Reduced Plasma Membrane Expression of Dysferlin Mutants Is Attributed to Accelerated Endocytosis via a Syntaxin-4-associated Pathway*

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Ferlins are an ancient family of C2 domain-containing proteins, with emerging roles in vesicular trafficking and human disease. Dysferlin mutations cause inherited muscular dystrophy, and dysferlin also shows abnormal plasma membrane expression in other forms of muscular dystrophy. We establish dysferlin as a short-lived (protein half-life ~4–6 h) and transitory transmembrane protein (plasma membrane half-life ~3 h), with a propensity for rapid endocytosis when mutated, and an association with a syntaxin-4 endocytic route. Dysferlin plasma membrane expression and endocytic rate is regulated by the C2B-FerI-C2C motif, with a critical role identified for C2C. Disruption of C2C dramatically reduces plasma membrane dysferlin (by 2.5-fold), due largely to accelerated endocytosis (by 2.5-fold). These properties of reduced efficiency of plasma membrane expression due to accelerated endocytosis are also a feature of patient missense mutant L344P (within FerI, adjacent to C2C). Importantly, dysferlin mutants that demonstrate accelerated endocytosis also display increased protein lability via endosomal proteolysis, implicating endosomal-mediated proteolytic degradation as a novel basis for dysferlin-deficiency in patients with single missense mutations. Vesicular labeling studies establish that dysferlin mutants rapidly transit from EEA1-positive early endosomes through to dextran-positive lysosomes, co-labeled by syntaxin-4 at multiple stages of endosomal transit. In summary, our studies define a transient biology for dysferlin, relevant to emerging patient therapeutics targeting dysferlin replacement. We introduce accelerated endosomal-directed degradation as a basis for lability of dysferlin missense mutants in dysferlinopathy, and show that dysferlin and syntaxin-4 similarly transit a common endosomal pathway in skeletal muscle cells.

Dysferlin belongs to the ferlin family of proteins, an ancient family with emerging roles in cellular trafficking and vesicle fusion. Caenorhabditis elegans FER-1 has been proposed as a calcium sensor or regulatory protein involved in the process of fusion of membranous organelles to the plasma membrane of spermatozoa (1). Impaired calcium-regulated fusion of vesicular organelles as seen in FER-1 mutants (1) is a theme that unites ferlin-related disease in humans.

Dysferlin was identified through linkage analyses of two families with an inherited form of muscular dystrophy (2, 3). Dysferlin is ubiquitously expressed, but with predominant expression in striated muscle. Dysferlin-deficient murine skeletal muscle fibers exhibit impaired calcium-mediated membrane repair (4), a process thought to involve calcium-activated vesicular exocytosis (5, 6) and/or endocytosis (7). Otoferlin is expressed in the brain, vestibular system, and cochlea, and underlies a form of human deafness (8, 9). Otoferlin-null mice are profoundly deaf, and exhibit a primary defect in calcium-mediated fusion of neurotransmitter-containing vesicles at the specialized ribbon synapse of cochlear inner hair cells (10). Myoferlin is broadly expressed in many tissues, with high expression in skeletal muscle during development and in regeneration (11). Myoferlin has not yet been implicated in human disease but has been shown to be important for myoblast fusion and myotube formation (12) and has an emerging role as a regulator of endocytosis in epithelial cells (13, 14). Mammalian Fer-1 L4, L5, and L6 GenBank™ sequences have been identified, but these proteins have not yet been characterized.

Ferlin proteins contain multiple C2 domains and have structural homology to the synaptotagmins. The synaptotagmins possess two calcium-binding C2 domains that lie on the cytoplasmic face of a lipid bilayer, anchored by an N-terminal transmembrane domain. The two C2 domains of synaptotagmin exhibit complex calcium-regulated binding to both SNARE (soluble N-ethylmaleimide-sensitive factor attachment protein receptors) protein machinery and membrane phospholipids, to regulate vesicular fusion (15). The ferlin proteins possess between four and seven tandem C2 domains within their cytoplasmic domain and are also anchored to lipid bilayers via their C-terminal transmembrane domain.

We are seeking to better understand dysferlin cell biology and its molecular role in membrane resealing. Defective membrane resealing is likely to be widely implicated in the muscular dystrophies, supported by recent findings of membrane reseal-
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Patients and Ethics—We identified the L344P mutation in two sisters referred to our Neuromuscular Clinic and laboratory. The elder sister first presented in her early twenties, with elevated CK (not precisely documented) and mild limb girdle weakness on examination. Muscle biopsy histology demonstrated variation in fiber size, several degenerate and necrotic fibers, and a moderate increase in lymphocytes and macrophages. The younger sister presented at age 16, with an elevated CK level of up to 4000 units/liter on occasion (normal range <230 units/liter), and with a painful swollen calf muscle, which was enlarged on MRI. She also presented with knee, hip, and shoulder pain and scoliosis. Routine muscular dystrophy was enlarged on MRI. She also presented with knee, hip, and shoulder pain and scoliosis. Routine muscular dystrophy weakness on examination. Muscle biopsy histology demonstrated variation in fiber size, several degenerate and necrotic fibers, and a moderate increase in lymphocytes and macrophages.

