Characterization of dysferlin's C2 domains reveals novel binding specificities to phospholipids and alpha-tubulin

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Abstract

Mutations in the dysferlin gene lead to a form of limb girdle muscular dystrophy (LGMD2B). Dysferlin is a transmembrane protein consisting of seven C2 domains and two DysF domains. Dysferlin is implicated in skeletal muscle membrane repair but its mechanisms in this process are poorly defined. To better understand dysferlin's function, this thesis sought to characterize the binding specificities of dysferlin's seven C2 domains to three phospholipids that compose mammalian lipid membranes, as well as to alpha-tubulin, which was identified by liquid chromatography-mass spectrometry as a novel dysferlin interacting protein. The following body of work showed that dysferlin's C2A domain binds to phosphatidylserine (PS), phosphatidylinositol 4-phosphate (PIP) and phosphatidylinositol 4,5-bisphosphate (PIP2) in a calcium-dependent manner. Dysferlin's other C2 domains each bound to PS in a weaker, calcium-independent manner. This study also revealed that dysferlin's C2A and C2B domains bound to alpha-tubulin in a calcium-independent manner. The possible implications of these interactions in patch membrane repair and dysferlin trafficking are discussed.

Résumé

La présence de mutations dans le gène de la dysferline est responsable du développement d'une forme de dystrophie musculaire des ceintures (LGMD2B). La dysferline est une protéine transmembranaire composée de sept domaines C2 et de deux domaines DysF. Pour mieux comprendre le fonctionnement de la dysferline, nous avons entrepris des études pour déterminer la spécificité d'interaction des domaines C2 avec des lipides trouvés dans les membranes des cellules eukaryotes. De plus, nous avons entrepris des études pour caractériser et valider l'interaction entre la dysferline et l'alpha-tubuline qui a été, préalablement, identifiée comme un ligand de la dysferline par chromatographie en phase liquide et spectrométrie de masse. Les résultats ont démontré que le domaine C2A de la dysferline se lie à la phosphatidylsérine (PS), le phosphatidylinositol-4-phosphate (PIP) et le phosphatidylinositol 4,5-biphosphate (PIP2) en présence de calcium. Quant aux autres domaines C2, ils se lient faiblement à la PS indépendamment de la présence de calcium. Ce travail a également révélé que les domaines C2A and C2B de la dysferline peuvent se lier à l'alpha-tubuline indépendamment de la présence de calcium. Les implications possibles de ces interactions sur la réparation de la membrane plasmique des muscles squelettiques et sur la ségrégation de la dysferline dans la cellule musculaire sont discutées.

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Abbreviations

AAV	Adeno-associated virus
AIP1	ASK1-interacting protein-1
BSA	bovine serum albumin
C2	second constant sequence
C2C12	mouse myoblast cell line
CD	circular dichroism
cPLA2	cytosolic phospholipase A2
DMEM	Dulbecco's Modified Eagle Medium
Doc2 (α, β and γ)	double C2 domain (α, β and γ isoforms)
EDTA	ethylenediaminetetraacetic acid
EGTA	ethylene glycol tetraacetic acid
EHD2	Eps15-homology domain protein 2
FBS	fetal bovine serum
GRIP2/PIP2 GRIP	PIP2 binding domain from phospholipase C delta 1
GST	glutathione S-transferase
HRP	horseradish peroxidase
IP4P	inositol polyphosphate 4-phosphatase
IPTG	isopropyl β-D-1-thiogalactopyranoside
LB	Luria broth
LC-MS/MS	liquid chromatography- tandem mass spectrometry
LGMD2B	Limb girdle muscular dystrophy type 2B
MG53 (TRIM72)	mitsugumin 53 (tripartite motif-containing protein 72)
MM	Miyoshi Myopathy
MCTP	Multiple C2 domain with two transmembrane regions
MTOC	microtubule organizing centre
nPKC Apl II	novel protein kinase C *Aplysia* II
PAO	phenylarsine oxide
PBS	phosphate buffered saline
PBST	phosphate buffered saline + Tween-20

PC	phosphatidylcholine
PI4K	phosphoinositol 4-kinase
PKC	protein kinase C
PLCδ1	phospholipase C delta 1
PS	phosphatidylserine
PtdIns3,5P_2	phosphaditylinositol 3,5-bisphosphate
PtdIns3P	phosphatidylinositol-3-phosphate
PtdIns4,5P_2 or PIP2	phosphatidylinositol 4,5-bisphosphate
PtdIns4P or PIP	Phosphatidylinositol-4-phosphate
PtdIns5P	phosphatidylinositol-5-phosphate
PTEN	phosphatase and tensin homologue
RBL	rat basophil leukemia
Slp3	synaptotagmin-like protein 3
SNAP	Soluble NSF attachment protein
SNARE	SNAP receptors
SytI	synaptotagmin I
Tac2-N	Tandem C2 domain in nucleus
TBS	Tris-buffered saline
TBST	Tris-buffered saline + Tween-20
WCB	whole cell body

