Intracellular Accumulation and Reduced Sarcolemmal Expression of Dysferlin in Limb–Girdle Muscular Dystrophies

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Dysferlin has recently been identified as a novel gene involved in limb–girdle muscular dystrophy type 2B (LGMD2B) and its allelic disease, Miyoshi myopathy. The predicted structure of dysferlin suggests that it is a transmembrane protein possibly involved in membrane fusion. Thus, unlike previously identified structural proteins in muscular dystrophy, dysferlin is likely involved in a novel pathogenic mechanism for this disease. In this study, we have analyzed the expression of dysferlin in skeletal muscle of patients with disruptions in the dystrophin–glycoprotein complex and patients with a clinical diagnosis of LGMD2B or Miyoshi myopathy. We show expression of dysferlin at the sarcolemma in normal muscle and reduced sarcolemmal expression along with accumulation of intracellular staining in dystrophic muscle. Electron microscopy in Miyoshi myopathy biopsies suggests that the cytoplasmic staining could be a result of the abundance of intracellular vesicles. Our results indicate that dysferlin expression is perturbed in LGMD and that both mutations in the dysferlin gene and disruption of the dystrophin–glycoprotein complex can lead to the accumulation of dysferlin within the cytoplasm.

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Autosomal recessive limb–girdle muscular dystrophy (LGMD2) is a heterogeneous group of myopathies characterized by a progressive muscle weakness involving the proximal muscles of the limbs and shoulder and pelvic girdles. Eight loci have been associated with LGMD2, and seven proteins have been characterized.[1–3] Commonly, LGMD2 is named by the gene primarily affected and its corresponding protein. Thus, α-, β-, γ-, and δ-sarcoglycanopathy (or LGMD2D, LGMD2E, LGMD2C, and LGMD2F, respectively) designate the LGMD2 cases that have mutations in one of the four sarcoglycan (SG) genes. Although mutations are present only in one of the four sarcoglycan genes, the other three sarcoglycans can be variably decreased or absent from the patient's skeletal muscle.[1,2] In striated muscle, sarcoglycans are associated with dystrophin, the protein responsible for Duchenne and Becker muscular dystrophy (DMD and BMD) and form the transmembrane dystrophin–glycoprotein complex (DGC)[1] along with dystroglycan[4] and sarcospan.[5]

Dysferlin is a recently identified transmembrane protein that has a high degree of homology with the vesicle-specific fertilization factor *fer-1* of *Caenorhabditis elegans*.[6,7] Dysferlinopathies account not only for LGMD2B but also for Miyoshi myopathy (MM),[6–9] an early adult–onset dystrophy manifesting with weakness and atrophy of the distal muscles of the lower limbs.

Although it has been shown that dystrophin and sarcoglycans are normally present at the sarcolemma of patients affected with dysferlinopathy,[10] no extensive studies have been reported about the expression of dysferlin in dystrophinopathies and sarcoglycanopathies. Herein, we detail dysferlin expression in skeletal muscle, and we show by immunohistochemical analysis that dysferlin is decreased at the sarcolemma in nearly half of sarcoglycanopathies and 20% of dystrophinopathies, suggesting a possible interaction between dysferlin and the DGC. We determine biochemically that dysferlin is not an integral component of the DGC, however.

Conversely, we demonstrate that dysferlin accumulates in the cytoplasm of fast fibers in dystrophic muscle in DMD, BMD, LGMD2C–F, and LGMD2B and

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MM with residual amounts of dysferlin. This accumulation of dysferlin seems to be extremely frequent among patients affected with muscular dystrophies, suggesting that abnormalities in the localization of dysferlin are common features of the myopathic process.

Finally, based on the evaluation of the residual amount of sarcolemmal dysferlin, we estimate that dysferlinopathies may account for over half of the cases of LGMD and generic muscular dystrophies excluded from other known loci. The finding of this unsuspected high frequency of dysferlinopathies should have a large impact on the molecular screening of LGMD, with consequences for genetic counseling, prenatal diagnosis, and the development of therapeutic approaches.

Materials and Methods

Dysferlin Antibody

The strategy for producing and purifying polyclonal antibodies has been previously reported.[11] Specifically, to generate a dysferlin polyclonal antibody we synthesized a peptide (YAFPNYAAMKLVKPFS), corresponding to amino acids 2065 through 2080 of the human dysferlin (GenBank accession no. AF075575), with an additional CAA peptide at the N-terminal for coupling purposes. Key-hole limpet hemocyanin-coupled peptide was injected into a New Zealand white rabbit. Affinity purification of dysferlin antibody R264 from the crude serum was accomplished using Immobilon-P (Millipore, Bedford, MA) strips containing the peptide coupled to bovine serum albumin.

Muscle Biopsies

The frozen skeletal muscle specimens used for this study were referred for diagnostic evaluation. Each sample was investigated by hematoxylin and eosin histological testing and by immunofluorescence using antibodies directed against laminin α2, dystrophin, α-SG, and, in some instances, β-, γ-, and δ-SG. Results from this approach were integrated with clinical information and genetic data (when available) to reach a final classification. All the biopsies reported here have normal expression of laminin α2. We classified as DMD and BMD 10 cases displaying abnormal expression of dystrophin and sarcoglycans. We considered as sarcoglycanopathies those 19 cases with normal dystrophin and reduced sarcoglycan expression, regardless of information on gene mutations. Among biopsies showing a normal amount and distribution of dystrophin and sarcoglycans, we identified 5 MM patients based on clinical information alone, 1 LGMD2B patient based on clinical information and a known missense mutation in the dysferlin gene, and 2 distal myopathy patients based solely on clinical features. The remaining cases with normal dystrophin and sarcoglycan expression were classified as LGMD or muscular dystrophy based on dystrophic histological features and clinical data clearly indicating LGMD versus more widespread signs and symptoms. Control cases included muscle tissue from patients with inflammatory myopathy, patients with myopathic clinical features but histologically normal biopsies, and patients without neuromuscular disease undergoing orthopedic surgical procedures.

