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Caveolin-3 in muscular dystrophy

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The dystrophin–glycoprotein complex (DGC) serves as a link between cytoplasmic actin, the membrane and the extracellular matrix of striated muscle. Genetic defects in genes encoding a subset of DGC proteins result in muscular dystrophy and a secondary decrease in other DGC proteins. Caveolae are dynamic structures that have been implicated in a number of functions including endocytosis, potocytosis and signal transduction. Caveolin (VIP-21) is thought to play a structural role in the formation of non-clathrin-coated vesicles in a number of different cell types. Caveolin-3, or M-caveolin, was identified as a muscle-specific form of the caveolin family. We show that caveolin-3 co-purifies with dystrophin, and that a fraction of caveolin-3 is a dystrophin-associated protein. We isolated the gene for human caveolin-3 and mapped it to chromosome 3p25. We determined the genomic organization of human caveolin-3 and devised a screening strategy to look for mutations in caveolin-3 in patients with muscular dystrophy. Of 82 patients screened, two nucleotide changes were found that resulted in amino acid substitutions (G55S and C71W); these changes were not seen in a control population. The amino acid changes map to a functionally important domain in caveolin-3, suggesting that these are not benign polymorphisms and instead are disease-causing mutations.

INTRODUCTION

Dystrophin is a membrane-associated protein in muscle and brain, and mutations in the gene encoding dystrophin yield a clinical spectrum that includes muscular dystrophy, cardiomyopathy and mental retardation. The C-terminus of dystrophin associates with a complex of transmembrane and membrane-associated proteins (1–3). This complex, known as the dystrophin–glycoprotein complex or DGC, has been characterized biochemically and can be divided into a number of subcomplexes that include dystroglycan, sarcoglycan, syntrophin, dystrobrevin and the recently identified, sarcospan (4, for reviews see refs 5,6). Dystroglycan is a heavily glycosylated, peripheral membrane protein that directly binds the extracellular matrix (ECM) protein, laminin (for review, see 7). Mice homozygously lacking dystroglycan have an embryonic lethality thought to arise from defects in extra-embryonic structures and their association with the ECM (8). Sarcoglycan, a second component of the DGC, is composed of at least four subunits, α, β, γ and δ (for review see ref. 9). Mutations in each of the sarcoglycan genes cause autosomal recessive muscular dystrophy phenotypically similar to Duchenne muscular dystrophy (DMD). Syntrophin, a 59 kDa cytoplasmic protein, directly binds residues within the C-terminus of dystrophin and has also been shown to associate with neuronal nitric oxide synthase (nNOS) (7,10–12). NOS is part of the DGC, and its association with the muscle membrane is altered in DMD (13,14).

Caveolae are small membrane invaginations on the surface of cells that participate in membrane trafficking, sorting, transport and signal transduction (for reviews see refs 15,16). Caveolin is a small molecular weight protein that has been shown to be associated in many instances with the caveolar structure. A muscle-specific form of caveolin, caveolin-3, was identified (17,18). The number and size of caveolae are specifically abnormal in DMD, and studies of smooth muscle, where dystrophin has a more restricted pattern at the sarcolemma, show specific co-localization with caveolae and caveolin (19,20). Immunoprecipitation of crude membrane preparations shows an association of caveolin and dystrophin (21). Recently, it was shown that caveolin-3 and NOS directly interact and that this binding results in the loss of NOS activity (22), suggesting that the caveolin-3–nNOS interaction may be one mechanism by which caveolin-3 participates in the DGC.

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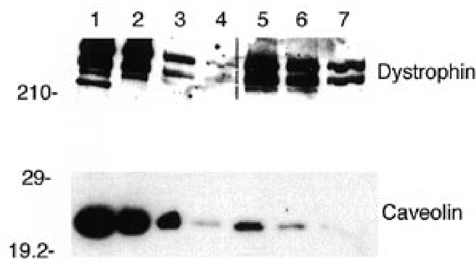

Figure 1. Caveolin-3 is a dystrophin-associated protein. The DGC was purified from rat skeletal muscle membranes using affinity and anion exchange chromatography (1–3). Fractions from the crude membrane preparation, the eluate from the WGA–Sepharose column and the low and high salt elutions from Q-Sepharose chromatography were separated by electrophoresis and immunoblotted with antibodies against dystrophin and caveolin-3. The upper panel represents those fractions separated on a 3–15% SDS–PAGE, while the lower panel represents those fractions separated by electrophoresis on a 15% SDS–PAGE. Lane 1, crude membranes; lane 2, WGA eluate; lane 3, low salt wash from the Q-Sepharose column; lanes 4–7, fractions 1–4, high salt wash from the Q-Sepharose column. The antibody against caveolin-3 is isoform-specific and has been characterized previously (21). The staining pattern of caveolin-3 in the identical fractions parallels that seen for dystrophin, suggesting that these proteins are both part of the DGC.

