The sarcolemmal proteins dysferlin and caveolin-3 interact in skeletal muscle

Chie Matsuda1,2,5, Yukiko K. Hayashi2, Megumu Ogawa2, Masashi Aoki3, Kumiko Murayama4, Ichizo Nishino4, Ikuya Nonaka4, Kiichi Arahata2,4† and Robert H. Brown, Jr5,*

1Molecular Neurobiology Group, Neuroscience Research Institute, AIST, Central 6, Tsukuba 305-8566, Japan, 2Department of Neuromuscular Research, National Institute of Neuroscience, National Center of Neurology and Psychiatry, Tokyo 187-8502, Japan, 3Department of Neurology, Tohoku University School of Medicine, Sendai 980-8574, Japan, 4Department of Ultrastructural Research, National Institute of Neuroscience, National Center of Neurology and Psychiatry, Tokyo 187-8502, Japan and 5Day Neuromuscular Research Laboratory, Massachusetts General Hospital-East, Charlestown, MA 02129, USA

Received April 2, 2001; Revised and Accepted June 24, 2001

Dysferlin is a surface membrane protein in skeletal muscle whose deficiency causes distal and proximal, recessively inherited, forms of muscular dystrophy designated Miyoshi myopathy (MM) and limb girdle muscular dystrophy type 2B (LGMD2B), respectively. The function of dysferlin is not defined. Caveolin-3 is another skeletal muscle membrane protein which is important in the formation of caveolae and whose mutations cause dominantly inherited limb girdle muscular dystrophy type 1C (LGMD1C). We report that dysferlin co-immunoprecipitates with caveolin-3 from biopsied normal human skeletal muscles. We also describe abnormal localization of dysferlin in muscles from patients with LGMD1C including novel missense mutation (T64P) in the human caveolin-3 gene (CAV3). The immunoprecipitation data are consistent with the parallel observation that dysferlin immunostaining is not normal in LGMD1C muscles. Amino acid sequence analysis of the dysferlin protein reveals seven sites that correspond to caveolin-3 scaffold-binding motifs, and one site that is a potential target to bind the WW domain of the caveolin-3 protein. This is the first description of a possible dysferlin interacting protein; it suggests the hypothesis that one function of dysferlin may be to interact with caveolin-3 to subserve signaling functions of caveolae.

INTRODUCTION

Dysferlin is a surface membrane protein in skeletal muscle whose deficiency causes distal and proximal, recessively inherited, forms of muscular dystrophy designated Miyoshi myopathy (MM) and limb girdle muscular dystrophy type 2B (LGMD2B), respectively. These diseases are characterized by early adult onset with progressive weakness and marked elevations of serum creatine kinase (CK) levels. Whereas MM affects distal, posterior calf muscles at onset, LGMD2B affects proximal muscles. Dysferlin is a member of a recently recognized family of homologous proteins described as ’ferlins’ (1–5). Little is known about the biological function of ferlin family members. That dysferlin might be important in some forms of signaling is suggested by the finding that each of these proteins has multiple so-called C2 domains. In many other proteins, C2 domains are believed to bind calcium and thereby trigger signal transduction and membrane trafficking events (6). A first step in defining the biological function of dysferlin is to identify proteins with which it interacts. To date, proteins that interact with dysferlin have not yet been reported.

Caveolin-3 is a cardiac and skeletal muscle protein which is a component of caveolae (7). Caveolin-3 is localized to the sarcolemma and believed to play an important role in the formation of caveolae membranes, serving as a scaffolding protein to interact with and organize lipid and protein constituents of caveolae. The importance of caveolin-3 to normal muscle function and viability is underscored by the observation that mutations in the CAV3 gene cause a dominantly inherited limb girdle muscular dystrophy type 1C (LGMD1C) (8). These mutations apparently behave in a dominant negative manner, forcing aggregation of both mutant and wild-type caveolin-3 proteins in the Golgi apparatus (9).

To further characterize the molecular properties of dysferlin, we have conducted studies seeking evidence of dysferlin interacting proteins. Here we present data showing that dysferlin interacts with caveolin-3 in normal human skeletal muscles. Moreover, we detect abnormalities in the expression of dysferlin in skeletal muscle biopsies from two LGMD1C patients who are deficient in caveolin-3. One of the two LGMD1C patients has a novel CAV3 mutation.

