Calcium-sensitive Phospholipid Binding Properties of Normal and Mutant Ferlin C2 Domains*

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Mutations in dysferlin, a novel membrane protein of unknown function, lead to muscular dystrophy. Myoferin is highly homologous to dysferlin and like dysferlin is a plasma membrane protein with six C2 domains highly expressed in muscle. C2 domains are found in a variety of membrane-associated proteins where they have been implicated in calcium, phospholipid, and protein-binding. We investigated the pattern of dysferlin and myoferlin expression in a cell culture model of muscle development and found that dysferlin is expressed in mature myotubes. In contrast, myoferlin is highly expressed in elongated “prefusion” myoblasts and is decreased in mature myotubes where dysferlin expression is greatest. We tested ferlin C2 domains for their ability to bind phospholipid in a calcium-sensitive manner. We found that C2A, the first C2 domain of dysferlin and myoferlin, bound 50% phosphatidylserine and that phospholipid binding was regulated by calcium concentration. A dysferlin point mutation responsible for muscular dystrophy was engineered into the dysferlin C2A domain and demonstrated reduced calcium-sensitive phospholipid binding. Based on these data, we propose a mechanism for muscular dystrophy in which calcium-regulated phospholipid binding is abnormal, leading to defective maintenance and repair of muscle membranes.

The ferlin family is an emerging group of mammalian proteins implicated in genetic disease. The ferlin family is named for its homology to the Caenorhabditis elegans protein fer-1 (1). fer-1 mutants exhibit fertility defects because of aberrant membrane fusion in developing sperm that results in an abnormal submembraneous vesicular accumulation (2). The mutations in a human ortholog of fer-1, dysferlin, have been associated with two different forms of muscular dystrophy in humans, Miyoshi myopathy (MM) and Limb Girdle muscular dystrophy 2B (LGMD2B) (2–4). The function of dysferlin and the mechanism by which dysferlin mutations lead to muscle dysfunction are unknown. It was recently reported (5, 6) that an abnormal accumulation of submembraneous vesicles is a feature of dysferlin mutant muscle.

Myoferlin is highly related to dysferlin and like dysferlin is also found at the plasma membrane in heart and skeletal muscle (7). To date, myoferlin has not been linked with any human disease, but its location on human chromosome 10q24 and its expression pattern make it a candidate gene for an autosomal dominant spastic paraplegia, SPG9, a disorder associated with muscle wasting and weakness (8). Based on homology to fer-1 and their predicted protein domain structure, the mammalian ferlins may have evolved specialized roles for cell type-specific membrane fusion. Myoferlin and dysferlin each have a single carboxyl-terminal transmembrane domain and six C2 domains that are predicted to reside in a large cytoplasmic domain (9).

Crystallographic studies have shown that the C2 domain is an independently folding domain composed of eight β strands forming a β sandwich structure (10, 11). Calcium binding loops reside at one end of the β sandwich structure, and calcium binding is mediated through a conserved group of aspartic acid residues. C2 domains are present in many membrane-associated proteins (12, 13). Proteins directly implicated in membrane fusion such as the synaptotagmins contain two C2 domains. The first C2 domain of synaptotagmin binds calcium and anionic phospholipids (14, 15). The second synaptotagmin C2 domain aids in protein-protein interactions and homo-oligomerization (16) and more recently was shown to bind phospholipids (17, 18). Most recently, data have emerged supporting the role of synaptotagmins as calcium sensors regulating the process of fast exocytosis (19, 20). In synaptotagmins, up to three calcium ions are known to bind at three loops at the end of the β sandwich structure.

Because of the predicted protein structure, domain function, and expression of dysferlin and myoferlin at the sarcolemma, we hypothesized that dysferlin and myoferlin are important for membrane fusion, because they occur during muscle development and during muscle repair. To assess this hypothesis, we studied the expression of ferlin proteins in an in vitro model of muscle development (21). Mature skeletal muscle is a syncytium that forms from the fusion of mononucleated myoblasts to multinucleate myotubes. We found that myoferlin was highly expressed in myoblasts that have elongated prior to fusion to myotubes. After fusion, myoferlin expression was decreased. Dysferlin expression increased concomitant with fusion and maturation of myotubes. Given that ferlins are expressed in the correct spatiotemporal pattern to play a role in membrane fusion events in muscle, we studied the biochemical properties of myoferlin and dysferlin C2 domains. We found that the first C2 domain, C2A, bound a negatively charged phospholipid mixture similar to the phospholipid composition of the inner surface of the plasma membrane (22, 23). Furthermore, C2A