A

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      1                                               ▼      50
mcav3 MTEEHTDLE ARIIKDIHCK EIDLVNRDPK NINEDIVKVD FEDVIAEPEGT
rcav3 MTEEHTDLE ARIIKDIHCK EIDLVNRDPK NINEDIVKVD FEDVIAEPEGT
hcav3 MAEEHTDLE AQIVKDIHCK EIDLVNRDPK NINEDIVKVD FEDVIAEPVGT

      51                                                    100
mcav3 YSFDGVWKV SFTTFTVSKY WCYRLLSTLL GVPLALLWGF LFACISFCHIW
rcav3 YSFDGVWRV SYTTFTVSKY WCYRLLSTLL GVPLALLWGF LFACISFCHIW
hcav3 YSFDGVWKV SYTTFTVSKY WCYRLLSTLL GVPLALLWGF LFACISFCHIW
          S                W

      101                                                   150
mcav3 AVVPCIKSY LIEIQCISHI YSLCIRTFCN PLFAALGQVC SNIKVVLRREG
rcav3 AVVPCIKSY LIEIQCISHI YSLCIRTFCN PLFAALGQVC SNIKVVLRREG
hcav3 AVVPCIKSY LIEIQCISHI YSLCIRTFCN PLFAALGQVC SSIKVVLRKEV
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B

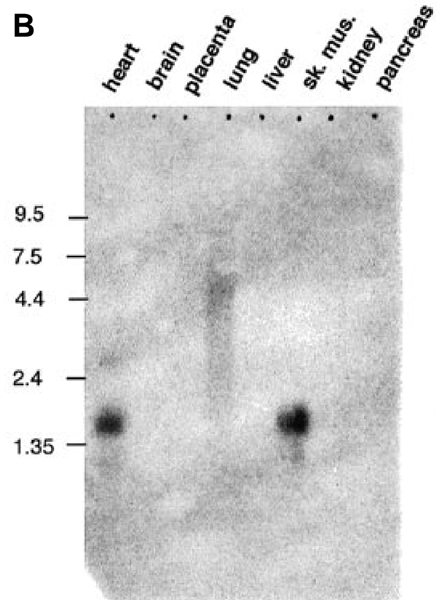


Figure 2. (**A**) The cDNA sequence of human caveolin-3 is shown on the lowest line (hcav3) and is compared with the sequences of mouse caveolin-3 (mcav3) and rat caveolin-3 (rcav3). The sequences are highly related. The two missense changes identified in patients with muscular dystrophy are shown in boxes, and the new amino acid is shown below. The solid line indicates one of the peptides recently shown by Venema *et al.* (22) to inhibit NOS activity. The dashed line indicates the transmembrane domain. It is thought that the N- and C-terminal domains of caveolin-3 are intracellular. The arrow indicates the placement of the single intron in the coding region of caveolin-3. The complete nucleotide sequence of the coding region of caveolin-3, exon 1 and exon 2 with the intronic flanking region has been deposited to GenBank under the accession nos AF036365–AF036367. (**B**) The expression pattern of caveolin-3 mRNA is shown in mRNA from multiple human tissues. A portion of the caveolin-3-coding region was labeled and hybridized to a blot containing 2 μg of poly(A)$^+$ mRNA from multiple human tissues. The caveolin-3 mRNA is 1.5 kb in size and is only present in striated muscle including cardiac and skeletal muscle.

To understand the relationship between caveolin and dystrophin better, we purified the DGC from skeletal muscle membranes using wheat germ agglutinin (WGA) and ion exchange chromatography, and found that caveolin-3 co-purifies with dystrophin. The human gene encoding caveolin was cloned and its chromosomal map position was determined by fluorescence *in situ* hybridization (FISH) as chromosome 3p25. Expression of caveolin-3 was studied and the organization of the caveolin-3 gene was determined. Primers were designed to amplify caveolin-3 sequences for single strand conformation polymorphism (SSCP) analysis and mutation detection. We analyzed patients with muscular dystrophy of an unknown genetic etiology and identified a single patient with a homozygous missense change (G55S) in caveolin-3 and a second patient with a single altered allele (C71W). These changes were not seen in a control population. Expression of dystrophin and the sarcoglycans is grossly normal in a skeletal muscle biopsy from the patient with a homozygous G55S change. G55 and C71 both fall within a cytoplasmic region of caveolin-3 that was implicated directly in inhibiting NOS activity. Given this, it is likely that these changes are disease-causing mutations and not neutral polymorphisms.