EXPERIMENTAL PROCEDURES

Antibodies and Fluorescent Reagents—Antibodies to the following proteins were used: dysferlin NCL-Hamlet (Novocastra, Leica Microsystems, Australia); anti-Myc (Santa Cruz Biotechnology); anti-Myc\textsuperscript{Alexa555} (Millipore); anti-His (Abcam, Cambridge, UK); anti-His\textsuperscript{Alexa488} (Qiagen Pty Limited); anti-HA; anti-EEA1 (BD Biosciences); anti-Syntaxin-4 (Synaptic Systems); Alexa Fluor\textsuperscript{®}-conjugated secondary antibodies (Invitrogen); Cy3-conjugated secondary antibodies (Jackson Immunoresearch Laboratories); HRP-conjugated secondary antibodies (Amersham Biosciences, Buckinghamshire, UK). Other fluorescent reagents were used: Cholera toxin\textsuperscript{Alexa647} and low density lipoprotein conjugated to lipophilic carbocyanine Dil (LDL\textsubscript{dil}) (Molecular Probes, Invitrogen). Flow cytometry isotype control staining was performed using LEAF\textsuperscript{TM} Purified Mouse IgG1 (κ Isotype, clone MGI-45, BioLegend).

Cell Culture—C2C12 cells (P22-P29) were provided by Dr Edna Hardeman, Children’s Medical Research Institute, Sydney. All cell culture and reagents were obtained from Invitrogen unless otherwise specified. Cells were grown in a humidified incubator containing 5% CO\textsubscript{2} and maintained at 37 °C. C2C12 cells were maintained in 1:1 F-12/DMEM containing 15% heat-inactivated fetal bovine serum (FBS) and 50 μg/ml gentamycin.

Dysferlin Expression Constructs—The dysferlin expression construct pcDNA4-EGFPdysferlin\textsubscript{MycHis} was kindly provided by Prof. Kate Bushby (23). \textbf{AC2C'B} (Dysferlin \textsubscript{ΔMet\textsuperscript{301-Ala\textsuperscript{497}}}) was derived through ligation of the dysferlin 5'-'fragment EcoRI/BspEI (Klenow fill-in to blunt), into the plasmid backbone pcDNA4-EGFPdysferlin\textsubscript{MycHis} restricted with EcoRI/BsfI (T4 blunt), resulting in deletion of the cDNA sequence between BspEI and BsfI. \textbf{AC2BC} (Dysferlin \textsubscript{ΔVal\textsuperscript{140-Ala\textsuperscript{497}}}) was derived through BsfI restriction, and ligation of annealed oligos (5'-AGCCGCTGCA-3', 5'-AGGCGCTTGA-3') to restore frame and introduce an AfeI site. \textbf{AC2BCD} (Dysferlin \textsubscript{ΔVal\textsuperscript{140-Pro\textsuperscript{1327}}}) was derived through AfeI digestion of \textbf{AC2BC}, and re-ligation. \textbf{C2EF-TM} contains the C-terminal dysferlin fragment from Asp\textsuperscript{1475} derived through EcoRI/ClaI restriction, and ligation of annealed oligos (5'-AGGCCTTTGCA-3', 5'-AGGCCCTTGA-3') to restore frame and introduce an AfeI site. \textbf{AC2BCD} was derived through ligation of a BamHI (Klenow blunted)/NotI fragment of pcDNA4-EGFPdysferlin\textsubscript{MycHis} religated into EcoRI (Klenow blunted)/NotI-digested vector backbone. \textbf{His-only} constructs were derived via NotI digestion and ligation of oligos (His: 5'-GGCCCGCGACAAAAACTCATCTCGAGAAGATCATGTAGCTGC-3'; 5'-GGCCGCTAGCTTTACAGATCTCGCTTAGAATGTTTTTGTCGCC-3'; \textbf{Myc} 5'-GGCCGGCAGAAACAATCTACATCGAAGAGGATGTAGCTGC-3'; 5'-GGCCGCTAGCTTTACAGATCTCGCTTAGAATGTTTTTGTCGCC-3'); \textbf{−EGFP} constructs were derived through KpnI (T4 blunt) and BsrG1 (T4 blunt) digestion and re-ligation of pcDNA4-EGFPdysferlin\textsubscript{MycHis}. The \textbf{L344P} construct was derived by amplification of the patient cDNA (Forward primer: 5'-ACCTTTCCAGGAGCCTTCC'3; Reverse primer: 5'-CAGATCTGAGGCTTTTGACAG-3') followed by digestion of this fragment by BsfI and ligation into the pcDNA4-EGFP full-length (FL)\textsuperscript{3} plasmid backbone digested with BsfI and de-phosphorylated. The syntaxin-4-HA construct was derived as a fusion construct, with the sequence coding for the C-terminal fragment of syntaxin-4-Alexa488 inserted between the N-terminal HA tag of the protein and vector backbone (C2BCD).
struct (pcDNA3-Syn4HA) was kindly provided by Prof. David James, Garvan Institute, Sydney.