Immunohistochemical Analysis

Seven-micron-thick cryosections of human muscle biopsies were preincubated for 30 minutes with 3% bovine serum albumin in phosphate-buffered saline. Primary and secondary antibodies were diluted in 1% bovine serum albumin in phosphate-buffered saline. Incubations with primary and secondary antibodies were carried out at room temperature for 90 minutes. The sections were observed under a laser scanning confocal microscope (BioRad MRC1024, Hercules, CA), and images were captured under identical conditions. For peptide competition purposes, dysferlin antibody was separately preincubated with 50 μg of the appropriate peptide for 1 hour at room temperature before proceeding with the immunohistochemical analysis.

With primary dysferlin antibody R264 (1:2 dilution), we used biotinylated anti-rabbit antibody as a secondary antibody and then the FITC- or CY3-conjugated streptavidin (Jackson ImmunoResearch Laboratories, West Grove, PA) following the manufacturer's instructions. Other primary antibodies used according to the manufacturer's instructions for this study were anti-caveolin 3 (anti-CAV-3) (Transduction Laboratories, Lexington, KY), anti-skeletal fast myosin (MyHC; Sigma, St Louis, MO), anti-human merosin (M-chain; Gibco-BRL, Rockville, MD), anti-dystrophin (VIA42), anti-α-SG (IVD3A9),[11] anti-β-SG, anti-γ-SG, anti-δ-SG (βSarc1/5B1, 35DAG/21B5, and δSarc3/12C1, respectively, generated in collaboration with L.V.B. Anderson, Newcastle General Hospital, Newcastle, UK), biotinylated anti-human IgG (Vector Laboratories, Burlingame, CA), and anti-human albumin (Dako, Santa Barbara, CA). The anti-glucose transporter (anti-GLUT4) antibody (1:100 dilution) was a kind gift of Dr Jeffrey Pessin's laboratory at the University of Iowa (Iowa City, IA). Appropriate FITC-conjugated secondary antibodies (Jackson ImmunoResearch Laboratories) were used according to the manufacturer's instructions.

Immunoblotting Analysis

Microsomes, KCl-washed microsomes, crude surface membrane, and DGC were isolated and fractionated from rabbit and mouse skeletal muscle as previously reported.[12] The DGC fractions tested for the presence of dysferlin were previously tested for the simultaneous presence of all the known components of the DGC. Proteins were resolved by 3 to 15% gradient sodium dodecyl sulfate–polyacrylamide gel electrophoresis and then transferred to a nitrocellulose membrane (Immobilon-NC; Millipore). Immunoblot analysis was performed as previously reported,[11] and membranes were detected with SuperSignal West Pico (Pierce, Rockford, IL) according to the manufacturer's instructions and after exposure to X-OMAT (Kodak, Rochester, NY) film. For peptide competition purposes, antibody was preincubated with 50 μg of appropriate peptide for 1 hour at room temperature before proceeding with the immunoblot analysis.

Primary antibodies used in this study were anti-dysferlin R264 (1:20 dilution) and anti-γ-SG (35DAG/21B5). Appropriate peroxidase-conjugated secondary antibodies (Boehringer Mannheim, Indianapolis, IN) were used according to the manufacturer's instructions.

Electron Microscopy

Fresh muscle tissue fragments measuring approximately 0.1 × 0.1 × 0.5 cm were dissected from patient biopsy specimens immediately on their receipt in the laboratory and fixed overnight in 2.5% glutaraldehyde and 0.02 M of cacodylate buffer at 4°C. After postfixation in 1% OsO_4 and 0.02 M of cacodylate buffer, the tissue was dehydrated and embedded in Poly-bed 812 (Polysciences, Warrington, PA). Regions of interest were selected from 1-μm thick sections stained with toluidine blue, and thin sections were evaluated on a Phillips CM10 electron microscope after uranyl acetate and lead citrate staining.

Results

Dysferlin Localizes at the Sarcolemma in Control Skeletal Muscle

The peptide selected to generate dysferlin antibody does not show any homology with proteins or expressed sequence tags (ESTs) present in the GenBank other than human dysferlin, suggesting that this peptide sequence is unique to dysferlin. By comparison of sequences, we also excluded any homology of the dysferlin peptide with the C-terminal of otoferlin (YSVPGYLVKKILGA, 4 of 14 identical amino acids), a dysferlin-like protein recently identified.[13] The dysferlin antibody showed sarcolemmal staining in human control biopsies by immunohistochemical analysis and identified a band at 230 kd in rabbit skeletal muscle membrane preparations by Western blot analysis (Fig 1A and B). The immunocompetition of dysferlin antibody with its antigenic peptide demonstrated complete inhibition of the antibody immunoreactivity by immunohistochemical and immunoblotting analysis (see Fig 1). Additionally, we verified that the immunoreactivity of the dysferlin antibody was not inhibited by the peptide corresponding to the C-terminal of myoferlin (YSLPNYLSMKIVKP, 9 of 14 identical amino acids) (see Fig 1), a second dysferlin-like protein recently described.[14] We also verified that the signal detected with dysferlin antibody was not caused by a background signal of the secondary antibody (see Fig 1). These data, taken together, indicate that dysferlin antibody does not cross-react with other known proteins homologous to dysferlin.

The immunohistochemical analysis of nine human normal control skeletal muscle biopsies with the dysferlin antibody showed that dysferlin localizes primarily to the sarcolemma (Fig 2). Dysferlin staining is discontinuous in a pattern more similar to that of the glucose transporter (GLUT4), a vesicle-associated membrane protein also present at the sarcolemma[15]) than to the homogeneous linear sarcolemmal labeling visualized for caveolin-3 (CAV-3), a muscle-specific component of caveolae[16] (see Fig 2). Dysferlin staining at the sarcolemma is also less uniform than the immunohistological pattern of δ-SG, dystrophin, and laminin α2 (data not shown). At magnifications lower than ×20, a faint cytoplasmic stain has occasionally been detected in control samples. When observed at a higher magnification (×100), a punctate internal pattern (see Fig 2) similar to that of GLUT4 (see Fig 2) is observed with the dysferlin antibody, suggesting that dysferlin may also be present in cytoplasmic vesicles, in particular near the sarcolemma. Additionally, in control biopsies,