*To whom correspondence should be addressed. Tel: +1 617 726 5750; Fax: +1 617 726 8543; Email: brown@helix.mgh.harvard.edu
Correspondence may also be addressed to C. Matsuda. Email: c-matsuda@aist.go.jp
†Deceased.
RESULTS

Dysferlin and caveolin-3 interact in vivo in normal muscles

Because dysferlin is a novel protein of unknown function, we have embarked on several studies to investigate its biological properties. We have used immunoprecipitation to define possible protein binding partners for dysferlin, starting with a family of proteins associated with the muscle membrane. In our initial experiments, we failed to find any evidence that dysferlin co-precipitated with dystrophin, α-, β- and γ-sarcoglycans, and neuronal nitric oxide synthase (nNOS). We also failed to find co-immunoprecipitation of dysferlin with myosin (the fast and slow isoforms) (data not shown). In contrast, co-immunoprecipitation studies did reveal an interaction of dysferlin with the membrane protein caveolin-3. Normal human muscle homogenates were immunoprecipitated with either anti-dysferlin co-precipitated with dystrophin, α-, β- and γ-sarcoglycans, and neuronal nitric oxide synthase (nNOS). We also failed to find co-immunoprecipitation of dysferlin with myosin (the fast and slow isoforms) (data not shown). In contrast, co-immunoprecipitation studies did reveal an interaction of dysferlin with the membrane protein caveolin-3. Normal human muscle homogenates were immunoprecipitated with either anti-dysferlin (Fig. 1A, SalI-1 and BamHI-2) or anti-caveolin-3 antibodies (Fig. 1B). All immunoprecipitates were then subjected to immunoblotting with either a monoclonal antibody to dysferlin or a monoclonal antibody to caveolin-3. As shown in Figure 1B, dysferlin (237 kDa) was specifically immunoprecipitated by anti-caveolin-3 antibody and, reciprocally, caveolin-3 (20 kDa) was specifically immunoprecipitated by the anti-dysferlin antibody, BamHI-2. When muscle was homogenized under mild conditions using a low concentration of detergent (0.1% CHAPS), the SalI-1 anti-dysferlin antibody immunoprecipitated a small amount of dysferlin. With a higher concentration of CHAPS (1%), this antibody immunoprecipitates much more dysferlin from homogenized muscle but fails to co-immunoprecipitate caveolin-3. Each of two different anti-caveolin-3 antibodies co-immunoprecipitates dysferlin (Fig. 1B, lanes 6 and 7), while normal rabbit or goat IgG do not (Fig. 1B, lanes 2 and 5).

Dysferlin expression is not normal in LGMD1C muscles

We next investigated the expression of dysferlin and caveolin-3 in muscles from two dystrophic patients with defined deficiencies in the caveolin-3 (CAV3) gene. Patient 1 has a novel missense mutation substituting proline for threonine at residue 64 (T64P), as described below; patient 2 has a previously reported missense mutation substituting glutamine for arginine at residue 27 (R27Q) (10) (M.Aoki, manuscript in preparation). As reported previously for LGMD1C (8), caveolin-3 staining is reduced but not absent at the muscle cell membrane in these patients (Fig. 2). Dysferlin staining is also present. However, it is patchy and variable in intensity in a pattern that is distinctly different either from normal or MM/LGMD2B muscle. A speckled pattern of cytoplasmic staining of dysferlin is observed in the LGMD1C patient with the T64P mutation but not in the R27Q patient. Immunostaining of α-sarcoglycan, a component of dystrophin–glycoprotein complex, is normal in these patients. Dystrophin is also normal in both LGMD1C and LGMD2B patients on immunofluorescence and immunoblot (data not shown).

In muscles from MM and LGMD2B patients, dysferlin staining is absent as reported previously (11,12). In contrast, caveolin-3 immunostaining is normal in the same muscle. There is no difference in the expression pattern of caveolin-3 between MM and LGMD2B.