Transfection—C2C12 cells were transfected overnight or for 24 h at 70–80% confluence with GeneJuice™ (Merck, Darmstadt, Germany), using 0.5 μg DNA/1.5 μl GeneJuice™ per 2 cm² culture area in 0.5 ml culture media. For FL-dysferlin and C2F-TM or FL dysferlin and syntaxin 4 -HA co-expression studies, plasmids were used at a ratio of FL: C2F-TM at 2:1 or FL: Syn4-HA at 2:1. For FL-dysferlin and △C2C co-expression, plasmids were used at a ratio of 1:1. Twenty-four hours post-transfection, cells were switched to low serum medium to induce differentiation (1:1 F12/DMEM, 3% horse serum, 50 μg/ml gentamycin).

Immunocytochemistry and Microscopy—Cells grown on Thermax™ coverslips (Nunc) were transfected and differentiated as described above. On the third day of differentiation, intact, live cells were immunolabeled at 4 °C with anti-MycAlexa555 (1:100) and/or anti-HisAlexa647 (1:100) diluted in Hank’s-buffered salt solution (HBSS) containing 20% FBS. Cells were washed in PBS and coverslips mounted on 22 × 50 mm glass coverslips using Fluorsave™ mounting reagent (Merck, Darmstadt, Germany). Confocal microscopy was performed using a 100× HCX Plan Apo oil immersion lens (1.4×) by imaging through the glass coverslip, as described (29), using a Leica SP2 Scanning Laser Confocal Microscope. Images (1024 × 1024) were captured without subtraction of glow-under. Background was subtracted postcapture using Adobe Photoshop software, through a single adjustment of the levels histogram. Deconvolution images were obtained on an inverted Olympus IX-71 microscope (Deltavision Image Restoration Microscope; Applied Precision/Olympus) and a Photometrics CoolSnap QE camera with a 100 × 1.4 NA oil immersion lens. Images were de-convolved and overlaid using DeltaVision SoftWoRx software version 3.7.1, with a single adjustment of the levels histogram.

Antibody and Fluorescent Reagent Uptake—C2C12 cells were grown on thermax coverslips, transfected, and differentiated as above. For dysferlin endocytosis studies, myotubes differentiated for 2 or 3 days were incubated with anti-MycAlexa555 (1:100), anti-HisAlexa647 (1:100), and/or anti-FLAG (1:100) in the culture medium at 37 °C. Coverslips were removed after 15 min or 3 h, acid washed (0.5 M glycerol, pH 2.2) to remove surface-bound antibody, washed briefly in HBSS, fixed in 3% PFA (paraformaldehyde), and mounted for confocal microscopy. Similarly, for studies using un-conjugated antibodies, cells were incubated in primary antibodies for 90 min or 3 h at 37 °C, acid washed, fixed, and resuspended in Ca/Mg-free PBS. Cells with compromised membranes were identified through addition of 10 μl (0.5 μg/ml) 7-amino-actinomycin D (7-AAD, BD Biosciences) to 300 μl of cell suspension. Flow cytometry was performed using a BD LSRII cytometer equipped with FACSDiva™ software (BD Biosciences). Postacquisition analysis was performed using FlowJo software (version 7.2.5) (Tree Star Inc.).
Results

The Effect of Dysferlin Domain Deletions upon Plasma Membrane Targeting and Vesicular Localization—Dysferlin’s modular structure, with seven putative cytoplasmic C2 domains, led us to consider the potential for specific roles of particular domains upon dysferlin trafficking and plasma membrane biology. We utilized an expression construct bearing a N-terminal EGFP dysferlin fusion protein with extracellular C-terminal Myc- and His-epitope tags (23), from which we derived a panel of dysferlin deletion mutants (Fig. 1a). Cell surface labeling of live cells using the extracellular epitope tag was used to assess plasma membrane targeting by confocal microscopy (Fig. 2a) and quantified by flow cytometry (Fig. 2b). Previous use of flow cytometry to analyze isolated myofibres (24) initiated our development of this quantitative approach, analyzing thousands of cells to produce a normal distribution of cell surface labeling, effectively normalizing cell-to-cell variability. All cells within a population of differentiated myotubes successfully transited the flow cytometer in hydrodynamic flow, producing a broad scatter profile of cells of increasing size with differentiation.