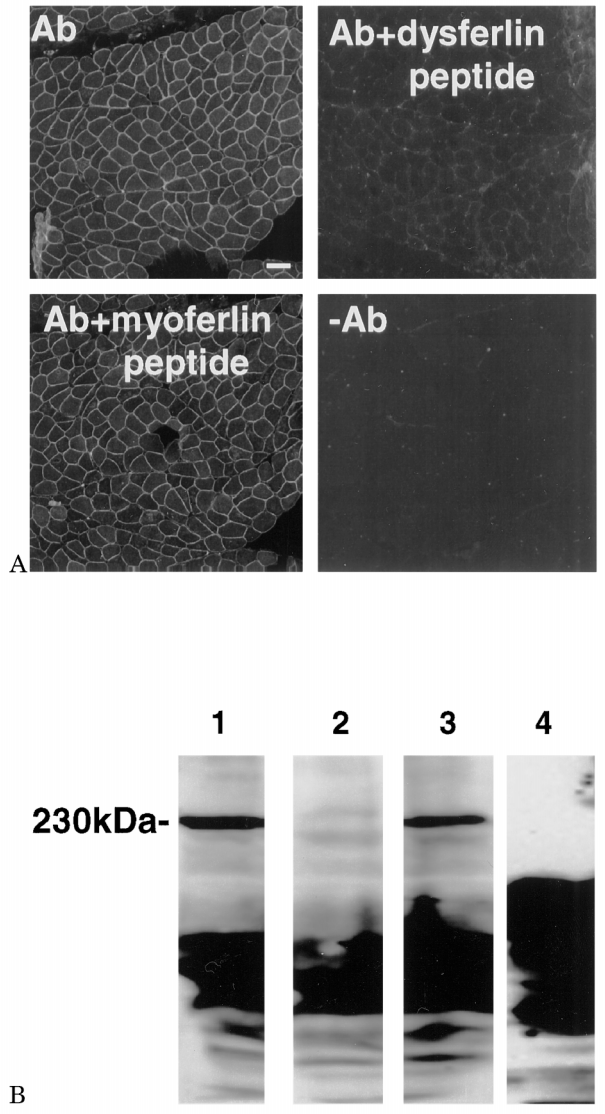

Fig 1. (A) Immunhistochemical analysis with dysferlin antibody (Ab) in control skeletal muscle. Ab immunocompetes with its antigenic dysferlin peptide. Ab does not immunocompete with myoferlin peptide. No background signal is detectable when dysferlin antibody is not applied. Bar = 100 μm. (B) Immunoblot analysis with dysferlin antibody in rabbit skeletal muscle. (1) Dysferlin is detected as a 230-kd band. (2) Ab immunocompetes with its antigenic dysferlin peptide. (3) Ab does not immunocompete with myoferlin peptide. (4) No band at 230 kd is detectable when dysferlin Ab is not applied.

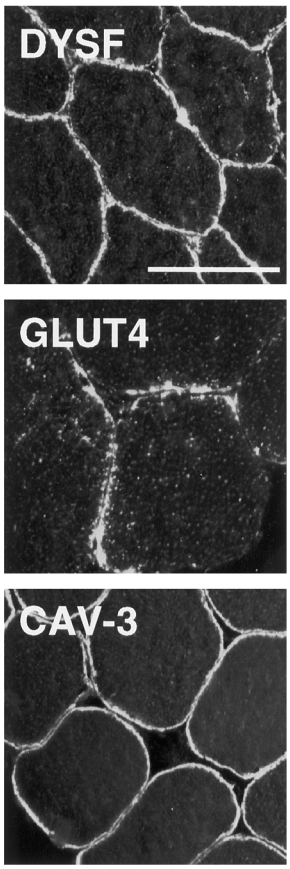

Fig 2. Dysferlin labeling in human control skeletal muscle compared with GLUT4 and CAV-3 labeling. Dysferlin labeling is discontinuous at the sarcolemma and punctate in the cytoplasm. GLUT4 shows the pattern of a vesicle protein that is also present at the sarcolemma. CAV-3 shows the pattern of a sarcolemmal protein. Bar = 50 μm.

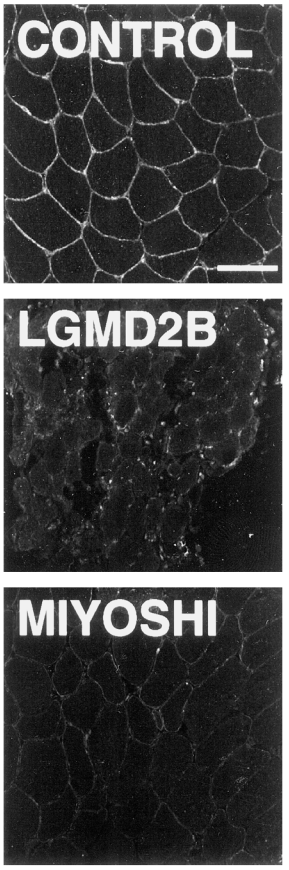

Fig 3. Dysferlin expression in limb–girdle muscular dystrophy type 2B (LGMD2B) and Mioshi myopathy (MM) dystrophic muscles. Dysferlin is absent from the biopsy of an LGMD2B case with a known homozygote missense mutation. Dysferlin is homogeneously reduced in an MM biopsy. Bar = 100 μm.

we have observed that dysferlin is present at neuromuscular junctions (data not shown).

Dysferlin Is Reduced in LGMD2B and MM

We investigated the biopsies of 6 patients with clinical diagnoses of LGMD2B or MM by immunohistological analysis with the dysferlin antibody. We found that dysferlin was totally absent in a case of LGMD2B with a known homozygous missense mutation (Dr Wrogemann, University of Manitoba, Manitoba, Canada, personal communication, 1999) (Fig 3 and Table) and in a case of MM (see Table), confirming that the dysferlin antibody was indeed directed against dysferlin and did not cross-react with other unknown proteins. In the remaining 4 cases, dysferlin was overall reduced, particularly at the sarcolemma (see Fig 3 and Table). In addition, we analyzed 2 cases of distal myopathy (not specified as MM), which also revealed a reduction of the amount of sarcolemmal dysferlin (see Table).

