Dissociation of the dystroglycan complex in caveolin-3-deficient limb girdle muscular dystrophy

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Limb girdle muscular dystrophy is a group of clinically and genetically heterogeneous disorders inherited in an autosomal recessive or dominant mode. Caveolin-3, the muscle-specific member of the caveolin gene family, is implicated in the pathogenesis of autosomal dominant limb girdle muscular dystrophy 1C. Here we report on a 4-year-old girl presenting with myalgia and muscle cramps due to a caveolin-3 deficiency in her dystrophic skeletal muscle as a result of a heterozygous 136G→A substitution in the caveolin-3 gene. The novel sporadic missense mutation in the caveolin signature sequence of the caveolin-3 gene changes an alanine to a threonine (A46T) and prevents the localization of caveolin-3 to the plasma membrane in a dominant negative fashion. Caveolin-3 has been suggested to interact with the dystrophin–glycoprotein complex, which in striated muscle fibers links the cytoskeleton to the extracellular matrix and with neuronal nitric oxide synthase. Similar to dystrophin-deficient Duchenne muscular dystrophy, a secondary decrease in neuronal nitric oxide synthase and α-dystroglycan expression was detected in the caveolin-3-deficient patient. These results implicate an important function of the caveolin signature sequence and common mechanisms in the pathogenesis of dystrophin–glycoprotein complex-associated muscular dystrophies with caveolin-3-deficient limb girdle muscular dystrophy.

INTRODUCTION

Limb girdle muscular dystrophy (LGMD) shows a wide range of clinical severity and age at onset (1). The mutated genes causing LGMD encode proteins with different localizations and functions within the skeletal muscle fibers (2,3). Proteins involved in the structure and function of the plasma membrane, the sarcomere and the nuclear envelope have all been reported to play a primary role in the pathogenesis of LGMD (2,3). A recently characterized sarcolemmal protein, caveolin-3, has been implicated in the pathogenesis of autosomal dominant LGMD 1C (4–6). A better understanding of the pathogenic process leading to muscle cell degeneration in caveolin-3-deficient LGMD originates from work on the functional analysis of caveolins (7–9).

Caveolin-3 is the muscle-specific protein product of the caveolin gene family (10) and a principal integral membrane component of caveolae, small invaginations of the plasma membrane proposed to play a role as endocytic vehicles and in signal transduction processes (7). Many signaling molecules are localized to the caveolae and interact with caveolins through a conserved 20 amino acid domain called the caveolin scaffolding domain (11,12). The interaction of caveolins and in particular the caveolin scaffolding domain with signaling molecules has been implicated in the activation and in the inactivation of signal transduction events. One of the best characterized examples of this interaction and its role in signal transduction is the binding of caveolins to nitric oxide synthases (13–16). Caveolin-1 has been shown to interact with endothelial nitric oxide synthase in endothelial cells and cardiac myocytes (17,18), whereas caveolin-3 directly interacts with neuronal nitric oxide synthase (nNOS) in skeletal muscle fibers (15). Interaction of caveolins with NOS is thought to hold the enzymes in an inactive conformation and only release their inhibitory constraints through caveolin disassociation, allowing the signaling to take place (7).

In skeletal muscle fibers, nNOS is closely associated with the dystrophin–glycoprotein complex (DGC) (19), which in muscle cells serves as a link between laminin in the extracellular matrix and the F-actin cytoskeleton (20). Even though caveolin-3 is not an integral component of the DGC (21), an association between caveolin-3 and the complex has been reported (22). Recent studies extended the numbers of muscular dystrophies associated with primary mutations affecting components of the DGC to six genetically distinct diseases (23). A key molecule of the complex is the basement membrane receptor dystroglycan, consisting of an extracellular α- and a transmembrane β-subunit. Dystroglycan has been shown to play an important role in the assembly and formation of basement membranes and in the pathogenesis of DGC-associated muscular dystrophies (24). The best known and

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most common form of muscular dystrophy associated with the DGC is Duchenne muscular dystrophy (DMD) caused by mutations in the dystrophin gene (25). In DMD the loss of dystrophin expression leads to the concomitant loss of other components of the DGC including dystroglycan and nNOS (19,26). Due to the interaction of caveolin-3 with nNOS and of nNOS with the DGC a similar pathogenic mechanism may underlie the DGC-associated muscular dystrophies and caveolin-3-deficient LGMD 1C.