All dysferlin deletion constructs targeted the plasma membrane, with varying efficiencies (Fig. 2a and b). C2C12 myotubes transfected with FL dysferlin showed punctate labeling of the surface membrane (Fig. 2a, FL, red), and a mixed pattern of cellular EGFP fluorescence, labeling intracellular networks, surface membranes (filapodia and lamellapodia), and a population of small vesicles (Fig. 2a, FL, green). The vesicle population was noted due to the striking abundance of vesicular labeling with several dysferlin expression constructs, particularly truncated mutants C2EF-TM and C2F-TM (shown for C2F-TM, Fig. 2a, red). Strong vesicular labeling for C2F-TM was similarly observed in both brightly fluorescent cells, and weakly fluorescent cells, suggesting that vesicular localization was not determined by protein overexpression. Prominent vesicle labeling was also observed with three constructs bearing different internal deletions within the dysferlin N-terminal domain (not shown): ΔC2BC dysferlin, ΔC2C*B dysferlin, and ΔC2BCD dysferlin (see Fig. 1a, upper panel), united by disruption of the region encompassing C2B and C2C. Thus, we derived four more expression constructs (see Fig. 1, a, lower panel, and b); three constructs bearing a specific microdeletion of C2A, C2B, or C2C, and one construct bearing missense mutation L344P, based on a patient from our Australian dysferlinopathy cohort (Fig. 1b). Leu344 is a highly conserved residue within the Fer1 domain, sandwiched between C2 domains C2B and C2C.

The C2B-Fer1-C2C Motif Critically Regulates Dysferlin Plasma Membrane Expression—Confocal microscopy revealed the prominent vesicular labeling observed with C2F-TM was recapitulated by any construct with a disruption within the C2B-Fer1-C2C motif (Fig. 2a, ΔC2B, ΔC2C, L344P). ΔC2C showed the most consistently profound vesicular localization (Fig. 2a, ΔC2C, green), and was also accompanied by the greatest reduction in plasma membrane expression levels (2.5-fold, Fig. 2b). Reduced plasma membrane expression levels (Fig. 2b), and prominent vesicular labeling (Fig. 2a) was also obvious, but less consistently dramatic, with L344P and ΔC2B. In contrast, ΔC2A showed normal plasma membrane expression levels without overt intracellular vesicular labeling. The trend for lower levels of plasma membrane expression correlating with increased vesicular labeling was also observed with severely truncated C2F-TM, though only to 60% levels of FL dysferlin (discussed in more detail in Fig. 7).
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**Intracellular Vesicles for C2F-TM Are Endocytic**—The correlation between prominent vesicular labeling and reduced levels of dysferlin plasma membrane expression raised the possibility of endosomal internalization of cell surface dysferlin. To address this, we performed antibody uptake assays in C2C12 myotubes transfected with either FL dysferlin or severely truncated C2F-TM, to determine if intracellular vesicles observed at steady state for C2F-TM were endocytic. After a three hour antibody uptake, all intracellular vesicles for both FL and C2F-TM dysferlin were co-labeled with endocytosed anti-Myc\(^{555}\) (red) and EGFP, indicating EGFP-dysferlin labeled vesicles were endosomal (Fig. 3a). When antibody uptake was limited to 15 min, considerably more anti-Myc\(^{555}\) uptake was observed for C2F-TM, relative to FL dysferlin (supplemental Fig. S2). Together, these results suggest truncated C2F-TM is more rapidly endocytosed following export to the plasma membrane, than is FL dysferlin.

C2F-TM and ΔC2C Co-endocytose with Full-length Dysferlin within Endosomal Compartments Labeled by Syntaxin-4 and Cholera Toxin—To determine whether dysferlin deletion constructs C2F-TM and ΔC2C highlight trafficking pathways also employed by FL dysferlin, we derived uniquely tagged expression constructs and performed pairwise co-transfections (i.e. C2F-TM\(_{\text{Myc}}\) with FL\(_{\text{His}}\), ΔC2C\(_{\text{Myc,His}}\) and FL\(_{\text{Flag,FL_{Myc,His}}}\) and FL\(_{\text{Flag}}\)). Antibody uptake assays were performed using two antibodies conjugated with different colored fluorophores, to uniquely label each dysferlin species within a pairwise co-transfection (Fig. 3b). FL dysferlin is co-endocytosed with ΔC2C and C2F-TM, within the same endocytic vesicle, as shown by dual labeling of endocytic vesicles with both red and blue antibody fluorophores (Fig. 3b, arrows). Thus, rapid endocytosis of mutant ΔC2C and C2F-TM does not appear due to abnormal targeting to atypical plasma membrane microdomains, nor usage of an alternate endosomal trafficking route. Co-endocytosis of FL dysferlin with ΔC2C or C2F-TM was similarly observed using FL dysferlin lacking the EGFP.

Of note, discreet disruption of ΔC2C (28 amino acid deletion) induced the same degree of vesicular labeling and reduction in plasma membrane expression as did ΔC2C\(^{\text{B}}\) (both by 2.5-fold, Fig. 2b), which bears a much larger deletion of 204 amino acids encompassing C2C, Fer1 and perhaps also disrupting C2B (depending on exact domain boundaries, see Fig. 1b). These results suggest a critical requirement for C2C, and the C2B-Fer1-C2C motif generally, for normal plasma membrane expression of dysferlin.