Dysferlin Is Reduced from the Sarcolemma of Sarcoglycanopathies and Dystrophinopathies but Is Not an Integral Component of the DGC

Because of its sarcolemmal distribution and its involvement in LGMD2B, we hypothesized that dysferlin might be a new component of the DGC. To begin to test this hypothesis, we analyzed 29 biopsies from patients characterized by a disruption of the DGC (sarcoglycanopathies [LGMD2C–F] and dystrophinopathies [DMD and BMD]). Although nearly half (10 of 19) of the biopsies from patients with sarcoglycanopathies showed a normal distribution of dysferlin at the sarcolemma (Fig 4B; see Table), the remaining 47% (9 of 19) of biopsies presented a patchy distribution or an overall reduction of dysferlin at the sarcolemma (see Fig 4C and D). Dysferlin was found to be secondarily reduced at the sarcolemma in α-sarcoglycanopathies, β-sarcoglycanopathies, and γ-sarcoglycanopathies, without obvious correlation between the reduction of dysferlin and the sarcoglycan gene primarily mutated (see Table). In dystrophinopathies, dysferlin was unevenly

Table. Summary of the Biopsies Submitted to Immunohistological Analysis with the Dysferlin Antibody

Diagnosis	Gene Mutated	Cases with Normal Sarcolemmal Dysferlin	Cases with Reduced Sarcolemmal Dysferlin	Total Cases
LGMD2B	Dysferlin	0	1	6
MM		0	5	
Distal myopathy	Unknown	0	2	2
DMD/BMD	Dystrophin	8	2	10
LGMD2D	α-SG	1	3	4
LGMD2E	β-SG	0	1	1
LGMD2C	γ-SG	2	1	3
LGMD2C-F	Unknown SG	7	4	11
LGMD	Unknown	6	10	16
Muscular dystrophy	Unknown	13	11	24
Total		37	40	77

LGMD2 = autosomal recessive limb–girdle muscular dystrophy; MM = Miyoshi myopathy; DMD = Duchenne muscular dystrophy; BMD = Becker muscular dystrophy; SG = sarcoglycan.

distributed at the sarcolemma in 20% (2 of 10) of the analyzed biopsies, with the same scattered pattern observed for sarcoglycanopathies (data not shown). The integrity of the sarcolemma of the fibers showing a decreased amount of dysferlin was verified by the normal immunolabeling of CAV-3 (see Fig 4 E–H) at their membranes in serial sections of the same biopsies. The irregular presence of dysferlin at the sarcolemma of patients affected with sarcoglycanopathy or dystrophinopathy indicated a possible interaction between dysferlin and the DGC.

To further analyze dysferlin's relation with the DGC, we performed an immunoblot analysis of membrane-enriched preparations from rabbit skeletal muscle with dysferlin antibody. We detected a band at 230 kd and sometimes minor bands considered to be degradation products in total microsomes, KCl-washed microsomes, and crude surface membranes, suggesting that dysferlin is a membrane protein (Fig 5). A similar pattern was observed for the membrane components of the DGC such as γ-SG (see Fig 5). Dysferlin was not detected in purified DGC fractions (see Fig 5), however, indicating that dysferlin is not an integral component of the DGC.

Dysferlin Accumulates in the Cytoplasm of Fast Fibers in Dystrophic Muscle

In contrast to control biopsies in which dysferlin localizes primarily at the sarcolemma, the immunohistological pattern in dystrophic muscle shows numerous fibers with cytoplasmic staining. We verified that the cytoplasmic labeling was not caused by the secondary antibody background and that it was not detectable when dysferlin antibody was immunocompeted with the dysferlin antigenic peptide (data not shown). The cytoplasmic stain was still present after immunocompetition of dysferlin antibody with the myoferlin peptide (data not shown). Furthermore, we verified that fibers presenting a cytoplasmic signal had not taken up IgG or albumin (data not shown) from the systemic circulation, confirming their sarcolemmal integrity. We also compared serial sections of muscle biopsies stained with hematoxylin and eosin and immunolabeled with dysferlin antibody and found that fibers with cytoplasmic labeling with dysferlin antibody had no distinguishing histological features. For example, dysferlin accumulated to a similar degree in small hypertrophic or normal-sized fibers. Cytoplasmic dysferlin was observed in necrotic fibers and only rarely in regenerating centrally nucleated fibers (data not shown).

Conversely, immunostaining of serial sections with dysferlin and fast myosin heavy chain antibodies indicated that dysferlin accumulates predominantly in fast-twitch fibers (Fig 6). The amount of cytoplasmic dysferlin, however, was quite variable among different fast fibers; some had little or no accumulation of dysferlin, which is similar to the pattern observed for the slow type fibers, although others had bright intracellular staining (see Fig 6B and C). Longitudinal sections showed that this cytoplasmic accumulation of dysferlin was uniform along individual fibers (data not shown). Interestingly, this cytoplasmic staining was observed in biopsies with normal as well as decreased amounts of sarcolemmal dysferlin. Besides a few biopsies with extensive areas of necrosis in which the cytoplasmic pattern of dysferlin was indeterminate, cytoplasmic dysferlin was present in dystrophinopathies (9 of 10 biopsies, 1 indeterminate biopsy), sarcoglycanopathies (17 of 19 biopsies, 2 indeterminate biopsies), and dysferlinopathies with a residual amount of dysferlin at the sarcolemma (4 of 5 biopsies, 1 indeterminate biopsy).

Dysferlin Is Irregularly Localized at the Sarcolemma in Half of the Unclassified Cases of LGMD and Other Muscular Dystrophies

Along with the analysis of known dystrophinopathies, sarcoglycanopathies, distal myopathies, and LGMD2B or MM presented here, we performed a random