RESULTS

Caveolin-3 is a dystrophin-associated protein

The relationship between the DGC and caveolin-3 was investigated by purifying dystrophin from rat skeletal muscle membranes. WGA affinity chromatography and anion exchange chromatography were used to fractionate skeletal muscle microsomes because it was these fractionation steps that were used originally in describing the DGC (1–3). The fractions from these chromatography steps were analyzed by immunoblotting using antibodies specific for dystrophin and caveolin-3, the muscle-specific form of caveolin, to demonstrate that both dystrophin and caveolin-3 were present in the DGC preparation (Fig. 1, lane 5). The degradation of dystrophin that produces the doublet pattern on immunoblotting did not interfere with its normal elution profiles. Furthermore, the dystrophin-containing WGA fraction was purified further with anion exchange chromatography, demonstrating that some caveolin-3 in muscle is dystrophin associated. It should be noted that a significant fraction of the total caveolin present in the skeletal muscle membrane sample did not co-purify with dystrophin (Fig. 1, lane 3), suggesting that caveolin is not associated exclusively with the DGC in muscle.

Table 1. Primer pairs for caveolin-3 gene amplification

	Size of PCR product (bp)	Forward 5′ to 3′	Reverse 5′ to 3′
Genomic DNA primers			
HC3-7F/HC3-E1-3	176 bp	agctcggatctcctcctgtgg	ccgaggcaggcctgcagagcc
exon 1		(1–20)	(156–176)
HC3-E2-5/HC3-2	186 bp	gagttgaggcttccccttgcc	cgaacaggaagccccagagca
exon 2		(1–21)	(174–194)
HC3-1/HC3-3UTR	267 bp	ctaccgtctgttgtccacgct	tgccaccgctgttgccccacc
exon 2		(133–153)	(379–399)
cDNA primers			
HC3-7F/ HC3-8R	222 bp	agctcggatctcctcctgtgg	ggtggtgtagctcaccttcca
		(10–30)	(219–239)
HC3-3/HC3-2	193 bp	ccgagaccccaagaacattaac	cgaacaggaagccccagagca
		(125–146)	(304–324)
HC3-1/HC3-3UTR	267 bp	ctaccgtctgttgtccacgct	tgccaccgctgttgccccacc
		(263–283)	(509–529)

Shown are the primers used for screening caveolin-3. Primers to the complete coding region in genomic or cDNA are shown, and the corresponding numbers of the nucleotide sequence are indicated in parentheses. The numbers correspond to the nucleotides in GenBank accession nos AF036366 (exon 1), AF036367 (exon 2) or AF036365 (cDNA sequence). In the case of the genomic DNA primers, the sequences lie in the flanking intron or untranslated sequence.

Characterization of the human gene encoding caveolin-3

cDNA and genomic phage encoding human caveolin-3 were isolated from a λgt10 human cardiac library and an EMBL-3 human genomic library, respectively. The cDNA sequence was determined and has been deposited in GenBank with the accession no. AF036365. The caveolin-3 cDNA encodes an open reading frame of 150 amino acids that is highly homologous to the rat and mouse caveolin-3 sequences (96% similarity to both) but less homologous to human caveolin-1 and caveolin-2 (82 and 58% similarity, respectively) (Fig. 2A). Genomic phage encoding caveolin-3 were partially characterized by restriction digest. Intron–exon border sequences were determined by direct sequencing using primers directed against the exonic sequences. Caveolin-3 is encoded by two exons separated by a single intron. The expression pattern of human caveolin-3 mRNA was studied using RNA from multiple human tissues and a radiolabeled fragment of the human caveolin-3 sequence. The caveolin-3 mRNA was found to be expressed exclusively in cardiac and skeletal muscle (Fig. 2B). Primers were designed to amplify both the complete caveolin-3-coding region and portions of the flanking introns from genomic DNA. The primers for this assay are shown in Table 1. The sequences of the caveolin exons and flanking intronic region contained within the PCR products were deposited in GenBank under the accession nos AF036366 (exon 1) and AF036367 (exon 2).

Human caveolin-3 is located at 3p25 and is mutated in patients with muscular dystrophy

Genomic phage encoding caveolin-3 were labeled with digoxigenin and hybridized to human chromosomes. Twenty different metaphase chromosome spreads were analyzed, and the caveolin signal was present just below the telomere of the short arm of chromosome 3 at 3p25 (Fig. 3). Since none of the known muscular dystrophy loci are in this region, we chose a sample of muscular dystrophy patients ($n = 82$) of unknown genetic etiology to screen for mutations in the caveolin-3 gene using PCR to amplify the caveolin-3 sequences and SSCP. Dystrophin abnormalities were excluded as the cause of muscular dystrophy in these patients by a normal dystrophin immunostaining pattern on muscle biopsy and/or a normal dystrophin locus by multiplex PCR analysis of the dystrophin gene. The selection of muscular dystrophy patients studied included 65 that had a normal α-sarcoglycan immunostaining pattern and 17 that had abnormal staining. Mutations in α-, β-, γ- and δ-sarcoglycan were excluded as the abnormality in the 17 patients with abnormal α-sarcoglycan immunostaining.

