Dysferlin Interacts with Annexins A1 and A2 and Mediates Sarcolemmal Wound-healing*

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Mutations in the dysferlin gene cause limb girdle muscular dystrophy type 2B and Miyoshi myopathy. We report here the results of expression profile analyses and in vitro investigations that point to an interaction between dysferlin and the Ca\(^{2+}\) and lipid-binding proteins, annexins A1 and A2, and define a role for dysferlin in Ca\(^{2+}\)-dependent repair of sarcolemmal injury through a process of vesicle fusion. Expression profiling identified a network of genes that are co-regulated in dysferlinopathic mice. Co-immunofluorescence, co-immunoprecipitation, and fluorescence lifetime imaging microscopy revealed that dysferlin normally associates with both annexins A1 and A2 in a Ca\(^{2+}\) and membrane injury-dependent manner. The distribution of the annexins and the efficiency of sarcolemmal wound-healing are significantly disrupted in dysferlin-deficient muscle. We propose a model of muscle membrane healing mediated by dysferlin that is relevant to both normal and dystrophic muscle and defines the annexins as potential muscular dystrophy genes.

Mutations in the dysferlin gene DYSF cause limb girdle muscular dystrophy (LGMD)\(^1\) type 2B and Miyoshi myopathy (1–3). Dysferlin is a 230-kDa muscle membrane protein (4, 5) that shows homology to the Caenorhabditis elegans FER1 protein thought to mediate fusion of intracellular vesicles to the sperm plasma membrane (6). Dysferlin has a single transmembrane domain at its C terminus and six C2 domains along the length of the cytoplasmic domain (7). Recent studies have illustrated Ca\(^{2+}\)-dependent binding of the first C2 domain of dysferlin to phospholipid; a mutation that causes muscular dystrophy negatively affects this binding (8). Dysferlinopathic muscle tissue reveals an accumulation of vesicles at the plasma membrane (9, 10). We have demonstrated previously a weak interaction between dysferlin and caveolin-3, a skeletal and cardiac muscle protein that organizes lipid and protein components of caveolae (11).

To elucidate the function of dysferlin, we examined gene expression patterns in normal and dysferlin-deficient mice at different ages and in different muscle compartments. We have employed a novel analysis algorithm, Relevance Networks (12), that correlates relative gene expression levels in high-density microarray samples. The algorithm identified a novel cluster of genes whose relative expression levels are highly correlated in all dysferlinopathic samples. Examination of this network prompted us to investigate further the roles of annexin A1 and annexin A2 in the dysferlinopathies.

Annexins are widely expressed Ca\(^{2+}\) and phospholipid-binding proteins that are implicated in membrane trafficking, transmembrane channel activity, inhibition of phospholipase A\(_2\) and cell-matrix interactions (13). The functions of many of the annexins are not clear. However, annexins A1 and A2 have been shown to aggregate intracellular vesicles and lipid rafts in a Ca\(^{2+}\)-dependent manner at the cytosolic surface of plasma membranes in many cell types (14, 15). Annexin A1 mediates this aggregation by forming a heterotetramer with S100A11, and annexin A2 has been postulated to have a similar relationship with S100A10 (p11) (16).

This investigation provides the first evidence for a Ca\(^{2+}\)-dependent interaction between dysferlin and annexins A1 and A2. After a membrane injury, there is disruption of dysferlin binding to annexin A1, Ca\(^{2+}\)-dependent vesicle aggregation, and fusion with the surface membrane. We show that this membrane repair process is severely upset in dysferlinopathic myotubes. These findings confirm the disrupted membrane healing seen in dysferlin knockout mice (10) and extend that work by suggesting possible interacting partners for dysferlin. We propose a central role for dysferlin in “patch” fusion events that compose a novel wound healing model in skeletal muscle sarcolemma.

MATERIALS AND METHODS

Microarray Sample Preparations—For the SJL/J and SWR/J samples, 10 animals of each species were killed at 6 weeks and at 8 months of age. Each group of 10 mice was divided into two groups of 5 mice each. RNA was extracted from one quadriiceps and one gastrocnemius from each mouse in the group, and a pooled RNA sample from each group was prepared for hybridization to the Affymetrix murine MG-U74Av2 arrays (Affymetrix, Santa Clara, CA), as described previously in detail (17). The remaining muscles were retained for subsequent immunohistochemical and Western blot analysis.