![Endocytic Regulation of Dysferlin Plasma Membrane Expression](image)

**FIGURE 2. Efficiency of plasma membrane targeting of dysferlin deletion constructs.** a, confocal analysis of surface anti-Myc\(^{555}\) and cellular EGFP fluorescence. Transfected and differentiated C2C12 myotubes were labeled with anti-Myc\(^{555}\) at 4 °C to specifically label plasma membrane dysferlin (red) with absent cytoplasmic immunoreactivity to surface applied antibodies confirming maintenance of an intact plasma membrane (not shown). Strikingly EGFP vesicles are apparent for C2F-TM (green, C2F-TM, arrows), and are also clearly observed with ΔC2B, ΔC2C, and patient missense mutant L344P. Scale bars, 10 μm. b, quantification of cell surface dysferlin by flow cytometry. Populations of transfected and differentiated C2C12 myotubes were surface labeled with anti-His\(^{555}\) and subject to flow cytometry. A histogram represents cumulative data of the relative efficiency of plasma membrane targeting for each dysferlin deletion construct over many experiments. For each experiment, transfected cells were selectively analyzed (supplemental Fig. S1), and levels of surface dysferlin labeled by anti-His\(^{555}\) versus levels of total EGFP fluorescence quantified (see "Experimental Procedures"). For each construct, results are presented relative to results observed for FL dysferlin within the same experiment. A highly significant reduction in plasma membrane dysferlin is observed for C2F-TM, ΔC2B, ΔC2C, ΔC2C\(^{\text{B}}\) and patient missense mutant L344P. Statistical significance was assessed using a Mann Whitney non-parametric test, with p < 0.001 represented by three stars (**

![](image)

**AF647 anti-His**

![Histogram representing cumulative data of the relative efficiency of plasma membrane targeting for each dysferlin deletion construct.](image)
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We also tracked dysferlin endocytosis from EEA1-labeled myotubes (Fig. 4), using a 90 min anti-His antibody uptake. Dysferlin transit to the late endosomal/lysosomal compartment was also confirmed using fluorescently conjugated low density lipoprotein (LDL, supplemental Fig. S3), a marker of the late endosomal/lysosomal pathway. More dextran and LDL positive vesicles were observed for C2F-TM compared with FL dysferlin (Fig. 4d). Thus, within 90 min, a large proportion of C2F-TM dysferlin transits from the early endosomal compartment through to late endosomes/lysosomes, suggesting that lysosomal degradation may be an end point for truncated C2F-TM following endocytosis.

Given the recent study associating otoferlin with syntaxin-1 (25), we explored the relationship between dysferlin and syntaxin-4, which localizes to the plasma membrane and is highly expressed in skeletal muscle (26). Antibodies recognizing endogenously expressed syntaxin-4 co-labeled FL and C2F-TM dysferlin endocytic vesicles in transfected myotubes (Fig. 5a). We also observed evidence for syntaxin-4/dysferlin co-labeled vesicles in untransfected myotubes (Fig. 5b); though in this case we could not confirm their endosomal origin via antibody uptake (an antibody raised to the short extracellular tail of dysferlin was tried unsuccessfully). To specifically examine endocytosed syntaxin 4, we co-transfected dysferlin with syntaxin-4-HA, which bears an extracellular HA tag (27). Antibody uptake experiments using anti-His and anti-HA antibodies revealed a striking co-labeling of dysferlin- and syntaxin-4-positive endosomal compartments (Fig. 5c), observed with all dysferlin mutants (including ΔC2C and patient missense L344P), and also with wild-type dysferlin lacking the N-terminal EGFP fusion protein (not shown). For truncated C2F-TM following a 90 min anti-His antibody uptake (where all endosomal compartments from early endosomes through to late endosomes/lysosomes are labeled by anti-His647), virtually every endosomal vesicle showed labeling for both dysferlin and syntaxin-4 (Fig. 5c, middle row), suggesting these two proteins similarly transit all maturation stages of this endosomal pathway.

Accelerated Endocytosis of C2F-TM Increases Protein Turnover and Lability—Protein half-life studies were used to assess whether the rapid transit of C2F to lysosomal compartments reflected greater protein lability, by using cycloheximide to block cytoplasmic protein synthesis. Studies of dysferlin protein decay over a time-course of cycloheximide treatment showed FL dysferlin to have an average protein half-life of 4.6 ± 0.7 h (Fig. 6, a and b), similar to endogenously expressed murine dysferlin (t1/2 = 4.8 ± 1.5 h). In contrast, more rapid decay was observed for C2F-TM (t1/2 = 2.4 ± 0.7 h), that could be blocked by preventing secretion of C2F-TM to the plasma membrane using brefeldin A (which collapses the Golgi complex and prevents export from the endoplasmic reticulum to the plasma membrane), and using bafilomycin to prevent endosomal acidification (a specific blocker of vacuolar ATPase). In contrast, MG132, a specific inhibitor of the ubiquitin proteasome (targeting misfolded proteins from the endoplasmic reticulum), did not protect C2F-TM from degradation. Therefore, rapid protein turnover of C2F-TM appears largely due to endosomal proteolysis, without clear evidence for misfolding and degradation from the endoplasmic reticulum (ERAD) (Fig. 6, c and d).

fusion protein, and using different combinations of extracellular epitope tags (not shown).