Novel mutation in the CAV3 gene

Patient 1, an 11-year-old Japanese girl, was evaluated for progressive, proximal muscle weakness and exercise-induced...
myalgia. She has no family history of any neuromuscular disorders. She showed floppiness of muscle tone at birth. Her motor milestones were marginally delayed: she walked at 1.5 years and did not run until 2 years of age. Serum CK levels were mildly elevated (736 IU/l; normal = 12–200 IU/l). A muscle biopsy demonstrated marked variation in the diameter of fibers from 20 to 100 µm, necrotic and regenerating fibers, increased numbers of central nuclei and a mild increase in fibrous connective tissue. Analysis of the CA\textsubscript{V3} gene revealed a heterozygous mutation substituting cytosine at position 228 for adenine (A228C) (Fig. 3A). This is predicted to produce a novel amino acid substitution, T64P. This change was not detected in members of the patient’s family (Fig. 3B) or in 50 normal control DNA samples as assessed by single strand conformation polymorphism (SSCP) analysis (data not shown). These changes in the CA\textsubscript{V3} gene and protein are novel and predicted to lie within the scaffolding domain (Fig. 3C).

Total levels of dysferlin are nearly normal in LGMD1C muscle

We also examined the expression level of dysferlin in muscle from patients with LGMD1C using immunoblot analysis. In these experiments, the final loading volume of SDS–PAGE was adjusted to normalize densitometric measurements of myosin heavy chain on the Coomassie brilliant blue R 250-stained gels. This analysis revealed normal levels of dysferlin in the T64P patient (Fig. 4, lane 6) and reduced levels in the R27Q patient (Fig. 4, lane 7).

We have also used immunoblotting to assess expression of caveolin-3 in MM and LGMD2B patients. In three of four patients with dysferlinopathies, caveolin-3 was normal (Fig 4, lanes 2–4), while in the one LGMD2B patient, it was reduced (Fig 4, lane 5).

DISCUSSION

The biological function of dysferlin has not yet been defined. Moreover, the fundamental mechanism whereby muscle degenerates in the absence of dysferlin is also unknown. That this differs from the pathophysiology of the most common muscular dystrophy, Duchenne muscular dystrophy (DMD), seems likely. Muscle degeneration in DMD typically begins at childhood (if not in utero) and is associated with marked elevations of the serum CK at birth. In contrast, MM/LGMD2B cases do not begin until after adolescence and, to the best of our understanding at present, do not show marked CK elevations until early adulthood (13).

As an approach to understanding the function of dysferlin and the pathogenesis of muscle cell death in MM/LGMD2B, we have begun studies to identify the molecular interactions of dysferlin. Immunoprecipitation studies using homogenates from normal human skeletal muscle show that dysferlin reproducibly interacts with caveolin-3. Immunoprecipitation of either protein co-precipitates the other.

Our data suggest that the dysferlin–caveolin-3 interaction is probably a low-affinity process. This is indicated in part by the fact that in each experiment, the protein that is immunoprecipitated primarily is considerably more abundant than the co-precipitated protein (e.g. Fig. 1B, lanes 3, 4, 6 and 7). Further, although dysferlin expression is abnormal in LGMD1C muscles by immunofluorescence, caveolin-3 expression appears normal in one of two studied cases of LGMD2B muscles, by both immunostaining (Fig. 2) and immunoblotting (Fig. 4, lane 4, the same case as in Fig. 2). The observation that caveolin-3 deficiency secondarily reduces dysferlin while there is no secondary change of caveolin-3 in dysferlinopathy is precluded. Thus, deficiency of dystrophin causes secondary reduction of sarcoglycans (14) but not vice versa (15). One interpretation of these findings is that caveolin-3 is
more strongly anchored to the membrane and thus does not change when dysferlin is absent, while dysferlin may be less tightly anchored and may, in consequence, be more significantly altered when caveolin-3 is absent.

Although we suspect the dysferlin–caveolin-3 interaction is a low-affinity process, we do not know the mechanism of the interaction. Our data do not distinguish between a direct interaction specifically between these two proteins and an indirect interaction mediated by intervening protein partners. In this context, we note that caveolin-3 has two well-defined binding domains, the scaffolding domain (16) and a WW domain (17). One may therefore predict that if the interaction of caveolin-3 with dysferlin is mediated by these domains, cognate binding sequences should be present in dysferlin. As in Table 1 and Figure 1A, such sequences are present in dysferlin. Dysferlin has seven sequences that conform to the micro-domains believed to bind the caveolin-3 scaffolding region (16,17); these are clustered in two groups, respectively, about a half the length of the protein from the N-terminus and at the transmembrane domain. In this context, we note that two of the predicted scaffolding-binding domains in dysferlin localize to the membrane spanning and extracellular regions; presumably, they are therefore inaccessible for binding to the scaffolding region in caveolin-3. Most molecules reported to be binding to caveolin contain cytoplasmically accessible caveolin-binding sites (16).