One female patient was found to have a homozygous missense change G55S (Fig. 4A). This patient was the only affected member of her family, and developed proximal muscle weakness in the first decade. Dystrophin mutations were excluded by immunostaining, western blotting showing normal size and content of dystrophin and multiplex PCR. A second muscular dystrophy patient was identified with a heterozygous change on one allele (C71W) (Fig. 4B). This patient also had symptoms of progressive proximal weakness that began in the first decade, but she remained ambulatory in the mid-second decade. A second abnormality was not identified in this patient's DNA and may reflect a limited sensitivity of our screening assay. Her mother and two siblings had the identical missense change but did not have symptoms of muscular dystrophy, arguing that a single abnormal allele is not sufficient to cause the phenotype and that the likely inheritance is autosomal recessive. The father's DNA was not available and we were unable to determine the nature of the second allele in the proband. The entire coding region and 20–30 bp of intronic flanking region were screened for mutations, but it is possible the mutation lies outside the region being screened either more distantly in the introns or in the 3′-untrans-

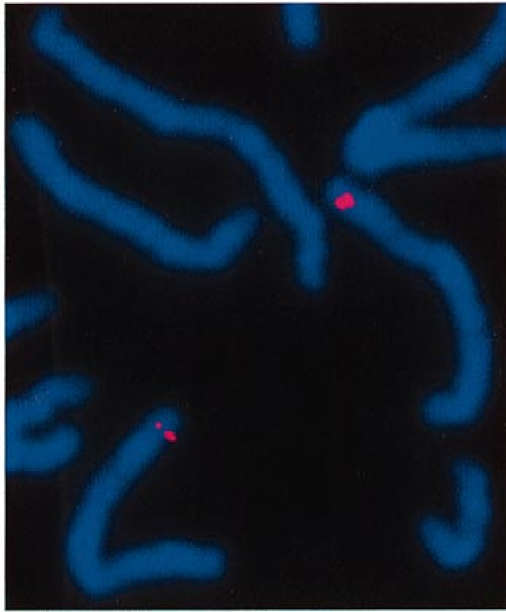
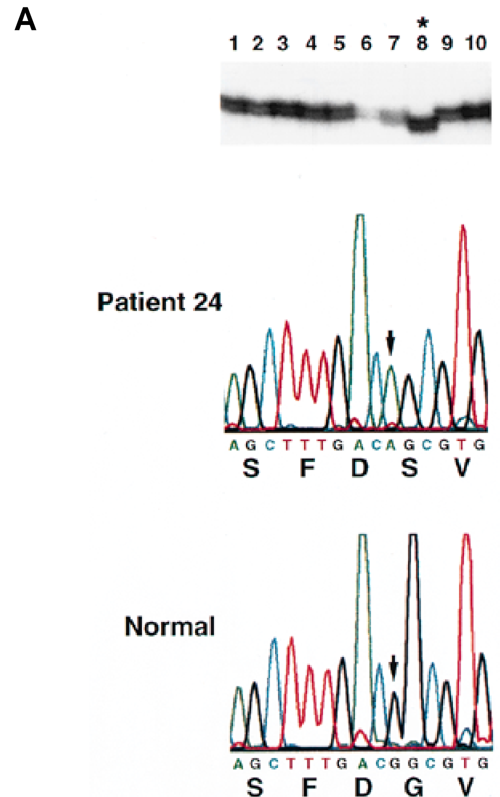


Figure 3. Fluorescence *in situ* hybridization (FISH) with genomic phage encoding caveolin-3. Genomic phage encoding caveolin-3 were labeled with digoxigenin and hybridized to human metaphase chromosomes. The map location of human caveolin-3 is 3p25.

lated region (3′UTR). Since both G55S and C71W were easily seen on SSCP, a control population of normal individuals of varied ethnicity was screened using SSCP. We chose this control population as representing the varied ethnicity of the patients identified with the mutation screen. Neither of these missense changes (G55S and C71W) was seen in 200 normal chromosomes screened by SSCP. The C71W missense change created a *Kpn*I site, and 50 normal chromosomes were also screened by restriction digest as well as SSCP and no new *Kpn*I sites were found. In a separate line of experiments, we tested patients with known mutations in the sarcoglycan genes ($n = 50$) and found no SSCP variants in caveolin-3 (data not shown). The patients identified with alterations in their caveolin genes (G55S and C71W) exhibited classical symptoms of progressive proximal muscular dystrophy characteristically seen in Duchenne/Becker muscular dystrophy or in the limb-girdle muscular dystrophies.