Affymetrix software was used to calculate the relative expression signal of each gene from the average difference of intensities between matching and mismatched probe-pairs designed to hybridize a particular sequence. Although we obtained gene expression data using vari-
our parameters such as age and muscle group, the distinction used for analysis of relevance networks in this study was whether the samples were normal (SWR/J) or dysferlin-deficient (SJL/J).

Relevance Networks—Relevance networks were generated (18) by using the ReNet software developed by Atul Butte of Children’s Hospital Bioinformatics Program, Boston.2 Raw data from microarrays run with samples from the gastrocnemius and quadriceps of 6-week- and 8-month-old SWR/J and SJL/J were uploaded to the ReNet software. The ReNet algorithm comprehensively compares all gene probe sets with each other in a pair-wise manner and generates networks of highly correlated genes. Networks were initially generated encompassing all samples with a correlation threshold of 0.95 and subsequently from control and dysferlin-deficient samples separately with a correlation threshold of 0.965.

Co-immunoprecipitation—SWR/J protein homogenate (1 mg) was resuspended in radioummode precipitation assay buffer (50 mm Tris-HCl, pH 8.0, 150 mm NaCl, 1% Nonidet P-40, 12 mm deoxycholic acid). Immunoprecipitations with the polyclonal annexin antibodies were performed using Biomag protein A magnetic beads (Qiagen, Valencia, CA). Immunoprecipitation experiments using the monoclonal dysferlin antibody, NCL-Hamlet, were performed using the Catch and Release kit (Upstate Biotechnology, Lake Placid, NY).

Cell Culture—Primary myoblast cells were cultured from 2-day-old SWR/J and SJL/J pups as described previously (19) in medium containing 20% fetal bovine serum and 2% chick embryo extract in Dulbecco’s modified Eagle’s medium at 37 °C in 5% CO2. Cells were induced to differentiate and fuse at 30–50% confluency by switching to serum-deprived medium (2.5% horse serum in Dulbecco’s modified Eagle’s medium).

Tissue Section Immunocytochemistry—Eight-micron-thick tissue sections from SWR/J and SJL/J quadriceps were fixed for 4 min with ice-cold methanol/acetone (1:1) and preincubated for 30 min with phosphate buffered saline containing 10% (v/v) goat serum prior to staining with primary antibodies using established methods. The primary antibodies were applied in three double-staining combinations as indicated. Images were collected with an Eclipse E800 M microscope (Nikon, Melville, NY) and Spot RT Software (Sterling heights, MI).

Fluorescence Lifetime Imaging Microscopy (FLIM)—SWR/J myotubes were preincubated for 10 min at 37 °C in PBS containing either 1.8 mm CaCl2 or 10 mm EGTA. Cells were injured by dragging a scalpel blade twice across the surface of the dish, in the presence of blue-fluorescent secondary antibody. Decay times were measured with a commercial multiphoton microscope (Radiance 2000, Bio-Rad) with attached Ti:Saphire laser (Tsunami, Spectra Physics) and a fast microchannel plate detector (Hamamatsu, Bridgewater, NJ) connected to high-speed time correlated single photon counting acquisition hardware (SPC-830; Becker & Hickl, Berlin; data was analyzed using SPCImage software (Becker & Hickl)). To investigate the interaction of two proteins, the cells were incubated with both the donor 488-nm-labeled dysferlin antibody and a 594-nm (annexins A1 and A2) or 568-nm (annexin A2) labeled acceptor antibody attached to the protein of interest.

Scrape Wounding and Expression of Lamp-1—SWR/J and SJL/J myotubes were preincubated for 10 min at 37 °C in PBS containing either 1.8 mm CaCl2 or 2 mm EGTA. Cells were injured by dragging a scalpel blade twice across the surface of the dish in the presence of Texas Red dextran (Molecular Probes). Dishes were transferred to ice and cells were fixed in PBS containing 4% paraformaldehyde for 15 min and blocked in PBS containing 10% goat serum for 30 min prior to incubation with primary antibodies diluted in PBS containing 0.5% Triton-X (v/v) for 60 min at 37 °C. Baseline decay times for FLIM were gathered as described (20) from injured and non-injured muscle sections (Fig. 2, a and b). Normal sarcosomal distribution of dystrophin in SJL/J mice confirms that the abnormal localization of annexin A2 is not an artifact of an already disrupted membrane (Fig. 2c). Although no dys-

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2 Available from www.chip.org/relnet/.
ferlin is evident on Western analysis of SJL/J muscle homografts (Fig. 1a), some expression of this protein is visible on immunofluorescent staining of tissue sections (Fig. 2, a and b) because of the higher sensitivity of this technique.