To our knowledge, no other studies have documented protein interacting partners for dysferlin. Indeed, the expression level and cellular localization of dystrophin and sarcoglycans are generally normal in muscles affected with MM/LGMD2B (11,18). However, it has been reported that patients with primary pathology of the sarcoglycanopathies have reduced sarcolemmal and increased cytosolic expression of dysferlin (19). The abnormality of localization of dysferlin in LGMD1C and the sarcoglycanopathies are similar. It has also been reported that dysferlin may interact with calpain 3 because there is a secondary reduction in calpain 3 expression in MM/LGMD2B patients as inferred from immunoblotting (20). It is not clear whether an analogous loss or abnormal cellular localization of calpain 3 in MM/LGMD2B is detected using immunostaining.

It is now well established that there are several sets of interacting membrane proteins in the skeletal muscle
membrane (21,22). For example, dystrophin interacts not only with β-dystroglycan and the syntrophins directly but also with intracellular proteins such as nNOS indirectly. Thus, there may be a secondary defect in expression levels of one or more of these proteins when a binding partner is deficient. For example, there is a secondary decrease in levels of α-dystroglycan and nNOS in some (23) but not all (Y.K.Hayashi and K.Arahata, unpublished data) patients with LGMD1C. Analogously, it is possible that there is an indirect interaction of dysferlin with one or more members of these protein families. We have therefore examined the expression levels of both dystrophin and nNOS in MM/LGMD2B muscles (from more than 20 patients) in more detail. Immunoblot analysis shows normal expression of both proteins in these patients (data not shown).

In this report, we also describe a novel mutation in the CAV3 gene. It has been reported that caveolin-3 may be virtually absent in muscle from LGMD1C patients by immunoblotting but not by immunostaining (8). Minetti et al. (8) have shown that a LGMD1C patient with a deletion of three amino acids (threonine-phenylalanine-threonine 64–66, or delTFT) reveals patchy residual membrane immunostaining for caveolin-3, although this protein was not detected on immunoblots of muscle from the same patient. Minetti et al. (8) contrasted the delTFT case results with those from an LGMD1C patient with the missense mutation substituting leucine 105 for proline. In the latter instance, caveolin-3 protein was not detected on immunostaining or immunoblotting. Because their caveolin-3 protein was absent on immunoblotting but weakly present on immunostaining, the two LGMD1C patients in our study are analogous to the delTFT case of Minetti et al. (8). Why there is this difference in the immunostaining patterns of patients with different CAV3 gene mutations is not yet clear. Presumably this reflects some difference in the physical properties (e.g. half-life, subcellular distribution) of the abnormal caveolin-3 proteins expressed by the different mutant CAV3 genes.

Regardless of the mechanism of interaction between dysferlin and caveolin-3, a question generated by these studies is whether there are morphological defects in caveolae in the absence of dysferlin. As recently reported by Selcen et al. (24), in two cases we have seen rather striking disruptions of the structure of the sarcolemma in muscle biopsies from patients with MM. Our cases also demonstrated subsarcolemmal vacuoles, as well as papillary projections of the sarcolemma (data not shown). It is difficult to know whether these vacuoles reflect abnormalities in the composition, positioning or trafficking of caveolae. Further studies are in progress to address this point in detail.

MATERIALS AND METHODS

Antibodies

Two polyclonal rabbit antisera, designated SalI-1 and BamHI-2, were raised against fusion proteins of GST and two human dysferlin fragments (Fig. 1A) (11). The antisera were affinity purified against the respective fragments and used for all experiments. Mouse monoclonal antibody against human dysferlin (NCL Hamlet) was purchased from Novocastra (Newcastle-upon-Tyne, UK) and used for the immunoblotting after immunoprecipitation. Goat monoclonal antibodies against mouse caveolin-3 were purchased from Santa Cruz (Santa Cruz, CA) and Research Diagnostics (Flanders, NJ) and used for immunoprecipitation. Mouse monoclonal antibody to caveolin-3 purchased from Transduction Laboratories (Lexington, KY) was used both for immunostaining and immunoblotting following immunoprecipitation.

