. After transfer, nitrocellulose sheets were stained with Ponceau S to visualize protein bands and subjected to immunoblotting. For immunoblotting, incubation conditions were as described by the manufacturer (Amersham Corp.), except we supplemented our blocking solution with both 1% BSA and 1% non-fat dry milk (Carnation).

Immunoprecipitation—Immunoprecipitations were carried out using protein A-Sepharose CL-4B (Pharmacia Biotech Inc.) as described previously (40). Briefly, differentiated C2C12 cells were lysed in a buffer containing 10 mM Tris, pH 8.0, 0.15 M NaCl, 60 mM octyl glucoside and subjected to immunoprecipitation with specific rabbit polyclonal IgG directed against dystrophin, glutathione S-transferase (an irrelevant rabbit polyclonal IgG), or protein A-Sepharose alone. After extensive washing, samples were separated by SDS-PAGE (15% acrylamide) and transferred to nitrocellulose. Blots were then probed with a mAb directed against caveolin-3.

Cardiac Myocytes and Smooth Muscle Cells—Rat cardiac myocytes were the generous gift of Drs. Douglas Sawyer, Thomas W. Smith, and Ralph A. Kelley (Brigham and Women’s Hospital, Division of Cardiology, Harvard Medical School, Boston, MA) (41). Rat aortic smooth muscle cells were the generous gift of Dr. Lee Graves (University of North Carolina, Chapel Hill, NC) and were isolated and characterized as described previously (42).

Characterization of a mAb Probe Specific for Caveolin-3—Caveolin-1, -2, and -3 are distinct gene products with different molecular masses, all in the range of ~18–24 kDa. Currently, there are no available antibody probes that selectively recognize caveolin-3. Comparison of the protein sequences of caveolin-1, caveolin-2, and caveolin-3 reveals that these proteins differ most significantly within their extreme N termini. (See Tang et al. (30) for an alignment.) Thus, a peptide derived from the unique N-terminal sequence of caveolin-3 was used to generate a caveolin-3-specific monoclonal antibody probe.

Fig. 1 (right panel) demonstrates the specificity of this novel mAb probe; it selectively recognizes caveolin-3, but does not recognize caveolin-1 or caveolin-2. In addition, mAb 2297 originally generated against caveolin-1 recognizes only caveolin-1 (Fig. 1, middle panel). Thus, these two selective mAb probes can be used to study the function and differential expression of distinct caveolin gene family members. Antibodies for caveolin-2 are not yet available; however, caveolin-1 and caveolin-2 have the same tissue distribution as revealed by Northern analysis (26).

Muscle-Specific Expression of the Caveolin-3 Protein—Fig. 2 (upper panel) shows the tissue distribution of the caveolin-3 protein. Caveolin-3 expression is detected only in muscle tissue types, i.e., heart and skeletal muscle. The tissue distribution of caveolin-1 is shown for comparison (Fig. 2, lower panel). In striking contrast, caveolin-1 is most abundant in adipose tissue; little or no caveolin-1 protein is expressed in muscle tissues. The low levels of caveolin-1 expression detected in skeletal muscle tissue derive from endothelial cells which line the blood vessels, but not skeletal muscle cells themselves (30). Thus, it appears that tissue-specific expression of caveolin-3 may be important for the functioning of muscle cells. This muscle-specific expression of caveolin-3 protein is in accordance with previous Northern analysis indicating that caveolin-3 mRNA is expressed only in muscle tissue types (30, 31).

Immunolocalization of Caveolin-3 in the Sarcotela of Skeletal Muscle Fibers—Fig. 3 shows the localization of caveolin-3 in skeletal muscle fibers. Caveolin-3 immunostaining is confined to the plasma membrane (i.e., the sarcotela) of these muscle cells. To confirm the nature of this localization, we performed double-labeling studies with antibodies to another muscle-specific plasma membrane protein, dystrophin. Our results indicate that caveolin-3 and dystrophin co-localize to the sarcotela (Fig. 3A). In addition, closer inspection of tange-
tial sections revealed punctate immunostaining for both caveolin-3 and dystrophin that closely coincided, suggesting that they co-localize to the same microdomains of the plasma membrane (Fig. 3B). In support of these findings, recent immunoelectron microscopy studies have directly localized dystrophin to caveolae in smooth muscle cells (32).

Up-regulation of Caveolin-3 Expression during the Differentiation of C2C12 Skeletal Muscle Myoblasts: Co-fractionation with Dystrophin and Dystrophin-associated Glycoproteins—Cultured C2C12 cells offer a convenient system to study skeletal myoblast differentiation. These cells can be induced to differentiate from myoblasts into myotubes bearing an embryonic phenotype in low mitogen medium over a period of 2 days (36, 37). Fig. 4 shows that caveolin-3 protein was undetectable in precursor myoblasts and strongly induced during myoblast differentiation. In contrast, no caveolin-1 expression was detected in either precursor myoblasts or differentiated myotubes (not shown). These results are consistent with the selective expression of caveolin-3 in skeletal muscle and other muscle tissues (Figs. 2 and 3) and suggest that caveolin-3 may function in muscle from the earliest stages of its development.

We next used differentiated C2C12 cells to examine the subcellular distribution of caveolin-3 and its potential association with other proteins. To separate C2C12 membranes enriched in caveolin-3 from the bulk of cellular membranes and cytosolic proteins, an established equilibrium sucrose density gradient system was employed (12, 13, 25, 26, 28, 30, 38, 39). In this detergent-free fractionation scheme (19), immunoblotting with anti-caveolin-3 IgG can be used to track the position of caveolae-derived membranes within these bottom-loaded sucrose gradients. Fig. 5 illustrates that in this fractionation scheme ~90–95% of caveolin-3 (fractions 5 and 6) is separated from the bulk of cellular proteins. In addition, caveolin-3 co-fractionated with known caveolin-1-associated proteins, such as G protein subunits and Src-family tyrosine kinases (Fig. 5, lower panels). This is in accordance with the idea that in muscle tissues caveolin-3 might subsume the functional role of caveolin-1 (30).