**Injury State and Ca\(^{2+}\)/H\(\text{1001}\) Modulate the Binding of Annexins to Dysferlin—**Dysferlin contains six calcium-responsive C2 domains; binding of calcium to at least one of these domains (C2A) is essential for the binding of dysferlin to phospholipid (8). Both annexins A1 and A2 are capable of binding to and aggregating phospholipids in a Ca\(^{2+}\)-dependent manner (15, 16). The aggregation and fusion of intracellular vesicles at the plasma membrane in response to a membrane injury event has been demonstrated in several non-muscle cell types (normal rat kidney, 3T3, L6E9, and Chinese hamster ovary) and has been shown to be Ca\(^{2+}\)-dependent (23). To investigate whether the interaction of dysferlin and the annexins is affected by the injury state of the sarcolemma, we performed immunofluorescent staining followed by fluorescence lifetime imaging microscopy on injured or intact SWR/J myotubes in the presence or absence of Ca\(^{2+}\) (Fig. 3). FLIM measures the decay half-life of fluorescent molecules immunologically attached to individual proteins. A shortening of the lifetime of a donor fluorophore in the presence of an acceptor indicates fluorescent resonance energy transfer between them. FLIM allows sensitive measurement of protein-protein interactions on a spatial scale of <10 nm (20).

A scalpel blade was used to injure SWR/J myotubes in culture in the presence of Ca\(^{2+}\) or EGTA. Injured cells were identified by the presence of membrane impermeable blue dextran (Fig. 3a). The fluorescence lifetime of the immunofluorescent label on the dysferlin antibody was measured alone or in the presence of a fluorescently labeled annexin antibody (Fig. 3, b–d). The faster decay time of the fluorophore attached to dysferlin in the presence of one attached to annexin A1 indi-
cates that annexin A1 associates with dysferlin in non-injured cultured myotubes in the presence of Ca\(^{2+}\) and that a disruption of the membrane destroys this association (Fig. 3b). In uninjured myotubes, independent of Ca\(^{2+}\), a 568-nm fluorophore attached to annexin A1 significantly reduced the decay half-life of the 488-nm fluorophore attached to dysferlin from a baseline of 2.2 ns \(\pm\) 0.28 ns to 1.8 ns \(\pm\) 0.07 ns. In injured myotubes, there is no significant FLIM-detectable association between dysferlin and annexin A1. Annexin A1 did not associate with dysferlin when intracellular Ca\(^{2+}\) was depleted by pre-incubation with EGTA (Fig 3b).

Annexin A2 is shown to associate with dysferlin in both non-injured and injured cells in the presence of Ca\(^{2+}\) (Fig. 3c). In these experiments, the directly labeled antibody attached to dysferlin has a baseline fluorescence lifetime of 2.3 \(\pm\) 0.27 ns. In the presence of Ca\(^{2+}\), this is significantly reduced to 1.6 \(\pm\) 0.07 ns in intact myotubes and 1.5 \(\pm\) 0.40 ns in the injured myotubes. No reduction is seen in the absence of Ca\(^{2+}\).

Annexin A6 did not demonstrate any significant interaction with dysferlin in cultured myotubes (Fig. 3d). These data combined suggest that annexins A1 and A2 may have significant but functionally distinct interactions with dysferlin, and this interaction may be mediated through the unique N-terminal domains of annexins A1 and A2 and not a conserved domain that would also be present on annexin A6. It is also possible that dysferlin and the annexins might form a complex with other as yet unidentified proteins to mediate their functions. The baseline decay times shown were unaffected by injury state or Ca\(^{2+}\) concentration.

Intracellular Vesicles Fuse with the Sarcolemma Post-injury—Reddy et al. (23) have reported that Ca\(^{2+}\)-regulated exocytosis of lysosomes follows plasma membrane injury in several cell types. To determine whether the same is true in skeletal muscle, we looked for the presence of the luminal domain of the lysosomal protein Lamp-1 on the surface of cultured myotubes post-injury. Injured myotubes were positively identified by uptake of the membrane-impermeable dye Texas Red dextran from the media during the injury event. Myotube membranes were not permeabilized during the immunofluorescent staining procedure, allowing the selective identification of surface-expressed protein. In the presence of Ca\(^{2+}\), sarcolemmal expression of Lamp-1 was detected on 86% of dextran-positive SWR/J (dysferlin-positive myotubes, line arrows) but not on uninjured cells (Fig. 4, a and d, open block arrows). Chelation of Ca\(^{2+}\) with EGTA in the SWR/J dishes significantly (p < 0.004)
reduces the amount of surface Lamp-1 seen post-injury with only 48% of dextran-positive cells with detectable Lamp-1 (Fig. 4, b and d). We found that, in the presence of calcium, 60% of cultured SJL/J myotubes (dysferlin-negative) stained positive for Lamp-1 expression at the sarcolemma post-injury (Fig. 4, c and d), indicating a significant ($p < 0.04$) reduction in the number of membrane repair events in these cells compared with control. The Ca$^{2+}$-independent repair process in these cells however remained active, with 41% of injured cells expressing Lamp-1 in dishes preincubated with EGTA (Fig. 4d).