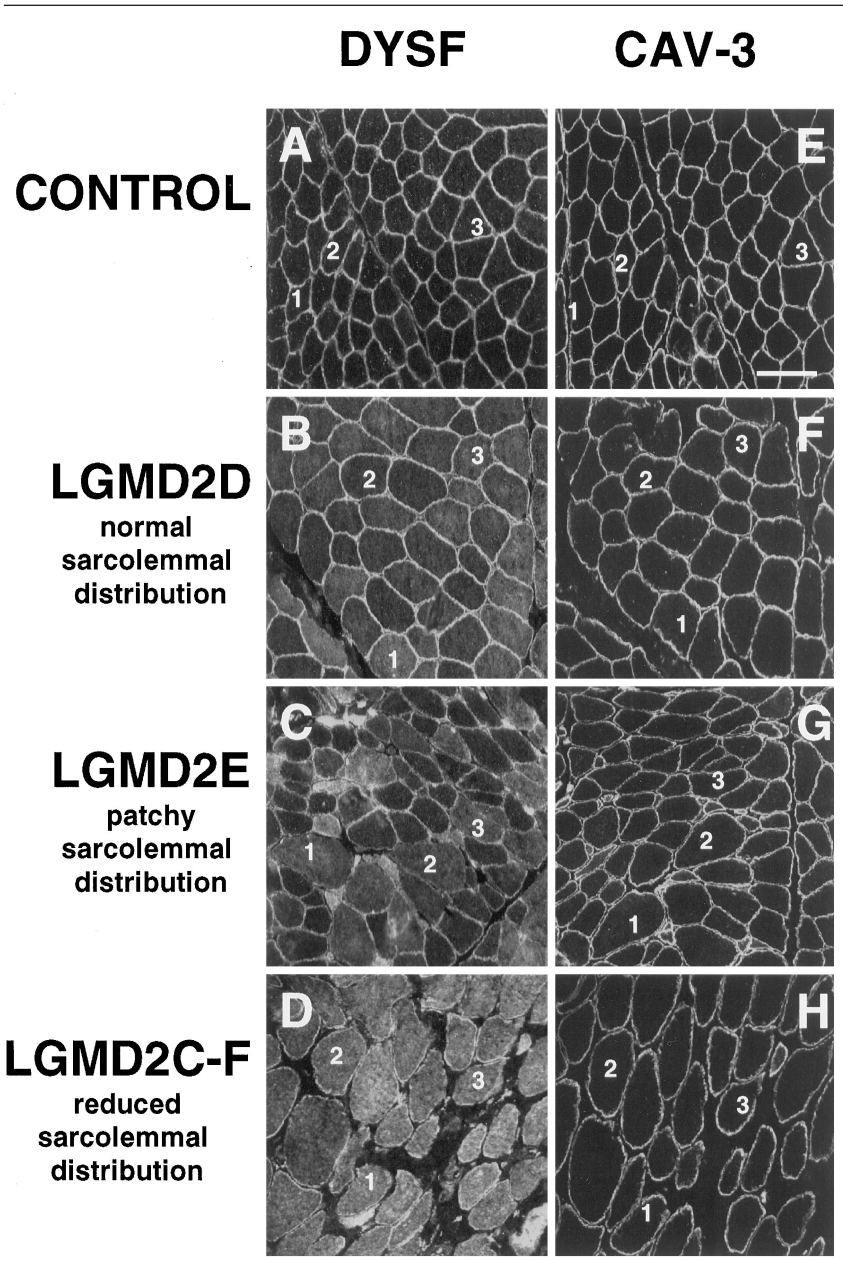

Fig 4. Dysferlin expression in sarcoglycanopathies (LGMD2C–F). (A) Dysferlin labels the sarcolemma in control skeletal muscle. (B) Dysferlin is normally distributed at the sarcolemma (53% of LGMD2C–F). (C–D) Dysferlin is irregularly distributed at the sarcolemma among different fibers (47% of LGMD2C–F). (E–H) Serial sections labeled with CAV-3 to verify the integrity of the sarcolemma. Numbers indicate identical fibers in serial sections. Bar = 100 μm.

screening of 40 additional index cases with dysferlin antibody, leading to a total of 77 biopsies (see Table). These 40 patients were clinically or histologically classified as having "LGMD" or "muscular dystrophy" because of their unclear phenotype. The diagnoses of merosin-deficient congenital muscular dystrophy (CMD), DMD, or BMD as well as LGMD2C–F were excluded by immunohistochemical analysis with antibodies directed against laminin α2, dystrophin, α-SG, and, in some instances, β-SG, γ-SG, and δ-SG, respectively. Fifty-one percent (21 of 40) of these 40 biopsies were discovered to have a reduction of dysferlin at the sarcolemma. We then subdivided these biopsies between LGMD and generic muscular dystrophies, and we observed that dysferlin was irregularly localized and overall decreased in 62% (10 of 16 biopsies) of the first group and 46% (11 of 24 biopsies) of the second.

Similar to our previous observations in dystrophinopathies, sarcoglycanopathies, and dysferlinopathies, cytoplasmic staining was also observed among these 40 patients; it was present in each biopsy with sarcolemmal reduction of dysferlin (21 of 21 biopsies) and in 11 of 19 cases with normal sarcolemmal staining for dysferlin. Conversely, the cytoplasmic labeling was not detectable in 7 cases with normal sarcolemmal dysferlin, and 1 case was indeterminate (data not shown). Biopsies from 3 patients with inflammatory myopathy were also evaluated for expression of dysferlin. Each

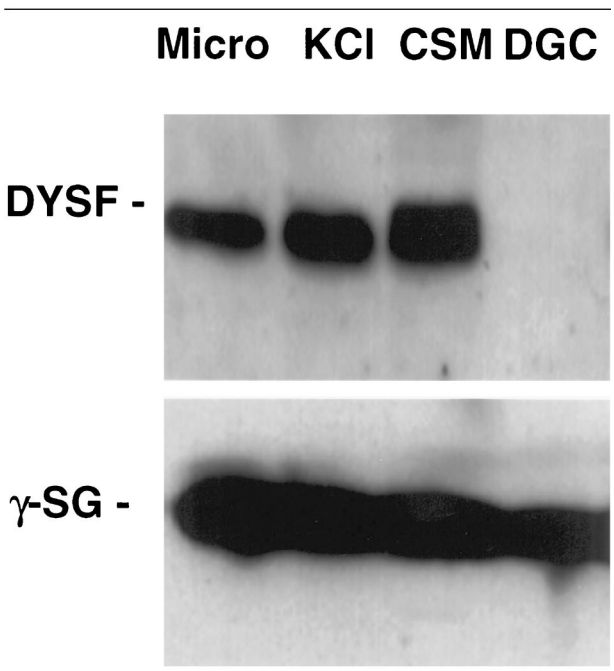

Fig 5. Dysferlin is not an integral component of the dystrophin–glycoprotein complex (DGC). (Upper panel) Dysferlin is present as a band at 230 kd in microsomes, KCl-washed microsomes, and crude surface membranes from rabbit skeletal muscle and is absent from the DGC. (Lower panel) γ-SG expression in equally loaded lanes as a control.

had decreased dysferlin staining at the sarcolemma and accumulation of dysferlin immunofluorescence in the cytoplasm (data not shown).