Immunoprecipitation

Normal human skeletal muscles were biopsied from biceps and femoral muscle. They were homogenized in a buffer containing 50 mM Tris–HCl pH 7.5, 150 mM NaCl, 0.15% CHAPS (3-[3-(cholamidopropyl)dimethylammonio]-1-propanesulfonic acid) and 1× protease inhibitor cocktail (Complete, Roche Molecular Biochemicals, Basel, Switzerland) on ice. Homogenates were precleared with protein A– or G–Sepharose (Amersham Pharmacia Biotech, Uppsala, Sweden) for 1 h at 4°C. Five hundred micrograms of proteins were incubated first with primary antibodies at 4°C overnight and then with 15 μl of protein A– or G–Sepharose for 1 h at 4°C. Immune complexes were eluted by boiling in 2× SDS–PAGE sample buffer and loaded on 4–20% gradient gels (Bio-Rad, Hercules, CA). Separated proteins in these gels were transferred to nitrocellulose membranes (Hybond ECL, Amersham Pharmacia Biotech) and incubated with specific anti-dysferlin and anti-caveolin-3 antibodies. Detection was performed using ECL detection reagent (Amersham Pharmacia Biotech).

Immunoblot analysis

Immunoblotting was performed as described previously (11).

Patients

MM/LGMD2B patients were diagnosed according to accepted clinical criteria (18). Muscle biopsies were obtained with informed consent following protocols approved by the respective institutions. LGMD1C patients were identified by virtue of having CAV3 mutations in the setting of clinical myopathy with dystrophic muscle pathology. Mutations in the dysferlin and CAV3 genes were identified by SSCP (1) and DNA sequence analysis. Mutations of the dysferlin gene in MM and LGMD2B examined in this study are summarized in Table 2.
Table 2. Mutations in dysferlin gene in MM and LGMD2B patients in this study

<table>
<thead>
<tr>
<th>Phenotype</th>
<th>Mutation</th>
<th>BP change</th>
<th>Consequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>LGMD2B, patient 1</td>
<td>3016 + 1 G→A</td>
<td>Altered 5' splice site</td>
<td>Altered protein</td>
</tr>
<tr>
<td>LGMD2B, patient 2</td>
<td>Y998C</td>
<td>TGG→TGT at 3370</td>
<td>Missense</td>
</tr>
<tr>
<td>MM, patient 1</td>
<td>3017 – 2 A→G</td>
<td>Altered 3’ splice site</td>
<td>Altered protein</td>
</tr>
<tr>
<td>MM, patient 2</td>
<td>Y522X</td>
<td>TAC→TAG at 1939</td>
<td>Nonsense</td>
</tr>
<tr>
<td>D1837N</td>
<td>GAC→AAC at 5882</td>
<td>Missense</td>
<td></td>
</tr>
</tbody>
</table>

SSCP analysis of the CAV3 gene

Genomic DNA was prepared from lymphocyte or skeletal muscle. Exon 2 of CAV3 was amplified using primers 5’-GGGATT- TTAGAAGCTGTAC-3’ [153–173, the numbers correspond to the nucleotides in the nmol/1:234 (8)] and 5’-GCTCTTATGT- CATTGCCACCA-3’ [365–346]. After initial denaturation at 94°C for 2 min, amplification was performed using 35 cycles at 94°C for 30 s, 60°C for 30 s and 72°C for 40 s. The PCR products were separated under non-denaturing conditions using GenePrep (Amersham Pharmacia Biotech) at 10°C. Segregated bands were visualized with PlusOne DNA Silver Staining Kit (Amersham Pharmacia Biotech).

Immunofluorescence

Immunostaining of frozen muscle sections was performed as described by Matsuda et al. (11). Primary antibodies were applied in two double-staining combinations: anti-dysferlin antibody (Safl-1) with caveolin-3 (Transduction Laboratories) or anti-dysferlin with α-sarcoglycan (Novocastra).

ACKNOWLEDGEMENTS

These studies were supported by the C. B. Day Foundation, Inc. (R.H.B. and C.M.), the Muscular Dystrophy Association (R.H.B.), Center of Excellence Research (C.M.), Grants-in-Aid from the Japan Foundation for Aging and Health and the Ministry of Health, Labour and Welfare (Y.K.H., M.A., I.N. and K.A.), Core Research for Evolutional Science and Technology (Y.K.H. and K.A.) and Grants-in-Aid for Scientific Research (B. 12557058) from Japan Society for the Promotion of Science (M.A.).

REFERENCES