This Lamp-1 detection level is not significantly different from that of normal (SWR/J) cells in the absence of Ca$^{2+}$.

**Dysferlinopathic Sarcolemma Has Significantly Reduced Healing Ability**—To determine whether a disruption of dysferlin expression has an effect on the efficiency of sarcolemmal repair, SWR/J and SJL/J myotubes were loaded with the fluorescent Ca$^{2+}$ indicator, Indo1-AM, and changes in [Ca$^{2+}$]$_i$ were monitored while the sarcolemma was disrupted with a micropipette. Several cells from each preparation of cultured myotubes were recorded (Fig. 5, a and b), and triplicate preparations were used to determine mean recovery times (Fig. 5d). SWR/J cells displayed a transient elevation of [Ca$^{2+}$]$_i$ in response to membrane disruption that returned to normal levels within a few seconds (Fig. 5a, red traces). This return to normal Ca$^{2+}$ levels was significantly delayed in myotubes cultured from SJL/J mice (Fig. 5a, green trace). Concurrent measurements taken in uninjured cells show that no photobleaching of the Ca$^{2+}$ indicator occurred for the duration of the experiments (Fig. 5b). Note that traces from injured myotubes (Fig. 5a) have been scaled to standardize baseline Indo1-AM ratios and times of injury for easier comparison, whereas traces from uninjured cells (Fig. 5b) have not been scaled to demonstrate the similarities in baseline ratios between separate dishes. From the individual traces, mean values for the recovery in each species were determined (Fig. 5c), and mean recovery half-times (time to recover to half maximal ratio) were calculated (Fig. 5d). Normal myotubes (SWR/J) have a mean recovery half-time of 4.7 ± 1.5 s. SJL/J myotubes have a mean recovery half-time of 16 ± 2.7 s, which is significantly longer than normal ($p < 0.006$). Also, measurements taken from injured SJL/J myotubes at regions distant from the sites of injury (traces not shown) indicate normal calcium sequestration and clearance in these cells, as the recovery half-time for these regions is 8 ± 1.7 s (SJL/J Away).

**DISCUSSION**

Here we report the differential patterns of gene expression between normal and dysferlin-deficient mouse muscle and the existence of a group of highly co-regulated genes in dysferlinopathic muscles. Analysis of this group has lead to the discovery...
of two novel functional binding partners of dysferlin, annexin A1 and A2, with significant implications for understanding the normal function of this protein in muscle.

Previous studies have examined the expression profiles of genes in both human and animal cases of muscular dystrophy (17, 24–27). In this study, the expression profiles of normal versus dysferlin-deficient mouse muscles were determined (supplementary Table I). The majority of gene changes reported here closely correlate with previous studies on muscular dystrophy as well as representing the inflammatory phenotype previously detailed in SJL/J mice (28, 29).

Much recent work has focused on making functionally relevant interpretations from high density microarray gene expression profiles. Clustering analysis of genes has become a standard method of extracting potentially useful interaction information from data sets (22). The guiding theory is that groups of genes that are clustered together (i.e., highly correlated in terms of relative expression levels over many samples) are likely to be co-regulated and/or involved in biologically related processes. Recent work in Saccharomyces cerevisiae has demonstrated a statistically significant link between genes that cluster together and known protein-protein interactions (30). In this study, we demonstrate the usefulness of a clustering algorithm, termed relevance networks (12), in extracting functionally related genes from mouse muscle microarray data, independent of species, muscle group, or disease-state. Further, we demonstrate that there exists a network of genes whose correlation is specific to the dysferlinopathic state. Members of this network represent strong candidates for further study into the pathogenesis of muscle atrophy in LGMD2B and Miyoshi myopathy. Among the genes in this network are markers of inflammatory processes and several modulators of the actin cytoskeleton, as well as the calcium-responsive phospholipid-binding annexins A1 and A2.