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‡ The abbreviations used are: MM, Miyoshi myopathy; LGMD2B, Limb Girdle muscular dystrophy 2B; GST, glutathione S-transferase; PC, phosphatidylcholine; PS, phosphatidylserine

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binding to phospholipids was calcium-sensitive. A point mutation in the C2A domain of dysferlin that causes muscular dystrophy showed abnormal calcium sensitivity and reduced phospholipid binding. Together, these data suggest a role for the mutated proteins in membrane fusion and a novel mechanism for muscular dystrophy.

**EXPERIMENTAL PROCEDURES**

**C2C12 Expression**—C2C12 cells were cultured and harvested at different timepoints in differentiation. Reducing serum content in the medium to 2% induced differentiation. Cells were collected, resuspended in lysis buffer (50 mM HEPES, pH 7.5, 150 mM NaCl, 2 mM EDTA, 10% glycerol, 1% Triton X-100) with protease inhibitors (Complete protease inhibitor mixture, Roche Molecular Biochemicals), and incubated on ice for 10 min. Cellular debris was removed by brief centrifugation at 8,000 g for 5 min at 4 °C, and the protein concentration of the supernatant was assayed with a Bio-Rad protein assay. Free calcium concentrations were calculated with WEBMAXC version 2.10 (www.porchlab/webmaxc2.htm) (24). All experiments were carried out in MilliQ water (Millipore) at room temperature, and a 0.1-M CaCl₂ was used for making all of the solutions. Proteins were washed three times in the specified calcium buffer, and 7.5 μM of [3H]phosphatidylcholine (Amersham Biosciences), lipids were pelleted by centrifugation at 15,000 rpm for 30 min. The supernatant containing soluble fusion protein was recovered. Fusion proteins were immobilized on glutathione-Sepharose, washed four times in buffer A (50 mM HEPES, pH 7.4, 0.1 M NaCl, 2 mM EGTA), and used for lipid binding assays essentially as described previously (15). Liposomes were made from a total of 1 mg of lipid (Avanti Lipids, Alabaster, AL) plus 10 μCi of [3H]phosphatidylcholine (Amersham Biosciences), lipids were mixed, dried under nitrogen, dried under vacuum overnight, and resuspended in 2 ml of buffer A. The lipid suspension was extruded through a 0.1-μm filter in an extruder (Avanti Lipids) to give uniform-sized vesicles. 7.5 × 10⁹ cpm of lipids were used per assay with 15 μg of protein. Protein was quantitated by Bio-Rad protein assay. Free calcium concentrations were calculated with WEBMAXC version 2.10 (www.stanford.edu/~cpuntun/webmaxc2.htm) (24). All experiments were carried out in MilliQ water (Millipore) at room temperature, and a 0.1 M calcium standard (Orion) was used for making all of the solutions. Proteins were washed three times in the specified calcium buffer, and lipid mixtures were brought to the appropriate free calcium concentration before mixing for the assay. Protein and lipid were incubated for 15 min at 24 °C with continuous vortexing. Sepharose fusion protein was collected by centrifugation at 14,000 rpm for 1 min and washed three times in the appropriate calcium buffer. ²H-Lipid was quantitated by
scintillation counting. Assays were performed in triplicate with at least two separate lipid and protein preparations. Assays performed in the presence of 1 mM KCl, 1 mM MgCl₂, or 1 M NaCl were treated in the same manner. Curve-fitting and binding constant calculations were performed using Origin 6.1 software (Origin Lab Corp., Northampton, MA). For dysferlin C2A lipid binding assays, 4 × 10⁵ cpm of lipids were used, and results were normalized to 15 μg of protein.

RESULTS

Expression of Dysferlin and Myoferlin in Myoblast Differentiation—To investigate the potential role of dysferlin and myoferlin in skeletal muscle membrane fusion, we examined the expression of dysferlin and myoferlin during myoblast differentiation in C2C12 cells, a cell line that undergoes differentiation from mononucleate myoblasts to multinucleate syncytial myotubes (21). Cultures representing different phases of fusion and differentiation in C2C12 cells were harvested and tested for dysferlin and myoferlin expression (Fig. 1A). Immunoblotting with an antibody specific to dysferlin (7) showed that dysferlin was expressed at the greatest levels in mature myotubes (Fig. 1A, bottom, and B, lane 3). An antibody specific to myoferlin (7) demonstrated that myoferlin is expressed at the greatest levels in cultures with high numbers of prefusion myoblasts (Fig. 1A, top, and B, lane 1). The level of myoferlin protein decreased after fusion of myoblasts to myotubes had occurred (Fig. 1B, lane 3).