Here we report on a novel mutation in the caveolin-3 gene in a 4-year-old girl with muscular dystrophy. The muscle biopsy showed a near-complete loss of caveolin-3 expression. In addition we found altered expression of nNOS and dystroglycan. Our results emphasize the importance of caveolin-3 in the pathogenesis of muscle fiber degeneration and suggest a common cascade of events in DGC-associated muscular dystrophies and LGMD 1C.

RESULTS

The skeletal muscle biopsy from the femoral quadriceps muscle of the 4-year-old girl revealed characteristic histopathological signs of muscular dystrophy. The biopsy showed a regular distribution and proportion of fiber types. Immunofluorescence analysis of cryosections was performed using antibodies against candidate proteins for muscular dystrophy. In contrast to sections of normal human skeletal muscle, a near complete loss of caveolin-3 expression was detected in the patient’s biopsy (Fig. 1). Most muscle fibers showed no caveolin-3 immunoreactivity whereas, in several others, partial expression at the plasma membrane was still detectable (Fig. 1).

The loss or reduction of caveolin-3 expression in skeletal muscle has so far been reported only as the primary cause of LGMD 1C and not as a secondary phenomenon in other forms of muscular dystrophy. We therefore searched for disease-causing mutations in the human caveolin-3 gene (CAV3) on chromosome 3p25 of the patient’s DNA. DNA was extracted from peripheral blood lymphocytes and amplified with exon-specific primers followed by single-stranded conformation polymorphism (SSCP) analysis. In exon 2 of the CAV3 gene, the patient showed an aberrant band compared with the normal human control DNA (Fig. 2A). The additional band was found neither in one of the parents nor in one of the two healthy brothers of the patient. Direct sequencing of the corresponding PCR product revealed a G→A substitution on one allele at position 136 (Fig. 2B) causing an amino acid change at position 46 from alanine to threonine (A46T). The heterozygous mutation generated a new BclI restriction site that would cut the fragment into two subfragments of 351 and 48 bp. Digestion with BclI confirmed the mutation in exon 2 of one of the patient’s alleles, as only the patient showed a 351 bp band in addition to the band of the normal allele solely found in the rest
of the family (Fig. 2C). Two hundred chromosomes from non-related individuals were also tested for the 136G→A mutation by BcI restriction analysis, but this mutation was never found (data not shown).

Both nNOS and caveolin-3 are located at the sarcolemma of skeletal muscle fibers and direct interactions between the two proteins have been suggested (15,16,27). Consequently, we looked for nNOS expression on skeletal muscle cryosections of the patient’s biopsy. In contrast to normal control muscle, no expression of the enzyme was found in the patient (Fig. 3). In skeletal muscle nNOS is associated with dystrophin and the DGC (19) and in addition, caveolin-3 has been reported to be associated with the DGC (22). As primary defects in several components of the DGC cause muscular dystrophy, the expression of the complex was studied in the patient’s skeletal muscle biopsy by immunohistochemical analysis. Using a newly generated polyclonal peptide antibody against α-dystroglycan we found a strong reduction of this peripheral membrane glycoprotein in the patient compared with normal control muscle (Fig. 4B). The antibody was raised against the C-terminal amino acid sequence of chick α-dystroglycan (M. Blank and S. Kröger, manuscript in preparation), which shows a high degree of sequence identity to dystroglycan from several mammalian species and crossreacts with mouse, rat, human and bovine tissue (S. Kröger, unpublished data). On western blots the antibody showed a broad band of ~156 kDa in muscle tissue and of 120 kDa in brain tissue, in accordance with the known molecular weight of α-dystroglycan (Fig. 4A).

The different molecular weight is due to tissue-specific differences in glycosylation (24). Using skeletal muscle preparations from mdx mice, an animal model for dystrophin deficiency characterized by a secondary loss of dystroglycan (28), no band was detected using the anti-peptide antiserum (data not shown). On transverse sections of control muscle α-dystroglycan showed a homogenous staining pattern around the muscle fiber surface (Fig. 4B). In the LGMD 1C patient, on the other hand, α-dystroglycan expression was almost completely lost (Fig. 4B); a residual staining signal was found in only a few fibers. In contrast to the loss of α-dystroglycan, we found a normal expression pattern for β-dystroglycan (Fig. 4B). A normal expression pattern was also found for α-, β-, γ- and δ sarcoglycan, dystrophin and the laminin chains α2 (Fig. 4B), α5, β1, β2 and γ1 (data not shown). Probably due to degeneration, some fibres showed reduced staining with the C-terminal dystrophin antibody (NCL-DYS2).