To define the endocytic route of dysferlin, we used characterized markers of various endocytic pathways. Dysferlin endocytic vesicles co-labeled with fluorescent choler toxin, known to bind a specific ganglioside of lipid rafts (Fig. 4a), and with caveolin-3 (Fig. 4b), but did not show significant co-labeling with transferrin (not shown). These results are in agreement with previous studies demonstrating caveolin-dependent, choler toxin-associated, but transferrin-independent dysferlin endocytosis in mouse embryonic fibroblasts (18).

We also tracked dysferlin endocytosis from EEA1-labeled (Fig. 4c) or Rab-5-labeled (not shown) early endosomes, through to dextran-loaded lysosomes (Fig. 4d), using a 90 min anti-His antibody uptake. Dysferlin transit to the late endosomal/lysosomal compartment was also confirmed using fluorescently conjugated low density lipoprotein (LDL,
ΔC2C and L344P showed greater protein lability than FL dysferlin, and were also partially protected from degradation by incubation with brefeldin A and bafilomycin. Interestingly, MG132 also partially protected ΔC2C and L344P from degradation, suggesting a role for the ubiquitin proteosome in their proteolysis. This contrasts with truncated mutant C2F-TM, unaffected by MG132, perhaps reflecting greater folding constraints of the much larger full-sized dysferlin mutants (Fig. 6d).

Disruption of the C2B-Fer1-C2C Motif Reduces Dysferlin Plasma Membrane Expression via Accelerated Endocytosis—We sought to quantify the plasma membrane half-life of dysferlin, and the effect of our specific mutations on accelerating dysferlin endocytosis. Previous studies with brefeldin A had demonstrated that dysferlin relied upon an intact Golgi apparatus for cell surface export (supplemental Fig. S4), and thus we employed a time course of brefeldin A treatment to examine the time-dependent loss of dysferlin from the cell surface across a large cell population using flow cytometry analysis. This approach presents the significant advantage of examining the rate of endocytosis, independently from antibody-induced or ligand-induced endocytosis, and independently of endosomal recycling (which requires an intact Golgi network).

Fig. 7a depicts a histogram of cumulative plasma membrane half-life data for each dysferlin expression construct over many experiments. Fig. 7b shows representative data from a single experiment examining the plasma membrane half-life of FL dysferlin, and deletion mutants ΔC2C and C2F-TM. The upper panels of Fig. 7b show the normal distributions of cell surface dysferlin labeling, which incrementally decrease as a function of time (t₀, t₃₀min, t₁h, t₂h), once all further export and recycling of dysferlin is blocked by treatment with brefeldin A. Normalizing cell surface dysferlin (anti-His⁶⁴⁷) relative to total dysferlin (EGFP) over time was then used to derive the plasma membrane half-life (half-life = log(2)/gradient, see “Experimental Procedures”).

These analyses were consistent and reproducible, and estimated the plasma membrane half-life (PM t₁/₂) of FL dysferlin to be ~3 h (PM t₁/₂ = 2.8 ± 0.2 h). In contrast, C2F-TM was endocytosed 3-fold faster (PM t₁/₂ = 0.9 ± 0.1 h) and ΔC2C 2.5-fold faster (PM t₁/₂ = 1.2 ± 0.2 h). Again, ΔC2C (28 amino acid microdeletion) and ΔC2C*B (206 amino acid deletion) behaved similarly, supporting a crucial role for the C2C domain for plasma membrane stability. ΔC2B and patient missense mutant L344P also showed a significant, but less marked reduction in plasma membrane half-life (PM t₁/₂ = 1.8 ± 0.2 h) relative to ΔC2C, supporting a general requirement for an intact C2B-Fer1-C2C domains for normal regulation of dysferlin plasma membrane expression.

Endocytic Rate Is a Major Determinant of Dysferlin Plasma Membrane Expression Levels—When we compared the levels of plasma membrane dysferlin (Fig. 2), with the plasma membrane half-life (Fig. 7), we observed a striking correla-
tion between the fold-acceleration in endocytic rate, and the fold-reduction in plasma membrane expression levels (Table 1). This relationship bore out for all full-sized dysferlin constructs, and suggests that even with some degree of misfolding (as suggested in Fig. 6d by partial MG132-mediated protection of ΔC2C and L344P proteolysis), endocytosis remains a primary regulator of dysferlin plasma membrane expression. This correlation did not hold true for truncated C2F-TM, where marked acceleration of endocytosis (3-fold) was not reflected in a similar fold-reduction in plasma membrane expression levels (reduced by only 40%). These results may yet be reasonable, whereby enhanced biosynthesis and export of much smaller C2F-TM assists plasma membrane expression levels, despite its extremely transient plasma membrane half-life.

Our results do not support that accelerated endocytosis results from excess plasma membrane dysferlin (because of overexpression); in this case, plasma membrane expression levels for each of the different constructs would be expected to be more broadly similar. Instead, each mutant construct consistently showed a similar fold reduction in efficiency of plasma membrane expression relative to wild-type dysferlin, over many experiments (see Fig. 2). Overall, the correlation between endocytic rate and plasma membrane expression levels for full-sized dysferlin proteins is striking and leads us to propose endocytosis as a major regulator of dysferlin plasma membrane expression.