To elucidate further the timing of myoferlin expression, we tested C2C12 cultures at different stages of myoblast differentiation for expression of myoferlin using indirect immunofluorescence and an antibody specific to myoferlin. We noted high magnification views of myoferlin expression in C2C12 cells during myocyte differentiation demonstrated that myoferlin is expressed in vesicles within the myoblasts (Fig. 2, E and F, arrow).

**DISCUSSION**

**Ferlin Phospholipid Binding**

A Single Myoferlin C2 Domain Binds to Phospholipids—All six myoferlin C2 domains were generated as GST fusion proteins (Fig. 4A). Each purified fusion protein was tested for its ability to bind to phospholipid vesicles containing lipids found in the cytoplasmic surface of the cell membrane in muscle (22, 23). Lipid vesicles containing 50% phosphatidylserine (PS) in phosphatidylcholine (PC) bound myoferlin C2A in the presence of 1 mM calcium (Fig. 4B). The lipids composed of 50% PS did not show binding to any of the other five myoferlin C2 domains, C2B, C2C, C2D, C2E, and C2F (Fig. 4B). None of the six myoferlin C2 domains demonstrated binding to 100% PC vesicles in the presence or absence of calcium (Fig. 4C). These findings are consistent with the phospholipid binding properties of a number of homologous C2 domains that similarly do

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**Fig. 3. Alignment of myoferlin and dysferlin C2A with synaptopagmin I C2A.** A, a schematic representation of dysferlin and myoferlin protein. Each of the six predicted C2 domains are shown in gray boxes, and the transmembrane domain is shown in black. B, Myoferlin and dysferlin C2A domains are aligned with a consensus sequence for C2 domains. This consensus sequence was obtained using evolutionarily divergent C2 domains (rat synaptopagmin II, IV, and VI, an Arabidopsis putative C2 domain protein, and rat protein kinase C) from the Conserved Domain Data Base (www.ncbi.nlm.nih.gov/Structure/cdd/cdd.shtml) using the SMART version 3.3 alignment. Because the secondary structure of a number of these C2 domains has been solved (13), the likely position of calcium binding is indicated (circles), and the positioning of the β strands is indicated (bars). Seven of the eight β strands are indicated; the eighth is positioned to the right of the diagram and is less conserved. The capital letters indicate those residues that are important for the C2 structure, particularly those of the β strands, and predicted calcium-coordinating residues. The letters in gray boxes are invariant in evolutionarily divergent C2 domains. The asterisk indicates a dysferlin point mutant responsible for muscular dystrophy (31) whose calcium and phospholipid binding properties we studied.

**Table 1.** Comparison of Predicted C2 Domains of Dysferlin and Myoferlin with Other C2 Domains.

| Domain | Dysferlin C2A | Myoferlin C2A | Other C2 Domains |
|--------|----------------|---------------|------------------|
| Length | 110 residues | 110 residues | 110 residues |
| homology | high | high | high |
| conserved residues | >75% | >75% | >75% |
| Predicted calcium binding residues | yes | yes | yes |
| Predicted phospholipid binding | yes | yes | yes |

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**Fig. 4.** Lipid binding properties of myoferlin C2A. A, primary structure of myoferlin C2A, shows the location of the predicted calcium binding residues. Based on sequence alignments, we predicted that these domains have type II topology similar to those seen in cytosolic phospholipase A₂, the non-classical protein kinase C isoforms δ, ε, η, and ζ, and phosphoinositide-specific phospholipase C isoforms. This topology prediction is based on the presence of the predicted calcium binding residues between the first and second β strands and between the fifth and sixth β strands as seen in all topology II C2 domains (13). The C2 domains at the amino terminus of proteins typically use topology II (13). The calcium binding sites of topology II C2 domains, similar to dysferlin and myoferlin C2A, have been noted to often lack one or more of the five conserved aspartic acid or glutamic acid residues and to contain alternative residues capable of coordination (13).