As previous immunoelectron microscopy studies have demonstrated that dystrophin is selectively localized to plasma membrane caveolae in smooth muscle cells (32), we also examined the distribution of dystrophin and dystrophin-associated glycoproteins (α-sarcoglycan/β-dystroglycan) in these sucrose density gradients. Fig. 6 shows that dystrophin and these dystrophin-associated glycoproteins also co-fractionate with caveolin-3. This is consistent with results demonstrating the co-localization of caveolin-3 and dystrophin in intact skeletal muscle fibers by fluorescence microscopy (Fig. 3).

To further examine whether caveolin-3 and dystrophin are physically associated as a discrete complex, co-immunoprecipitation experiments were performed. Lysates from differentiated C2C12 were immunoprecipitated with anti-dystrophin IgG, an irrelevant IgG or protein A-Sepharose beads alone.
Immunoprecipitates were then subjected to immunoblot analysis with a mAb directed against caveolin-3. Fig. 7 shows that caveolin-3 specifically co-immunoprecipitates with dystrophin; little or no caveolin-3 was found associated with an irrelevant IgG or beads alone.

Expression of Caveolin-3 in Cardiac Myocytes and Smooth Muscle Cells—Primary cultures of cardiac myocytes and smooth muscle cells were used to further examine the muscle-specific expression of caveolin-3. Fig. 8 shows the differential expression of caveolin-1 and caveolin-3 in these cell types. While only caveolin-3 is expressed in cardiac myocytes (left panel), both caveolin-1 and caveolin-3 are co-expressed in smooth muscle cells (right panel). Thus, it appears that striated muscle cell types (cardiac myocytes and skeletal muscle fibers) express predominantly caveolin-3.

Discussion

Duchenne and Becker muscular dystrophies result from mutations in the dystrophin gene (43). Dystrophin is a high molecular mass cytoskeletal protein that co-purifies with a complex of dystrophin-associated proteins which are thought to anchor dystrophin to the cytoplasmic face of the muscle cell plasma membrane (44). Several dystrophin-associated proteins have been identified and cloned (reviewed in Ref. 45). These include: (i) the dystroglycan complex (156- and 43-kDa glycoproteins, termed α- and β-dystroglycan); (ii) the sarcoglycan
complex (50, 43, and 35 kDa, termed α-, β-, and γ-sarcoglycan); and (iii) an unknown ~20–25 kDa nonglycosylated integral membrane protein. Mutations in α-, β-, and γ-sarcoglycan have been detected in either autosomal recessive muscular dystrophy, limb-girdle muscular dystrophy, or severe childhood autosomal recessive muscular dystrophy (45). To date, no genetic lesions have been described in α- or β-dystroglycan. The function of the dystrophin complex remains unknown, although some have suggested that the dystrophin complex may form part of a stretch-activated calcium channel that is defective in Duchenne muscular dystrophy.

Two related morphological observations seemingly implicate muscle cell caveaæ in the pathogenesis of Duchenne muscular dystrophy: (i) dystrophin has been localized to plasma membrane caveaæ in smooth muscle cells using immunoelectron microscopy techniques (32) and (ii) another electron microscopy study demonstrates that skeletal muscle caveaæ undergo characteristic changes in size and their distribution in patients with Duchenne muscular dystrophy, but not in other forms of neuronally based muscular dystrophies examined (33). However, little or no caveolin-1 is expressed in cardiac myocytes and skeletal muscle fibers (30). As such it has been difficult to assess the potential role of caveolin proteins and caveaæ in muscle cell membrane biology, as no caveolin probes exist for skeletal and cardiac muscle. However, recent studies indicate that skeletal and cardiac tissues express mRNA encoding a muscle-specific caveolin gene, termed caveolin-3 (30).

Here, we have generated and characterized a mAb probe that recognizes the caveolin-3 protein, but not other known members of the caveolin gene family. Undoubtedly, this novel probe will greatly facilitate the study of caveolin in striated muscle cells. Immunolocalization of caveolin-3 in skeletal muscle fibers reveals that caveolin-3 is localized to the sarcosome (muscle cell plasma membrane) and this immunostaining coincides with the distribution of another muscle-specific plasma membrane marker protein, dystrophin. In addition, detergent-free subcellular fractionation studies indicate that caveolin-3 co-fractionates with members of the dystrophin complex (dystrophin, α-sarcoglycan, and β-dystroglycan) and cytoplasmic signaling molecules (G-proteins and Src-like kinases), but is clearly separated from the bulk of cellular proteins. This is consistent with previous reports demonstrating that in non-muscle cells caveolin-1 copurifies with components of the membrane cytoskeleton and cytoplasmic signaling molecules (12–19).

Several independent lines of evidence suggest that caveolin-3 may represent the “unknown 20–25 kDa integral membrane component of the dystrophin complex” mentioned above. Caveolin-3 is a 20–25 kDa integral membrane protein (i) that is selectively expressed in muscle tissue types, (ii) co-localizes with dystrophin to the sarcosome of muscle fibers, (iii) co-fractionates with dystrophin and dystrophin-associated proteins, and (iv) co-immunoprecipitates with dystrophin. In this regard, we are currently searching for mutations within the human caveolin-3 gene in patients with unknown causes of muscular dystrophy.