**DISCUSSION**

Four different mutations in the CAV3 gene (Fig. 2D) have so far been shown to cause autosomal dominant LGMD 1C (4–6). Here we demonstrated a new de novo mutation in one of the CAV3 alleles of a 4-year-old girl with myalgia, muscle cramps and a dystrophic pattern in the skeletal muscle biopsy. A mutation in only one allele is consistent with the idea of a dominantly inherited disease. In previous LGMD 1C cases, two mutations have been reported within the scaffolding domain of caveolin-3 (5,6), one in the transmembrane domain (6) and one in the N-terminal domain of the protein (4). An additional mutation in the scaffolding domain has been described in one patient with proximal muscle weakness in a homozygous state, suggesting a possible autosomal recessive inheritance (5) (Fig. 2D).

Analysis of genomic DNA in our patient revealed a missense mutation that changed an alanine to a threonine (A46T) in the caveolin signature sequence. The caveolin signature sequence
is a stretch of 8 amino acids (FEDVIAEP) within the hydrophilic N-terminal domain of caveolin-1 (α- and β-isoforms), caveolin-2 and caveolin-3 in mammals (10,29). The fact that all known mammalian caveolins contain this invariant sequence points to an essential but so far unknown functional role of the caveolin signature sequence, suggesting that the identified base exchange is not a benign polymorphism but rather a disease-causing mutation.

Although only one of the caveolin-3 alleles was mutated in our patient, caveolin-3 expression in the plasma membrane was almost completely lost (Fig. 1). It has previously been shown that caveolin-3 molecules form a homomeric complex that is localized to the plasma membrane after its assembly (9,30,31). Dissociation of the assembly process by a mutated protein leads to degradation of the entire complex within the Golgi system and lack of sarcolemmal caveolin expression (31). It was therefore postulated that in the autosomal dominant inherited forms of caveolin-3 defects the mutations must cause the formation of a dominant-negative form of caveolin-3 (31). Therefore, screening of muscle biopsies from patients affected by muscular dystrophy of unknown origin with antibodies against caveolin-3 is a suitable tool to diagnose LGMD 1C. Loss of caveolin-3 expression from the plasma membrane caused by a missense mutation in the caveolin signature sequence indicates a role for this sequence in the assembly of the homomeric caveolin-3 complex or in its translocation from intracellular compartments to the plasma membrane. The muscle biopsy of our patient revealed no increase in the intracellular caveolin-3 staining signal by immunohistochemical analysis that would indicate enrichment of the protein in the Golgi system (Fig. 1).

The phenotype of our patient was fairly mild and an obvious muscle weakness was not detected by the age of 4 years. This finding is in accordance with previous reports on LGMD 1C patients, who all showed a mild clinical phenotype (4–6). This may be one explanation why so far only four different missense mutations have been characterized. One might speculate that only missense mutations leading to a conformational change of the protein that interferes with the assembly of caveolin-3 oligomers develop a muscular dystrophy. A nonsense mutation in one allele might not affect the assembly process in a dominant-negative fashion.

Interestingly, we found an altered expression pattern of two of the major components of the DGC in the patient’s muscle biopsy. α-dystroglycan, the peripheral membrane protein linking the basement membrane protein laminin 2 with the transmembrane protein β-dystroglycan, was almost completely lost from the fiber surface. Even though a direct interaction between α-dystroglycan and caveolin-3 is unlikely, loss of the caveolin network seems to affect the expression of the peripheral membrane protein. A similar finding has already been established for several muscular dystrophies, including DMD and sarcoglycan-deficient LGMD 2C–F (32,33). The loss of α-dystroglycan has not only been reported in the human diseases but also in the corresponding animal models (28,34,35). Chimeric mice generated with embryonic stem cells targeted for both dystroglycan alleles have skeletal muscles essentially devoid of dystroglycan and develop a progressive muscle pathology with changes emblematic of muscular dystrophies in humans. The central role of α-dystroglycan in the pathogenesis of muscular dystrophy has been shown in several reports emphasizing the role of α-dystroglycan as a linker between the extracellular matrix and the cytoskeleton (24,36,37). Once this crucial structural link is disrupted muscle fibers seem to become more vulnerable for mechanical stress. How does the loss of caveolin affect expression of α-dystroglycan? It has been previously reported that patients affected by muscular dystrophy show an increased protease activity in their skeletal muscle (38–42). Unspecific degradation of proteins in the extracellular matrix may be a result of the increased protease activity supporting the vicious circle of fiber degeneration. This might explain why several forms of muscular dystrophy show the same secondary loss of α-dystroglycan expression. Overexpression of caveolin-3 in patients suffering from DMD and in dystrophin-deficient mdx mice (43,44) points to the fact that there is a functional correlation between the DGC and caveolae.