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**Fig. 5.** The primary structure of myoferlin C2A, shows the location of the predicted calcium binding residues. Based on sequence alignments, we predicted that these domains have type II topology similar to those seen in cytosolic phospholipase A₂, the non-classical protein kinase C isoforms δ, ε, η, and ζ, and phosphoinositide-specific phospholipase C isoforms. This topology prediction is based on the presence of the predicted calcium binding residues between the first and second β strands and between the fifth and sixth β strands as seen in all topology II C2 domains (13). The C2 domains at the amino terminus of proteins typically use topology II (13). The calcium binding sites of topology II C2 domains, similar to dysferlin and myoferlin C2A, have been noted to often lack one or more of the five conserved aspartic acid or glutamic acid residues and to contain alternative residues capable of coordination (13).
not bind neutral phospholipid (15) but instead demonstrate binding to the negatively charged PS (14, 27). The lipids containing 25% PS in PC showed no binding to myoferlin C2A, C2C, or C2F regardless of calcium concentration (data not shown), suggesting that a significant presence of negatively charged phospholipid is required for the interaction with myoferlin C2A. Also, 50% phosphatidylinositol and 50% phosphatidylethanolamine vesicles were similarly tested and did not bind to any of the six myoferlin C2 domains (data not shown).

**Binding Properties of Myoferlin C2A to Phosphatidylserine**—A range of calcium concentrations was tested to determine the binding constant of myoferlin C2A for 50% PS. C2A was unable to bind PS-containing vesicles in the presence of normal intracellular calcium levels (0.1 μM); however the ability to bind rapidly increased with half-maximal lipid binding observed at 1 μM (Fig. 5A). The interaction of myoferlin C2A with PS is abolished in the presence of high salt, consistent with the predicted electrostatic interaction (Fig. 5B). The requirement for calcium is also specific, because in the presence of Mg2+ or K+, phospholipid binding did not occur (Fig. 5B).

**Mutation in Dysferlin C2A Abolishes Lipid Binding**—Mutations in dysferlin lead to two forms of muscular dystrophy, MM and LGMD2B (3, 4). A large number of mutations have been described to date (28–34), and there is a phenotypic range of symptoms that varies from mild to severe associated with these mutations. We tested the ability of dysferlin C2A to bind lipid vesicles containing 50% PS and found that dysferlin C2A demonstrated similar binding properties to myoferlin C2A (Fig. 6A). Dysferlin C2A bound 50% PS with a half-maximal lipid binding at 4.5 μM calcium. A mutation in dysferlin was recently described (V67D, GenBank™ accession number AF075575) that is associated with LGMD (31). Individuals with the V67D mutation exhibited a range of phenotypic severity consistent with both MM and LGMD2B. This mutation alters a residue within the C2A domain of dysferlin and introduces an acidic residue for a conserved non-polar residue within a β strand.
A point mutation in dysferlin (V67D) was described as being associated with muscular dystrophy (31). Dysferlin C2A was expressed as a GST fusion protein and purified from E. coli. A, dysferlin C2A binds 50% PS in a calcium-dependent manner (solid line). A missense amino acid substitution (V67D) that causes muscular dystrophy was engineered and tested for its ability to bind phospholipids through a range of calcium concentrations (dashed line). Partial phospholipid binding was detected at lower calcium concentrations and was diminished at higher calcium concentrations (dashed line). Abnormal calcium-dependent phospholipid binding combined with an abnormal accumulation of submembraneous vesicles in dysferlin mutant muscle (5, 6) suggests that membrane fusion is abnormal in dysferlin-mediated muscular dystrophy. B, the expression and purification of the normal and V67D dysferlin C2A domain are shown in lanes 1 and 2, respectively.

**DISCUSSION**

Similar to most C2 domain-containing proteins, myoferlin and dysferlin are found associated with membranes. The predicted topology of dysferlin and myoferlin places the six C2 domains within the cytoplasm anchored by their carboxyl-terminal transmembrane regions. In a cell culture model of myoblast differentiation, dysferlin was expressed at low levels in myoblasts and increased its expression upon differentiation as myoblasts fused to myotubes. In contrast, myoferlin was expressed highly in the prefusion myoblast. These prefusion myoblasts were distinguished from quiescent myoblasts by their elongated appearance and an accumulation of myoferlin-containing vesicles. The fusion of myoblasts to myotubes is known to involve vesicular structures (35). Similarly, the repair of torn surface membranes uses a number of mechanisms that may also include vesicular structures (36). Because of their intracellular location, the spatiotemporal pattern of expression, and domain structure, we hypothesize a coordinated role of myoferlin and dysferlin where myoferlin may be specialized for myoblast fusion and dysferlin may be important for membrane fusion and repair in the mature myotube. To examine the potential role of ferlin proteins in membrane fusion events, we studied the biochemical properties of ferlin C2A domains and found that myoferlin and dysferlin C2A domains bind phospholipid in a calcium-sensitive fashion.