Immunohistological Pattern of Dysferlinopathies

Hereafter, we designate as "dysferlinopathies" the biopsies presenting an abnormal distribution of dysferlin at the sarcolemma, keeping in mind that this term encompasses "primary dysferlinopathies" as is likely the case in LGMD2B and MM as well as "secondary dysferlinopathies" as is the case for the dystrophinopathies and sarcoglycanopathies reported here.

The overall result of our immunohistological approach on 77 biopsies is that the distribution of dysferlin can be quite different among dystrophic biopsies. Among the 40 dysferlinopathies identified, we found only 3 cases with a complete absence of dysferlin. Most patients had some residual membrane-associated dysferlin and presented the following immunohistochemical patterns. In the type I pattern, the biopsy seemed to be brightly spotted as a result of a mild reduction of dysferlin at the sarcolemma and an abundant accumulation of the protein in the cytoplasm of fast fibers (Fig 7). In these cases, some fibers showed a punctate and continuous labeling at the sarcolemma, which was indistinguishable from that of a control muscle fiber, whereas other fibers did not display any peripheral staining, and still others presented an intermediate dotted labeling. It is worth noting that fibers presenting a cytoplasmic signal could be grouped in some areas of the biopsy, although only a few of these fibers were present in other areas. In the type II pattern, the biopsy looked like a pale mosaic, with an equal reduction of dysferlin at the sarcolemma of each fiber and a modest accumulation of the protein in fast-type fibers (see Fig 7). In some cases, the same biopsy displayed both type I and II patterns in different areas. In the type III pattern, biopsies presented faint and scattered peripheral staining of dysferlin and strong cytoplasmic labeling, which was more evident in fast-type fibers but also present in slow-type fibers (see Fig 7). The MM case presented in Figure 7 is the most noteworthy, because it showed a total absence of dysferlin at the sarcolemma, despite a clear accumulation of cytoplasmic protein. The biopsy of this MM patient is also characterized by the presence of several rimmed vacuoles such as has been reported in other subjects affected by distal myopathy.[17] In fibers with vacuoles, dysferlin either seemed to be homogeneous in the cytoplasm or exclusively concentrated around the vacuole. Nevertheless, dysferlin was absent from the vacuole rim membrane and its lumen. This observation suggests that the vacuoles are not a cytoplasmic pool for dysferlin accumulation or degradation. This last distribution of dysferlin has been observed exclusively in 4 patients classified as having MM, distal myopathy, or generic muscular dystrophy and has never been observed in dystrophinopathies or sarcoglycanopathies.

Electron Microscopy of MM Biopsies

To further investigate the origin of the intracellular labeling detected with the dysferlin antibody, we proceeded to electron microscopic analysis of the biopsies of 2 MM and 2 control cases. In the MM biopsies, no abnormality was found in the sarcomeric structure or in the distribution and structure of organelles such as mitochondria, nuclei, and the Golgi apparatus. The amounts of glycogen and neutral lipid were also similar to those of the controls. One striking difference from controls was the presence in the MM muscles of an increased number of vesicles. Electron micrographs in Figure 8 contrast the subsarcolemmal regions of control and MM muscle, showing accumulation of vesicles under the plasma membrane and elsewhere in the cytoplasm in MM fibers. The signal detected by immunofluorescence with dysferlin antibody, apparently at the sarcolemma in this MM biopsy, could actually come from the "ring" of docking vesicles accumulating under the plasma membrane. The technical limits of immunohistochemical analysis did not allow us to discriminate between signal originating from the sarcolemma or from the subsarcolemmal vesicles.

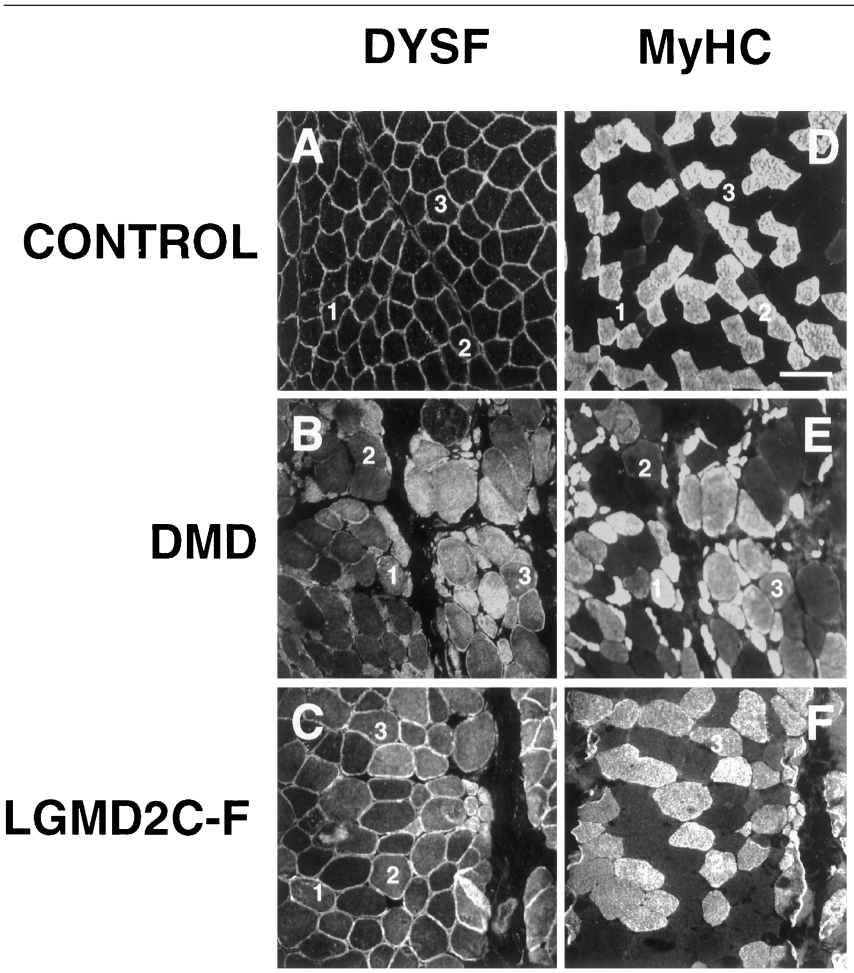

Fig 6. Immunohistological pattern for dysferlin in slow- and fast-type fibers. (A and D) In control skeletal muscle, the dysferlin pattern is equal in slow- and fast-type fibers (labeled with anti-skeletal fast myosin-MyHC). (B and E) Dysferlin accumulates predominantly in fast-type fibers in dystrophinopathies. (C and F) Dysferlin accumulates predominantly in fast-type fibers in sarcoglycanopathies. Serial sections are displayed in each row. Numbers indicate identical fibers in serial sections. Bar = 100 μm.