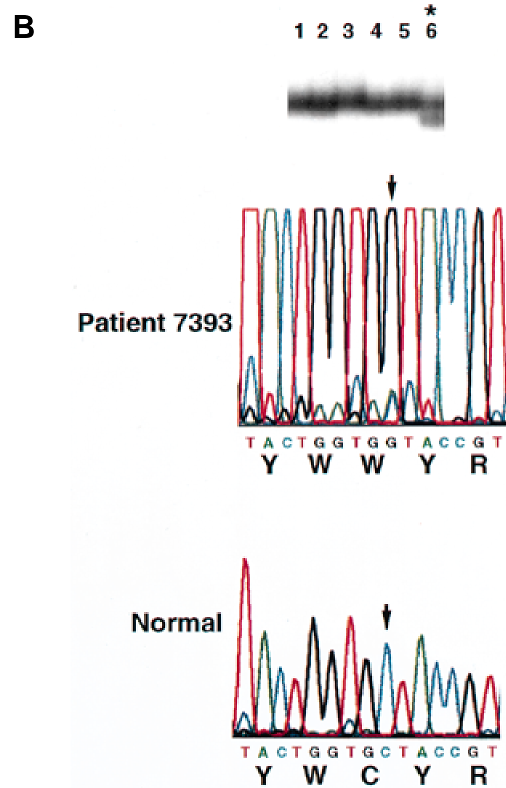


Figure 4. Mutation analysis of human caveolin-3 in patients with muscular dystrophy. DNA from patients with muscular dystrophy who had no identifiable mutation in dystrophin, α-, β- or γ-sarcoglycan was studied for mutations in caveolin-3. Primers were used to amplify cDNA prepared from a muscle biopsy (A) or genomic DNA (B). (**A**) The SSCP variant and sequence associated with the G55S substitution. (**B**) The SSCP variant associated with the C71W missense change. The lower band was excised from the SSCP gel, reamplified and directly sequenced to show the sequence associated with that SSCP variant. This C71W change also creates a *Kpn*I site that was used to test the members of the proband's family for the same mutation. Two unaffected siblings and her mother were found to carry a single copy of C71W, suggesting that a single abnormal allele is not sufficient to cause muscular dystrophy and the autosomal recessive inheritance. However, a second abnormal allele could not be detected in this patient, and the father's DNA was not available for study. Neither missense substitution was detected in 200 chromosomes from normal, unrelated controls nor in an additional 100 chromosomes from patients with muscular dystrophy of known genetic etiology.

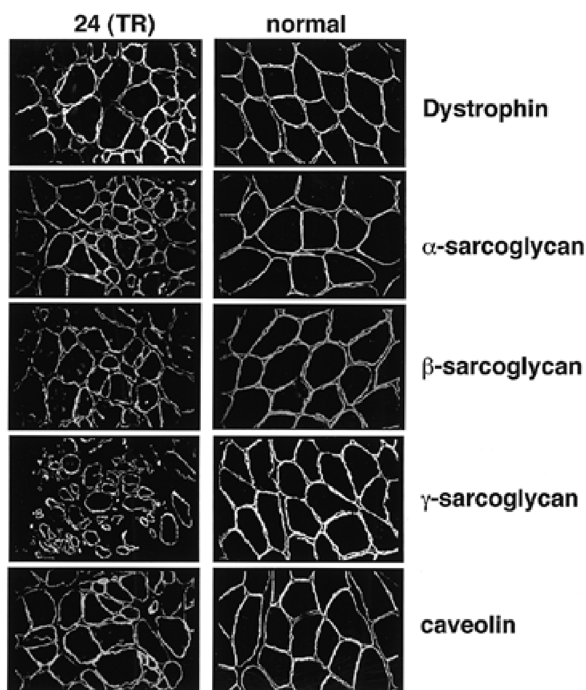


Figure 5. Immunostaining of the muscle biopsy of the patient with the G55S substitution. Sections of the patient's muscle biopsy were made and stained with antibodies to dystrophin and the components of sarcoglycan. The dystrophin and sarcoglycan patterns appeared slightly patchy but intact, and this pattern may have been due to freeze artifact. The sarcoglycan staining, as well as staining with a caveolin-3-specific antibody showed that these proteins were associated with the muscle membrane.

The muscle biopsy from the patient with the homozygous G55S mutation was stained with antibodies directed at dystrophin, components of the sarcoglycan complex and caveolin-3. The staining pattern for dystrophin and the sarcoglycans appeared slightly patchy (Fig. 5), and this may be due to freeze artifact present in the muscle since it was not present in all muscle sections. The staining for caveolin-3 also appeared largely normal. The G55S amino acid substitution may not alter the intracellular location of caveolin yet may interfere with the normal function of caveolin in the membrane.

DISCUSSION

A number of lines of evidence point to the association of caveolae and the DGC. Early ultrastructural characterization of muscle from patients with DMD showed abnormal size and numbers of caveolae (19). Co-localization of caveolin and dystrophin was seen in immunohistochemical staining of smooth muscle (20). Smooth muscle differs from striated muscle in that the dystrophin staining pattern is discontinuous at the membrane, and therefore it is ideal for demonstrating co-localization of dystrophin and caveolin. In the present work, we isolated dystrophin from skeletal muscle membranes and, using an antibody specific to caveolin-3, determined that caveolin-3 co-purifies with dystrophin in a manner similar to other components of the DGC.