Contribution of authors to phospholipid binding studies:

C. Therrien and M. Sinnreich were responsible for the majority of the theoretical and technical advances in this project. Experimental works presented in Figures 2.1, 3.1 to 3.6 were published in Biochemistry 48 (11): 2377-84 (2009):

Characterization of lipid binding specificities of dysferlin C2 domains reveals novel interactions with phosphoinositides.

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C. Therrien made the predictions for the topologies of dysferlin's C2 domains [Therrien et al (2006) J Neurol Sci 250(1-2):71-8] and designed the GST-C2 domain constructs used in this study. S. Pickles and S. Di Fulvio optimized the expression and purification procedures for these constructs. All proteins used in this study were expressed and purified by S. Di Fulvio. Experiments were performed by C. Therrien and S. Di Fulvio, as indicated below. Experiments were designed and interpreted by C. Therrien and S. Di Fulvio. The article was written by C. Therrien, with assistance by S. Di Fulvio and M. Sinnreich.

Contributions to figures:
C. Therrien Figures 2.1 and 3.1
S. Di Fulvio Figures 3.1 to 3.6

Contribution of authors to alpha-tubulin binding studies:

B.A. Azakir made the initial prediction that dysferlin interacts with alpha-tubulin based on liquid chromatography-mass spectrometry analysis results and protein binding studies. S. Di Fulvio mapped the interaction to dysferlin's C2 domains and the results are presented as Figure 3.7 in this thesis. This study was published in PLoS ONE 5(4): e10122 (2010):

Dysferlin interacts with tubulin and microtubules in mouse skeletal muscle

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C. Therrien made the predictions for the topologies of dysferlin's C2 domains [Therrien et al (2006) J Neurol Sci 250(1-2):71-8] and designed the GST-C2 domain constructs used in this study. GST-C2 domain fusion proteins used in this study were expressed and purified by S. Di Fulvio. Experiments were designed and interpreted by B.A. Azakir, C. Therrien and S. Di Fulvio. Experiments presented in Figure 3.7 of this thesis were performed by S. Di Fulvio. The article was written by B.A. Azakir with assistance by C. Therrien, S. Di Fulvio and M. Sinnreich.

1. Introduction

1.1 Limb girdle muscular dystrophies and dysferlinopathies

Limb girdle muscular dystrophy type 2B (LGMD2B) (Liu et al 1998), Miyoshi Myopathy (MM) (Liu et al 1998) and distal anterior compartment myopathy (Illa et al 2001) are all caused by mutations in the sarcolemmal protein dysferlin. These dysferlinopathies are clinically characterized by progressive weakness and wasting of the affected limb muscles. As the muscles deteriorate and atrophy, the muscle tissues are replaced by fatty and connective tissues. The degenerating muscle fibers at times exhibit inflammatory infiltrates, as seen on muscle biopsies, and release large amounts of the muscle-specific enzyme, creatine kinase, into the serum. The onset of these diseases is in the late teens to early twenties. Compared to other muscular dystrophies, these myopathies have a slower rate of progression. However, there is currently no cure or treatment available for dysferlinopathies.

In 1998, two independent laboratories discovered that these myopathies were caused by mutations in the dysferlin (*DYSF)* gene (Bashir et al 1998; Liu et al 1998). *DYSF* encompasses 55 exons that span over 150kb of genomic DNA. In 2006, there were over two hundred and fifty unique dysferlin mutations reported in the Leiden muscular dystrophy database (Therrien et al 2006). There is no apparent mutational "hot spot", since mutations have been found throughout the 55 exons of the *DYSF* gene. Most mutations (72%) are single nucleotide substitutions, of which 40% are missense mutations, 19% are nonsense mutations and 13% are intronic mutations, nucleotide deletions, insertions or duplications (Glover & Brown 2007). A large number of the missense mutations (74%) involve amino acids that are predicted to be buried inside the dysferlin protein (Glover & Brown 2007), and 49% of these are found within the seven C2 domains and two DysF domains of dysferlin (Bashir et al 1998; Therrien et al 2006). These mutations result in a loss of functional dysferlin protein. Except in rare instances,

there is no correlation between disease severity and the genotype (Glover & Brown 2007).