Discussion

In the past few years, it has been demonstrated that the disruption of the link between the cytoskeleton and the extracellular matrix underlies apparently unrelated muscular dystrophies such as DMD, LGMD, and congenital muscular dystrophies. The dramatic consequences of the disruption of the DGC on membrane integrity have been well documented recently in several animal models[18–23] and in vitro systems.[24]

When we initially studied dysferlin expression in healthy and dystrophic skeletal muscle, we hypothesized that dysferlin could be a new component of the DGC for three reasons. First, there is a phenotypic similarity between LGMD2B and sarcoglycanopathies (LGMD2C–F). Second, dysferlin primary sequence predicts a single transmembrane domain,[6,7] which is a common characteristic of the sarcoglycans. Third, we observed an overlap between dysferlin and dystrophin/sarcoglycan subcellular localization at the sarcolemma and neuromuscular junction in skeletal muscle. This hypothesis was at first reinforced by the finding that nearly half of Sarcoglycanopathies and 20% of dystrophinopathies have a patchy distribution or a decreased amount of dysferlin at the sarcolemma by immunohistochemical analysis. Nevertheless, we formally demonstrated by DGC purification and fractionation that dysferlin is not an integral component of the DGC. Consequently, dysferlin does not seem to be involved in the dystrophic process through the direct disruption of the link between the cytoskeleton and the extracellular matrix as is the case for dystrophin and sarcoglycans, and a new pathogenetic mechanism should therefore be hypothesized.

Dysferlin primary sequence predicts a homology with the *C. elegans fer-1* factor, a vesicle-specific protein present in spermatozoa, and a role for dysferlin in membrane fusion has been hypothesized.[6,7] Here, for the first time, we report evidence that dysferlin can accumulate within muscle cells, possibly in vesicles, and we suggest a potential mechanism leading to muscular dystrophy.

In two MM biopsies, in which the dysferlin antibody revealed an accumulation of the protein in the cytoplasm and an absence or extreme reduction of dysferlin at the sarcolemma (see Figs 7 and 8), we found an excess of vesicles in the cytoplasm by electron mi-

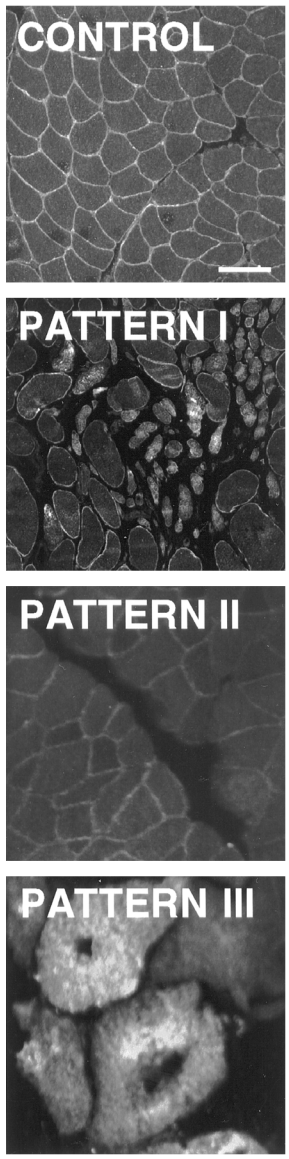

Fig 7. Dysferlin immunohistological pattern in biopsies with residual amount of protein. Dysferlin is equally distributed at the sarcolemma of control skeletal muscle fibers. (Pattern I) Some fibers have bright cytoplasmic staining in a field of fibers with a reduced or normal amount of sarcolemmal dysferlin (limb–girdle muscular dystrophy type 2D shown as an example). (Pattern II) Dysferlin is homogeneously reduced at the sarcolemma of each fiber and equally accumulated in the fibers displaying cytoplasmic staining (unknown muscular dystrophy shown as an example). (Pattern III) Dysferlin is nearly absent from the sarcolemma of most of the fibers and accumulates unequally in their cytoplasm (Miyoshi myopathy shown as an example). Bar = 100 μm.

croscopy (see Fig 8). Although this excess of vesicles does not formally prove that the cytoplasmic immunofluorescence detected with the dysferlin antibody comes from these vesicles, it is reasonable to hypothesize that dysferlin is a component of these vesicle membranes. Dysferlin primary sequence indicates the presence of three full and two incomplete C2 domains at the N-terminal.[13] C2 domains interact with negatively charged phospholipids and proteins to trigger the fusion of vesicles to the plasma membrane.[25] In control biopsies, we found that the amount of cytoplasmic dysferlin is just above the threshold of resolution of light microscopy. Indeed, the presence of dysferlin at the sarcolemma could indicate a dynamic status of transit during the physiological process of membrane turnover. In dystrophic muscle, dysferlin-carrying vesicles could accumulate in the intracellular compartment of the fiber, responding to a still unknown stimulus related to the dystrophic pathway. A potentially similar process has been observed in cardiomyocytes, where GLUT4, a vesicle protein, is translocated from the storage vesicles to the transverse tubules and the sarcolemma after muscle contraction or insulin secretion.[26] In the case of LGMD2B and MM patients with presumed primary mutations in the dysferlin gene, our antibody directed against the C-terminal of the protein still detected an intense cytoplasmic signal. Thus, we speculate that abnormal dysferlin molecules could be present at the vesicle membrane but that fusion between the vesicle and the sarcolemma does not occur. This results in substantially decreased immunofluorescent staining of the sarcolemma. An analogous situation has been observed in *fer-1* mutants, where the vesicles contributing membrane and glycoproteins to the plasma membrane of spermatozoa[27] are retained in the cytoplasm and are unable to fuse with the plasma membrane.[28] We also observed that dysferlin is decreased at the sarcolemma and accumulates in the cytoplasm of some patients characterized by the disruption of the DGC. Analogous to what we observed in MM biopsies, an excess of unidentified vesicles has also been reported in γ-sarcoglycanopathies[29] and DMD.[30] In these cases, we hypothesize that dysferlin is correctly localized in vesicle membranes but that the sarcolemmal interacting partner for the C2 domains of dysferlin is destabilized by the disruption of the DGC, blocking the vesicle fusion process. Further investigations are likely to arise if this sarcolemmal dysferlin-interacting partner is a component of the DGC or other, as yet unknown, membrane proteins. Alternatively, dysferlin could be a membrane component of the sarcoglycan-carrier vesicles in skeletal muscle, which are secondarily eliminated during the degradation of vesicles carrying abnormally assembled sarcoglycan complexes.[31] From this point of view, the normal amount of sarcolemmal dysferlin observed in most DMD and BMD biopsies would be consistent with the fact that the decrease of sarcoglycans at the membrane of DMD and BMD patients is unrelated to the sarcoglycan complex translocation and is secondary to the loss of the dystrophin and the consequent instability of the DGC.