While caveolin co-purifies with dystrophin, only a fraction of the intracellular caveolin is associated with dystrophin, consistent with the other cellular functions attributed to caveolin and caveolae. Future experiments aimed at addressing the ratio of dystrophin to caveolin and the proportion of intracellular caveolin that is associated with the DGC are needed to assess better the nature of this interaction. For example, whether a direct interaction between caveolin and dystrophin exists is unknown. However, recent data suggest that the caveolin–nNOS association is one mechanism by which caveolin-3 binds the DGC (22). It was shown previously that nNOS binds syntrophin, a 59 kDa component of the DGC (13). Caveolin-3 recently was shown to bind nNOS and result in the inhibition of NOS activity (22). Several peptides representing caveolin-3 sequences were shown to inhibit NOS activity *in vitro* (22). One of these, a peptide of 20 amino acids, including the G55 and C71 amino acids, was shown to inhibit NOS activity *in vitro* (22). This suggests that the interaction between caveolin-3 and dystrophin may be indirect and mediated through nNOS. These data further support that G55S and C71W are pathogenic mutations and not neutral polymorphisms. Moreover, these missense changes were not seen in a control population of 200 chromosomes from normal individuals and 100 chromosomes from muscular dystrophy patients with known mutations.

Interestingly, immunostaining of the muscle from the patient with the G55S change shows a nearly normal pattern for dystrophin and selected components of the DGC. This pattern stands in contrast to that seen in primary dystrophin mutations or in the primary sarcoglycan mutations where components of the sarcoglycan complex may be virtually absent from the muscle membrane. This suggests that caveolin normally is placed within the muscle membrane but is functioning abnormally, perhaps disrupting the normal activity of NOS. An improved understanding of the interaction of caveolin and caveolae in dystrophic muscle may shed light on the inherent membrane instability present in these disorders. The availability of caveolin-3 gene sequence, organization and a screening strategy to detect mutations should facilitate the study of additional muscular dystrophy patients and better assess the role of this protein in the dystrophic process.

MATERIALS AND METHODS

Purification of the DGC

Microsomal membranes were prepared from rat skeletal muscle by the pyrophosphate variant (1–3) with the addition of E64 at a final concentration of 2 μg/ml, 1 mM benzamidine, 77 nM aprotinin, 2 μg/ml leupeptin, 1 mM iodoacetamide and 0.2 mM phenylmethylsulfonyl fluoride (PMSF). The microsomes were washed twice with 0.6 M KCl and solubilized in 1.0% digitonin, and the DGC purified by WGA affinity chromatography as described (3). The dystrophin-containing fractions eluted from WGA–Sepharose were then separated further using Q-Sepharose fast flow chromatography; the column was washed extensively with 0.27 M NaCl, and the DGC was eluted with 0.35 M NaCl. The Q-Sepharose column fractions were analyzed for the presence of dystrophin and caveolin-3 using SDS–PAGE and immunoblotting. A monoclonal antibody specific for caveolin-3 was described previously (21). A polyclonal antibody directed at the rod of dystrophin, anti-6–10, was described previously (23). Secondary antibodies conjugated to horseradish peroxidase were from Jackson Immunochemicals and were imaged using ECL (Amersham).

Isolation of human caveolin-3 cDNA clones

Primers were designed based on the rat and mouse caveolin-3 sequences (GenBank accession nos U31968 and U36579, respectively), and used to amplify cDNA reverse transcribed from human skeletal and cardiac muscle. The primers used for the initial amplification were RC-F1 5′ATCATCAAGGACATT-CACTGCAAGGAGATAGA3′ and RC-R2 5′CGGGTTGCA-GAAGGTGCGGATACA3′. These primers produced a 420 bp product from human cardiac and skeletal muscle cDNA that was directly sequenced and found to contain human caveolin-3 sequences. The PCR product was reamplified in the presence of ^{32}P to generate radiolabeled fragments for screening a human cardiac cDNA library. λgt10 cDNA clones were isolated and subcloned into Bluescript (Stratagene). All sequencing was performed with Taq cycle sequencing on an ABI 373 sequencer, and all analyses were performed with Sequcencher (Gene Codes), MacVector and the GCG sequence analysis package.

Isolation and characterization of human caveolin-3 genomic clones

The same 420 bp fragment representing human caveolin-3 was used to screen an EMBL-3 human genomic library (Clontech, catalog no. HL1006d). Six positively hybridizing clones were isolated and characterized by restriction digest and direct sequencing. The intron–exon structure of human caveolin-3 was determined by direct sequencing using the primers listed in Table 1. Phage DNA was prepared by cesium gradient centrifugation followed by Qiagen lambda prep. and directly sequenced using Taq cycle sequencing and an ABI 373 sequencer.