**MATERIALS AND METHODS**

**Subjects**

A 3.5-year-old girl of healthy, non-consanguineous parents was referred to the Düsseldorf University Hospital due to elevated serum creatine kinase levels (max. 800 U/l). At the age of 4 years she developed myalgia and muscle cramps in the lower limbs. Due to the persistent elevated creatine kinase serum levels a needle biopsy of the femoral quadriceps muscle was performed. The patient had normal motor milestone development and showed no skeletal muscle weakness. Investigations revealed normal electro- and echocardiogram and nerve conduction, but the electromyography showed myopathic features.

The muscle biopsy from the *vastus lateralis* showed replacement of muscle fibers by fat tissue associated with an increase in endomysial and perimysial connective tissue. There was a wide variation in fiber size. Both atrophic and markedly hypertrophic fibers were observed along with a few hyalin fibers. In addition, there were degenerating, regenerating and split fibers.

**Immunohistochemistry**

Skeletal muscle sections were prepared from normal control tissue, the caveolin-3-deficient patient, a patient diagnosed with DMD and from control and *mdx* mice. Sections of 6 μm were cut at −20°C using a cryostat. Slides were blocked with 5% bovine serum albumin (BSA) in 1× phosphate-buffered saline (PBS). The antibodies used for immunocytochemistry include the monoclonal antibodies NCL-a-SARC, NCL-b-SARC, NCL-g-SARC and NCL-d-SARC directed against α-, β-, γ- and δ-sarcoglycan, respectively, NCL-b-DG against β-dystroglycan, NCL-DYS1, -DYS2 and -DYS3 against dystrophin (Novocastra, Newcastle upon Tyne, UK), and anti-caveolin-3 (Transduction, Lexington, KY). Monoclonal antibodies directed against the human laminin α2, α5, β1, β2 and γ1 chain were purchased from Chemicon (Temecula, CA). Staining for nNOS was performed using a rabbit polyclonal anti-nNOS antibody (Upstate Biotechnology, Lake Placid, NY). After blocking for 30 min sections were incubated with primary antibodies for 90 min in 1% BSA/PBS. After washing with 1% BSA/PBS, sections were incubated with biotin-conjugated secondary antibodies (Amersham, Braunschweig,
Germany) for 30 min, then washed with 1% BSA/PBS. Slides were then incubated with Texas Red-conjugated streptavidin (1:200) for 30 min (Amersham). After a rinse with PBS, sections were mounted with Aquatex (Merek, Darmstadt, Germany). Sections were observed under a Zeiss Axiosplan fluorescence microscope (Carl Zeiss, Jena, Germany).

Genetic analysis
Genomic DNA (50–100 ng) extracted from peripheral blood lymphocytes from each member of the family was used as a template for PCR amplification. Reactions with exon-specific primers were performed as previously described (5). Thirty-five cycles of amplifications were performed as described above with an annealing temperature of 55°C (5). PCR products were digested with Bcl I (LifeTechnologies, Karlsruhe, Germany) and products were analyzed by electrophoresis on a 2% Seakem LE agarose (FMC, Rockland, ME) gel stained with ethidium bromide. Direct sequencing of PCR products was performed using the dye terminator cycle sequencing chemistry and analyzed on a 373A Stretch Fluorescent Automated Sequencer (Applied Biosystems, Weiterstadt, Germany).

Generation of anti-α-dystroglycan antiserum
For immunization of two sheep the 20 amino acid peptide, PDRSHVGKHEYFMYATDKGG, was coupled to keyhole limpet hemocyanin via an additional N-terminal cystein. This peptide, For immunization of two sheep the 20 amino acid peptide, PDRSHVGKHEYFMYATDKGG, was coupled to keyhole limpet hemocyanin via an additional N-terminal cystein. This peptide was used as an immunogen for the preparation of antiserum overnight in PBS containing 3% milk powder and subsequent extensive washing. The signal was developed using the enhanced chemiluminescence system as described by the manufacturer (Amersham).

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