Our experiments suggest a novel Ca\(^{2+}\)-dependent interaction between dysferlin and annexins A1 and A2 that may play a role in the aggregation and fusion of intracellular vesicles in response to membrane injury (Fig. 6). This model is highly analogous to the patch fusion models of membrane healing proposed for non-muscle cells (31, 33). In this patch fusion model, dysferlin sits, while in the resting state, on the sarcolemma, while annexins A1 and A2, in monomeric or oligomeric forms, are localized to the subsarcolemmal region where they can interact with the dysferlin cytoplasmic domain (Fig. 6a). Membrane injury resulting from mechanical stress on the muscle fiber allows the influx of Ca\(^{2+}\) along a steep concentration gradient. The increased intracellular Ca\(^{2+}\) concentration sets in motion a sequence of events (as yet not elucidated) that results in the aggregation of intracellular vesicles, such as lysosomes, to form a hydrophobic "patch," which then translacates to and fuses with the sarcolemma to prevent further damage to the cell (Fig. 6b). Dysferlin is proposed to act as a Ca\(^{2+}\)-dependent "hook" that then enables the efficient fusion of the repair patch with the sarcolemma.

Our data support this model in at least four regards. First, we have shown for the first time the co-localization of both annexins A1 and A2 with dysferlin at the sarcolemma of normal muscle and the disruption of this localization in dysferlinopathic muscle sections. Our co-immunoprecipitation experiments demonstrate the ability of dysferlin to pull down both annexins in vitro. The Ca\(^{2+}\) and injury dependence of this association is evident from our FLIM studies. By using fluorescein as a donor molecule immunologically attached to dysferlin, we were able to measure a significant interaction between dysferlin and annexin A1 in resting, uninjured cells at physiological Ca\(^{2+}\) levels but not in injured cells. Annexin A2 associated with dysferlin in a calcium-dependent manner in both resting and injured cells. Second, as predicted by previous studies and confirmed by us, the repaired sarcolemma of an injured myocyte contains markers of the intracellular vesicles (such as Lamp-1) that have fused with it. That this vesicle fusion process is disrupted in dysferlin-deficiency is shown by the reduction of post-injury Lamp-1 expression in cultured SJL/J myotubes. In the absence of Ca\(^{2+}\), there is a significantly reduced but measurable amount of Lamp-1 expression in both normal and dysferlin-deficient myotubes post-injury. This suggests that there is an alternate mechanism of membrane repair that is independent of Ca\(^{2+}\) influx and dysferlin deficiency.

Third, our kinetic analyses of Ca\(^{2+}\) flux reveal a consistent retardation of membrane healing in myotubes in the absence of dysferlin. In our view, this strongly supports the model proposed above while at the same time demonstrating that patch fusion membrane repair can nonetheless occur, albeit more slowly, without dysferlin. This assay confirms recent work in a dysferlin knockout mouse that also shows slower than normal membrane healing (10).

Finally, we note that these experimental findings are supported by clinical reports that (i) electron microscopy of dysferlin-deficient LGMD muscle reveals membrane disruption and...
unfused vesicle populations (32), and (ii) serum CK levels in dysferlin-deficient patients are extremely high, indicative of "leaky" membranes (1). Two intriguing aspects of our model are noted. First, calpain might be implicated in this repair process, as annexins A1 and A2 are susceptible to cleavage by this enzyme, and mutations in the calpain gene have also been associated with limb-girdle muscular dystrophy type 2A (33). The mechanism whereby calpain mutations cause muscular dystrophy remains to be defined. In our model, calpain cleavage of annexins A1 and A2 may be critical for patch formation and/or membrane insertion. The second interesting aspect of our model is the possible involvement of caveolin-3. Mutations in caveolin-3 cause muscular dystrophy (LGMD1C) by an unknown mechanism (34). A weak interaction between dysferlin and caveolin-3 has been reported (11). Annexin A2 is known to organize lipid microdomains; mutant forms of caveolin-3 may disrupt annexin A2 localization and perturb the patch fusion repair process. Our model also has implications for the normal roles of members of the ferlin family in cells other than muscle where cellular membranes are frequently distended and flexibility and efficient healing are likely to be critical. Examples are otoferlin in sensory hair cells (35), FER1 in spermatocytes (6), and dysferlin in monocytes (36), which, when differentiated into macrophages, extend and retract expansive membrane processes. Finally, annexins A1 and A2, which respectively map to human chromosomes 9q21.3 and 4q31.3, must now be considered strong candidates in the search for mutations leading to proximal limb girdle weakness in patients with no apparent mutations in DYSF or any other LGMD gene.

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