Northern analysis

The same 420 bp PCR product was generated in the presence of ^{32}P and used as a probe against a multiple tissue northern blot (Clontech) that contained 2 µg of poly(A)$^+$ mRNA from each tissue listed. Conditions for hybridization were those previously described (24).

Fluorescence *in situ* hybridization (FISH) with genomic sequences encoding caveolin-3

Two overlapping genomic phage encoding human caveolin-3, HC3-G1 and HC3-G6 were nick translated and labeled with digoxigenin as described (25). Human cells arrested at metaphase were prepared from primary lymphoblastoid cells using standard cytogenetic techniques. Nuclei were visualized by fluorescence microscopy using a Zeiss Axiophot microscope equipped with a triple band pass filter set [fluorescein/Texas red/DAPI (4′,6-diamidino-2-phenylindole); Omega Optical] and recorded as described (25).

Mutation analysis

Total RNA was prepared from muscle biopsies, and cDNA was synthesized with reverse transcriptase primed with oligo(dT) as described (24). The primers that generate overlapping products were used to amplify the coding region of human caveolin-3 and are listed in Table 1. The PCR products were separated by electrophoresis under non-denaturing conditions using SSCP. Gels were run at 600 V under constant voltage at room temperature for 8–10 h or until the fragments of interest had migrated to the center of a 40 cm gel. Gels were run under two conditions that included 0.5× MDE (FMC Bioproducts, Rockland, ME) without glycerol and 0.5× MDE with 4% glycerol. Additionally, 0.5× MDE with 10% glycerol was used on the PCR product amplified by the first pair of caveolin-3 cDNA primers since this condition was found to be more favorable for detecting SSCP variants. Gels were dried and autoradiographed for 4–24 h at –80°C. SSCP variants were excised from the gels and eluted in 100 µl of dH_2O. Five µl of the eluted fragment was used for reamplification under the conditions described above except that ^{32}P was omitted and the reaction volume was 50 µl. The reamplified PCR products were purified using Wizard PCR Clean up (Promega, Madison, WI), and 2 µl was used for sequencing. Taq cycle sequencing was performed with an ABI 373 sequencer.

Immunocytochemistry

Muscle biopsies sections of 7 µm of were cut at –20°C using a cryostat and briefly fixed in acetone. Slides were blocked with 5% fetal calf serum in 1× phosphate-buffered saline (PBS). The antibodies used for immunocytochemistry include the monoclonal antibodies, NCL-DYS2 and NCL-50DAG directed against dystrophin and α-sarcoglycan, respectively (Novocastra, Newcastle upon Tyne, UK) and rabbit polyclonal antibodies anti-β-sarcoglycan and anti-γ-sarcoglycan. The secondary antibodies, anti-rabbit and anti-mouse IgG were coupled to Cy3 (Jackson Immunochemicals). The results were visualized on a Leitz microscope equipped with epifluorescence and filters.

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REFERENCES

1. Campbell, K.P. and Kahl, S.D. (1989) Association of dystrophin and an integral membrane glycoprotein. *Nature*, **338**, 259–262.
2. Yoshida, M. and Ozawa, E. (1990) Glycoprotein complex anchoring dystrophin to sarcolemma. *J. Biochem. (Tokyo)*, **108**, 748–752.
3. Ervasti, J. and Campbell, K.P. (1991) Membrane organization of the dystrophin–glycoprotein complex. *Cell*, **66**, 1121–1131.
4. Crosbie, R.H., Heighway, J., Venzke, D.P., Lee, J.C. and Campbell, K.P. (1997) Sarcospan, the 25-kDa transmembrane component of the dystrophin–glycoprotein vomplex. *J. Biol. Chem.*, **272**, 31221–31224.
5. Ozawa, E., Yoshida, M., Suzuki, A., Mizuno, Y., Hagiwara, Y. and Noguchi, S. (1995) Dystrophin-associated proteins in muscular dystrophy. *Hum. Mol. Genet.*, **4**, 1711–1716.
6. Campbell, K.P. (1995) Three muscular dystrophies: loss of cytoskeleton–extracellular matrix linkage. *Cell*, **80**, 675–679.
7. Kramarcy, N.R., Vidal, A., Froehner, S.C. and Sealock, R. (1994) Association of utrophin and multiple dystrophin short forms with the mammalian M(r) 58,000 dystrophin-associated protein (syntrophin). *J. Biol. Chem.* **269**, 2870–2876.
8. Williamson, R.A., Henry, M.D., Daniels, K.J., Hrstka, R.F., Lee, J.C., Sunada, Y., Ibraghimov-Beskrovnaya, O. and Campbell, K. P. (1997) Dystroglycan is essential for early embryonic develoment: disruption of Reicher's membrane in DAG1-null mice. *Hum. Mol. Genet.*, **6**, 831–841.
9. Bönnemann, C.G., McNally, E.M. and Kunkel, L.M. (1996) Current progress in the muscular dystrophies: beyond dystrophin. *Curr. Opin. Pediatr.*, **8**, 569–582.
10. Ahn, A.H. and Kunkel L.M. (1995) Syntrophin binds to an alternatively spliced exon of dystrophin. *J. Cell Biol.*, **128**, 363–371.