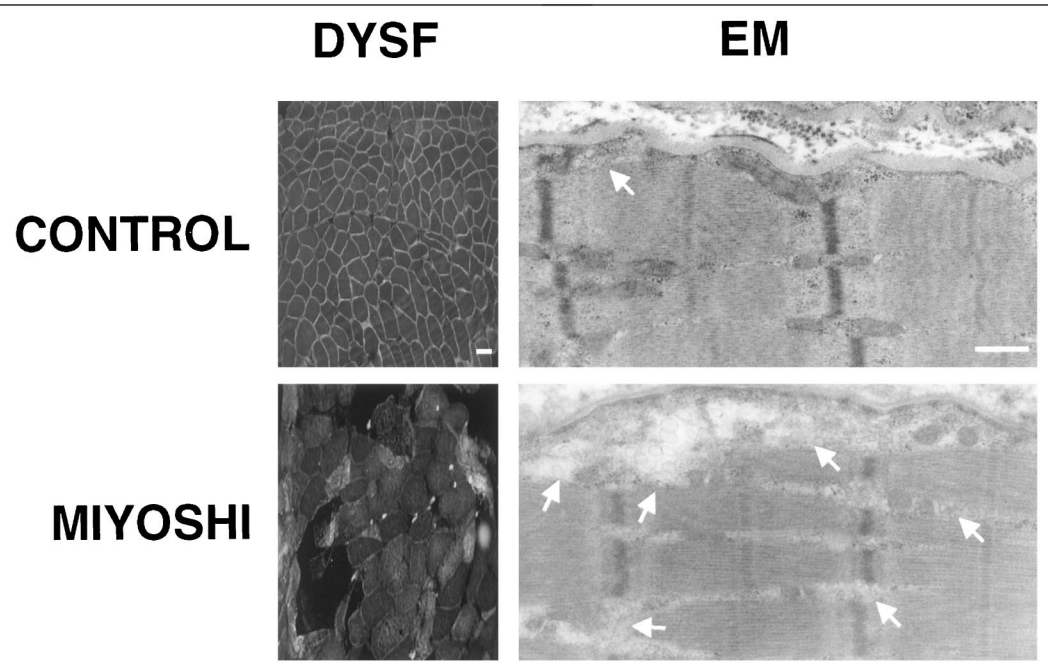

Fig 8. Immunohistochemical analysis and electron microscopy. In control skeletal muscle, dysferlin is present at the sarcolemma, and few vesicles localize under the plasma membrane. In a Miyoshi myopathy (MM) biopsy, dysferlin is reduced at the sarcolemma and accumulates unequally in the cytoplasm, although a large number of vesicles (indicated by arrows) are present under the plasma membrane and in the cytoplasm. Bar for immunohistochemical analysis = 100 μm. Bar for electron microscopy = 1 μm.

The variable amount of dysferlin accumulating in DMD and LGMD2C–F fast fibers and the scarcity of highly bright fibers (usually unequally distributed in the biopsy) are in accord with the results of a previous Western blot analysis reporting that a normal amount of dysferlin was present on a few LGMD2C–F biopsies analyzed.[10] At the same time, in LGMD2C–F biopsies, the uneven distribution of dysferlin at the sarcolemma and cytoplasm suggests that dysferlin could have a local modifier effect, which could partially explain the phenotypic variability observed in patients.

As we reported, the fibers accumulating cytoplasmic dysferlin in dystrophic biopsies are predominantly fast-twitch fibers. It has been demonstrated in DMD that the fast fibers undergo extensive necrosis and regeneration before the slow fibers are affected.[32] Nevertheless, in the late stages of DMD as well as in LGMD2B and MM, the fast and slow fibers seem to be equally affected. This suggests that dysferlin could play a role in the early stages of the disease rather than in the late stages. Although dysferlin is expressed early in human development,[10] LGMD2B and MM are early adult–onset myopathies without evidence of muscle development abnormalities. Additionally, the SJL mouse, an animal model of dysferlinopathy,[33] has been known for a decade for its increased muscle regeneration capacity.[34] Furthermore, we found dysferlin variably reduced at the sarcolemma and accumulated in the cytoplasm of three inflammatory myopathy biopsies used as controls for non-DGC–related myopathies. Thus, assuming that dysferlin plays a role in membrane fusion in mammalian skeletal muscle as *fer-1* does in *C. elegans*,[35] we expect dysferlin to be more likely involved in a repair activity, which is exacerbated in damaged fibers, than in a growth or regeneration process of the fiber.

As we mentioned, a diagnosis of "dysferlinopathy" based on the residual amount of sarcolemmal dysferlin is complicated by the cytoplasmic labeling. Nevertheless, we analyzed a cohort of 40 patients with undefined diagnoses of LGMD or muscular dystrophy, and we estimated that dysferlin was reduced in half of them. Further investigations of patients with dysferlin deficiency are likely to occur if these patients have mutations in the dysferlin gene itself or elsewhere. Indeed, the loss of dysferlin could be a secondary effect caused by the mutations in another gene(s) as has been demonstrated for the sarcoglycanopathies. Because of the presence of uncharacterized human ESTs homologous to dysferlin in the databases, we can anticipate that the family of "fer-like" genes is likely to be enlarged in the coming years. Some of these gene products could compensate for the reduction of dysferlin in LGMD2B and MM, thus accounting for their phenotypic variety. Finally, more information on dysferlin function and its relation to other proteins is likely to lead to the discovery of additional candidate genes for muscular dystrophies.

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