The ferlin proteins are the only known C2 domain-containing proteins that have more than three C2 domains, and the ferlin proteins are unique with six C2 domains. In other C2 domain-containing proteins, a single C2 domain is often responsible for calcium-dependent phospholipid binding. We tested all six C2 domains of myoferlin and found that only C2A demonstrated the binding to phospholipids under our experimental conditions. The role of the remaining ferlin C2 domains is unknown, but similar to other C2 domain-containing proteins, these domains may be involved in protein-protein interactions. The requirement for the negatively charged phospholipid may be influenced by the number of positively charged residues in the calcium binding loops of the dysferlin/myoferlin C2A domain. A negatively charged lipid membrane domain is necessary for membrane fusion to occur (37, 38). Additionally, the myoblast membrane is associated with an unusual composition of phospholipids that is distinct from fibroblasts and consistent with the phospholipid binding profile of C2A (22, 23). The requirement for 50% PS for C2A binding suggests that C2A may respond to lipid clustering in the membrane or to specific regions of the membrane with higher concentrations of PS.

Myoblast fusion is critical during muscle development and in the repair of existing muscle fibers damaged through regular use and exercise and in degenerative disorders like muscular dystrophy. Myoblast fusion has been shown to require an influx of calcium from the extracellular space (39). It has been estimated that the fusion of myoblast membranes requires calcium concentrations in the range of 1.4 μM (40), and localized pools of increased calcium are present near the regions of membrane fusion (41). Dysferlin and myoferlin C2A phospholipid binding occurred at calcium concentrations above 1 μM, consistent with calcium requirements for myoblast fusion. The repair of membrane tears also requires calcium (36). It is known that in animal models for muscular dystrophy, these membrane tears occur more frequently (42, 43). An elevation of myoferlin has been observed in microsomal fractions from this same animal model (7), potentially reflecting an increased need for membrane repair machinery in this damage-susceptible muscle.

The first C2 domain of dysferlin demonstrated similar calcium and phospholipid binding properties to myoferlin C2A. The cooperativity of binding of these two C2 domains is consistent with binding multiple calcium ions. The C2A domains are found at the extreme amino termini of dysferlin and myoferlin, most remote from the transmembrane domain at the carboxyl terminus. We speculate that this placement may indicate a role for the C2A domain in localizing the amino terminus to the plasma membrane or possibly in the attraction of ferlin-containing vesicles to the plasma membrane. In support of this finding, it has been noted that dysferlin mutant muscle displays an increase in vesicle accumulation under the membrane (5, 6). Additionally, dysferlin interacts with caveolin, another membrane-associated protein important for intracellular vesicular trafficking (44).

Dysferlin missense mutations may alter dysferlin function.
Illarioshkin et al. (31) described a mutation associated with both mild and severe phenotypes that changed a single amino acid (V67D) within the C2A domain of dysferlin. This amino acid substitution occurs within one of the eight β strands and inserts a novel charge residue. Molecular modeling suggests that this residue is important to the core structure of the C2 domain (data not shown). Dysferlin C2A-V67D showed partial phospholipid binding at lower calcium concentrations but minimal phospholipid binding at higher calcium concentrations. The peak intracellular calcium concentration within a myofiber can be as high as 14 μM (45), thus it is highly probable that this mutation alters phospholipid binding of dysferlin in vivo.

There are two important membrane fusion events in skeletal muscle. Myoblasts fuse into myotubes during muscle development in the embryonic state, and in the adult, this process occurs to repair and regenerate muscle fibers that have been damaged. The increased expression of myofillin specifically in the prefusion myoblast suggests that this protein plays a critical role in this event. By demonstrating the ability of myofillin and dysferlin to bind calcium and phospholipids, two of the key players in muscle membrane fusion, we have now placed ferlin family members in the correct temporal and spatial position for a role in membrane fusion in the development and repair of muscle.

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