11. Suzuki, A., Yoshida, M. and Ozawa, E. (1995) Mammalian alpha 1- and beta 1-syntrophin bind to the alternative splice-prone region of the dystrophin COOH terminus. *J. Cell Biol.*, **128**, 373–381.
12. Peters, M.F., Adams, M.E. and Froehner, S.C. (1997) Differential association of syntrophin pairs with the dystrophin complex. *J. Cell Biol.* **138**, 81–93.
13. Brennan, J.E., Chao, D.S., Xia, H., Aldape, K., and Bredt, D.S. (1995) Nitric oxide synthase complexed with dystrophin and absent from skeletal muscle sarcolemma in Duchenne muscular dystrophy. *Cell*, **82**, 743–752.
14. Brenman, J.E., Chao, D.S., Gee, S.H., McGee, A.W., Craven, S.E., Santillano, D.R., Wu, Z., Huang, F., Xia, H., Peters, M.F., Froehner, S.C. and Bredt, D.S. (1996) Interaction of nitric oxide synthase with the postsynaptic density protein PSD-95 and alpha1-syntrophin mediated by PDZ domains. *Cell*, **84**, 757–767.
15. Anderson, R.G.W. (1993) Plasmalemmal caveolae and GPI-anchored membrane proteins. *Curr. Opin. Cell Biol.*, **5**, 647–652.
16. Lisanti, M.P., Tang, Z., Scherer, P.E., Kubler, E., Koleske, A.J. and Sargiacomo, M. (1995) Caveolae, transmembrane signalling and cellular transformation. *Mol. Membr. Biol.*, **12**, 121–124.
17. Way, M., and Parton, R.G. (1995) M-caveolin, a muscle-specific caveolin-related protein. *FEBS Lett.*, **376**, 108–112.
18. Tang, Z., Scherer, P.E., Okamoto, T., Song, K., Chu, C. D., Kohtz, S., Nishimoto, I., Lodish, H.F. and Lisanti, M.P. (1996) Molecular cloning of caveolin-3, a novel member of the caveolin gene family expressed predominantly in muscle. *J. Biol. Chem.*, **271**, 2255–2261.
19. Bonilla, E., Fischbeck, K. and Schotland, D.L. (1981) Freeze-fracture studies of muscle caveolae in human muscular dystrophy. *Am. J. Pathol.*, **104**, 167–173.
20. North, A.J., Galakiewics, B., Byers, T. J.,Glenney, J. R. Jr and Small, J.V. (1993) Complementary distribution of vinculin and dystrophin define two distinct sarcolemma domains in smooth muscle. *J. Cell Biol.*, **120**, 1159–1167.
21. Song, K.S., Scherer, P.E., Tang, Z.L., Okamoto, T., Li, S., Chafel, M., Cju, C., Kohtz, D.S. and Lisanti, M.P. (1996) Experession of caveolin-3 in skeletal, cardiac, and smooth muscle cells. *J. Biol. Chem.*, **271**, 15161–15165.
22. Venema, V.J, Ju, H., Zou, R. and Venema, R.C. (1997) Interaction of neuronal nitroc-oxide synthase with caveoin-3 in skeletal muscle. *J. Biol. Chem.*, **45**, 28187–28190.
23. Lidov, H.G.W., Byers, T.J., Watkins, S.C. and Kunkel, L.M. (1990) Localization of dystrophin to postsynaptic regions of central nervous system cortical neurons. *Nature*, **348**, 725–728.
24. Noguchi, S., McNally, E.M., Ben Othmane, K., Hagiwara, Y., Mizuno, Y., Yoshida, M., Yamamoto, H., Bönnemann, C.G., Gussoni, E., Denton, P.H., Kyriakides, T., Middleton, L., Hentati, F., Ben Hamida, V., Nonaka, I., Vance, J.M., Kunkel, L.M. and Ozawa, E. (1995) Mutations in the dystrophin-associated protein γ-sarcoglycan in chromosome 13 muscular dystrophy. *Science*, **270**, 819–822.
25. Gussoni, E., Wang, Y., Fraefel, C., Miller, R.G., Blau, H.M., Geller, A.I. and Kunkel, L.M. (1996) A method to codetect introduced genes and their products in gene therapy protocols. *Nature Biotechnol.*, **14**, 1012–1016.