DISCUSSION

Dysferlin is a tail-anchored protein, with multiple tandem C2 domains that are likely to underlie its role in calcium-dependent and vesicle-mediated membrane resealing. We sought to further define the basic cell biology of dysferlin, as a reference to study the consequences of patient-derived mutations, and to define the roles of calcium and C2 domains upon dysferlin trafficking and membrane biology. The ferlins are an ancient family of proteins, and it is likely that they possess primordial functions in vesicular trafficking that can be revealed through studies of dysfunction in human disease. Synaptotagmin 1 possesses two C2 domains, with each showing distinct calcium-regulated binding to different phospholipid membranes (28, 29), and different SNARE proteins (30, 31). Dysferlin has seven putative C2 domains, of which four show potential calcium sensitivity (32), suggesting even more modular complexity.

Our studies reveal dysferlin to be a transitory transmembrane protein in cultured myotubes, with an average plasma membrane half-life of ~3 h, and a reasonably short-lived protein half-life of ~4–6 h. Transfected myotubes provide a good myogenic model to derive baseline information of dysferlin cell biology. This information is highly relevant for the development of gene replacement therapies to treat muscular dystrophy caused by loss of dysferlin, and perhaps also relevant to otoferlin replacement strategies in human deafness. However, it is important to acknowledge that dysferlin behavior may be differentially regulated in different cellular compartments, and in different cell types.

To study domain-specific roles for dysferlin, we constructed a panel of dysferlin deletion constructs. We identified a requirement for the dysferlin C2B-Fer1-C2C region for stable plasma membrane expression. Disruption or

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**FIGURE 6.** Half-life analysis of dysferlin expression constructs. Transfected and differentiated C2C12 myotubes (on the third day of differentiation) were incubated with or without cycloheximide (100 μg/ml) and other drugs for the time periods indicated. Duplicate or triplicate wells for each condition were harvested in RIPA buffer and 50 μg of total protein (for C2F-TM) was separated on a 3–8% gel and Western blotted. Blots were probed with NCL-Hamlet, and levels of dysferlin expression assessed through densitometry. α treated cells were harvested at t0, t6, t2, and t12; t6 shown. eDysf represents endogenous dysferlin, detected by both NCL-Hamlet antibodies (not shown). eDysf has a lower apparent molecular mass than transfected FL-dysferlin due to conjugation of the 29 kDa EGFP tag. The doublet band observed for C2F-TM is presumed to be a breakdown product. b, graphed data represents averaged half-life calculations based on four or five separate experiments. For each individual experiment, density readings for duplicates or triplicates were averaged, and half-life calculations were performed as described under “Experimental Procedures.” c, brefeldin A protects C2F-TM from degradation (*asterisks), mutation of each domain within the C2B-Fer1-C2C motif resulted in reduced plasma membrane expression due to accelerated endocytosis, and, increased protein lability from endosomally driven proteolysis. Targeted disruption of C2C-mutant dysferlin plasma membrane expression most profoundly accelerating dysferlin endocytic rate by 2.5-fold. Our previous collaborative studies have shown that caveolin deficiency results in more rapid dysferlin endocytosis via a caveolin-independent pathway in mouse embryonic fibroblasts (18, 19). In our current study, rapidly endocytosed dysferlin mutants were shown to maintain an association with caveolin-3-positive endosomes in cultured myotubes. Thus, employing a caveolin-independent endocytic route does not easily explain our findings, though altered dysferlin/caveolin-3 endocytic regulation cannot be excluded. Importantly, we show that a patient missense mutation also displays reduced plasma membrane expression due to accelerated endocytosis, which also increases protein lability. This is the first evidence for endosomally driven proteolysis of dysferlin as the mechanism underlying dysferlin lability in dysferlinopathy. We are now exploring whether accelerated endocytosis is a feature of other patient missense mutants, particularly those affecting the C2B-Fer1-C2C motif.

On the basis of an identified association between otoferlin and syntaxin I in cochlear inner hair cells (25), we explored a relationship between dysferlin and the muscle counterpart of syntaxin-1, syntaxin-4. Syntaxin-4 positively labeled dysferlin endocytic vesicles, from early endosomes to late endosomes/lysosomes, suggesting both proteins similarly transit a common endosomal pathway. This dysferlin/syntaxin-4 endocytic pathway may be relevant to studies of known syntaxin-4-dependent cargo; syntaxin-4 is involved in the trafficking of GLUT4 receptors in response to insulin (33–35), and has also been associated with cytokine release in macrophages (36). This inflammatory association is particularly interesting given the marked inflammatory response in dysferlinopathy (37) and the abnormal immune signaling in the dysferlin-deficient mouse (38–41). Of note, the dysferlin/syntaxin-4 endosomal association is maintained even when dysferlin is severely truncated (C2F-TM). The syntaxin-1 interactive domain of otoferlin was mapped to C2F (25), highly conserved between paralogues, and our ongoing studies are exploring the potential of a direct interaction mediated by dysferlin C2F and syntaxin-4.

In summary, our data establish dysferlin as a transient plasma membrane protein subject to endocytic regulation of cell surface expression levels relevant to a potential plasma membrane- or vesicle-based role in membrane resealing, and a likely basis for abnormal dysferlin localization in muscle of patients with muscular dystrophy (21, 22). We identify whereas MG132 has little effect on protein levels. d, ΔC2C2, L344P and C2F-TM dysferlin are all more labile then FL dysferlin after 6 h of cycloheximide treatment (lanes 3 and 4), with partial protection of degradation by brefeldin A (lanes 5 and 6) and bafilomycin (lanes 9 and 10). MG132 treatment of cells transfected with ΔC2C2 and L344P dysferlin also affords partial protection from cycloheximide-induced protein degradation (lanes 7 and 8).
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a key role for the C2B-FerI-C2C motif for dysferlin plasma membrane expression, and propose that increased protein lability due to accelerated endocytosis may underpin disease pathogenesis in some cases of dysferlinopathy. Significantly, we identify a dysferlin and syntaxin-4 endocytic pathway, relevant for known syntaxin-4 dependent cargo, and perhaps also more widely significant in the pathology of dysferlinopathy. Our results raise questions as to whether acceler-

![Diagram](image_url)

FIGURE 7. Determination of the plasma membrane half-life of dysferlin. Transfected and differentiated C2C12 myotubes (on the third day of differentiation) were incubated with or without brefeldin A for the times indicated. Cells were harvested and labeled with anti-His<sup>AF647</sup> as described under “Experimental Procedures.” Immediately prior to flow cytometry analysis, cells were incubated in the presence of actinomycin D (7-AAD), to identify any cells with compromised membranes. Cells labeled by 7-AAD were excluded and cells positive for EGFP fluorescence analyzed. a, histogram representing cumulative data of the calculated plasma membrane half-life for dysferlin expression constructs. Plasma membrane half-lives were calculated using the geometric mean of the normal distribution of the anti-His<sup>AF647</sup> fluorescence, relative to the geometric mean of the normal distribution of the EGFP fluorescence, for each cell population, at each time point. Data from between 3 and 23 experiments were averaged, as indicated. Error bars are 95% confidence intervals. Statistical significance was assessed using a Mann Whitney non-parametric test, with \( p < 0.001 \) represented by three stars (***) 。b, representative graph of flow cytometry data demonstrating surface labeling for transfected cells expressing EGFP-FL dysferlin, C2F-TM dysferlin, and \( \Delta C2C \) dysferlin, as annotated. Note the incremental decline in plasma membrane expression for expressed dysferlin constructs with increasing periods of brefeldin A treatment. The Alexa647/EGFP fluorescent ratio for each time point was plotted on a semi-log plot as described under “Experimental Procedures.” Only experiments with a line-of-best-fit of \( r^2 \geq 0.9 \) were included.
ated endocytosis of dysferlin mutants reflects abnormal exocytic delivery of dysferlin-rich vesicles that are then endocytosed, active removal of malfunctioning dysferlin, or dysregulation of a primary endocytic role that may also influence other endocytic cargo. Distinguishing between these possibilities is now essential to understanding the molecular role dysferlin plays in membrane resealing and the pathology of dysferlinopathy.

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REFERENCES


TABLE 1

<table>
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<tr>
<th>Dysferlin rate is a primary regulator of dysferlin surface expression</th>
<th>Predicted steady-state plasma membrane dysferlin (based on half-life)</th>
<th>Actual steady-state plasma membrane dysferlin</th>
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<tr>
<td>EGFP-FL dysferlin</td>
<td>1.00 (PM&lt;sub&gt;t&lt;/sub&gt;=2.8 ± 0.2h)</td>
<td>1.00</td>
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<td>EGFP-2CF dysferlin</td>
<td>0.32 (PM&lt;sub&gt;t&lt;/sub&gt;=0.9 ± 0.1h)</td>
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<td>EGFP-ΔC28 dysferlin</td>
<td>0.71 (PM&lt;sub&gt;t&lt;/sub&gt;=0.4 ± 0.1h)</td>
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<td>EGFP-ΔC2C dysferlin</td>
<td>0.43 (PM&lt;sub&gt;t&lt;/sub&gt;=1.2 ± 0.2h)</td>
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<tr>
<td>EGFP-ΔC2C&lt;sup&gt;B&lt;/sup&gt; dysferlin</td>
<td>0.40 (PM&lt;sub&gt;t&lt;/sub&gt;=1.1 ± 0.4h)</td>
<td>0.37</td>
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<tr>
<td>EGFP-L344P dysferlin</td>
<td>0.64 (PM&lt;sub&gt;t&lt;/sub&gt;=1.8 ± 0.2h)</td>
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Reduced Plasma Membrane Expression of Dysferlin Mutants Is Attributed to Accelerated Endocytosis via a Syntaxin-4-associated Pathway
Frances J. Evesson, Rachel A. Peat, Angela Lek, Fabienne Brilot, Harriet P. Lo, Russell C. Dale, Robert G. Parton, Kathryn N. North and Sandra T. Cooper

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