1.2 The ferlin family of proteins

Dysferlin is a member of the ferlin family, which is composed of one *Caenorhabditis elegans (C. elegans)* orthologue and six known human homologues.

Dysferlin shares homology with the *fer-1* gene of *C. elegans* (Bashir et al 1998). Fertility factor-1 (Fer-1) is a transmembrane protein expressed in primary spermatocytes of *C. elegans*. Fer-1 mRNA has also been detected in *C. elegans* muscle cells, but was absent from neurons (Krajacic et al 2009). Fer-1 is required for the calcium-dependent fusion of specialized membranous organelle vesicles with the spermatid's plasma membrane (Washington & Ward 2006). Membranous organelles contain stacks of membranous material that when fused to the spermatid's plasma membrane provides the extra membrane and proteins needed for the formation of the pseudopod, which provides motility to the sperm (L'Hernault February 20, 2006). Mutations in Fer-1 lead to the formation of a shorter pseudopod, inadequate sperm motility and worm infertility (L'Hernault February 20, 2006). Mutations in Fer-1 also alter the gene expression of muscle-enriched genes known to regulate the structure and function of muscles (Krajacic et al 2009).

The six human ferlin homologues are, in order of discovery, Dysferlin (Fer1L1), Otoferlin (Fer1L2), Myoferlin (Fer1L3), Fer1L4, Fer1L5, and Fer1L6. All ferlin homologues share a conserved protein structure and have in common 183 strictly conserved residues, multiple cytoplasmic C2 domains and a single C-terminal transmembrane domain (See Figure 1.1).

Dysferlin is a 230kDa type II transmembrane protein containing seven C2 domains and two DysF domains. Dysferlin is found predominately in skeletal muscle and cardiac muscle, and can also be found in kidney, placenta, lung and brain (Bansal & Campbell 2004). The dysferlin protein is located at the plasma membrane (sarcolemma) of muscle fibers and in the t-tubule system, as well as cytoplasmically, presumably in a vesicular compartment.

Myoferlin also contains seven C2 domains and two DysF domains, and is highly expressed in cardiac and skeletal muscle (Davis et al 2000), and in the placenta (Robinson et al 2009). Whereas dysferlin is found predominantly in mature muscle cells, myoferlin is principally expressed in myoblasts (the undifferentiated form of skeletal muscle cells) that are undergoing fusion into myotubes (the differentiated form of skeletal muscle cells) (Doherty et al 2005). Myoferlin is located at the plasma membrane but, unlike dysferlin, myoferlin was also reported to associate with the nuclear membrane (Davis et al 2000). Myoferlin deficiency has not yet been linked with human pathology, although a myoferlin-deficient mouse model showed an abnormal skeletal muscle phenotype with small muscle fibers, decreased muscle mass, and impaired regeneration following muscle injury (Doherty et al 2005).

Otoferlin contains six C2 domains, no DysF domains and an additional coiled-coil domain. Otoferlin is essential for the calcium-dependent exocytosis of synaptic vesicles in the ribbon synapse of auditory inner hair cells (Roux et al 2006), in immature outer hair cells (Beurg et al 2008) and in vestibular hair cells (Dulon et al 2009). Mutations in the otoferlin gene (*OTOF*) lead to a recessive form of human deafness called nonsyndromic prelingual deafness DFNB9 (Yasunaga et al 1999).

Little is known about the other three human ferlin homologues, and they have not yet been associated with human or animal pathology.

1.3 C2 domains: structure and function

All members of the ferlin family contain multiple C2 domains. Dysferlin contains seven, which are named C2A through C2G, located from the N-terminus to the C-terminus, respectively. Initially defined as the second constant sequence (i.e. C2) in protein kinase C (PKC), C2 domains have been found in a plethora of signaling proteins, particularly those involved in membrane trafficking and signal transduction pathways.

C2 domains are independently folded protein domains containing 120-140 amino acid residues. C2 domains are composed of a β-sandwich motif containing eight anti-parallel β-sheets (See Figure 1.2).

C2 domains are assigned one of two types of topologies (Type I or Type II) depending on the connectivity of their β-strands (See Figure 1.2A-B). Type I C2 domains have their N and C termini at the top of β-sheets 1 and 8, whereas Type II C2 domains have their N and C termini at the bottom of β-sheets 1 and 8. Classical examples of Type I and Type II topologies are synaptotagmin I (SytI) and protein kinase C-δ (PKCδ), respectively. The functional significance of the two topologies is unknown; however, one possibility is that it could influence the orientation of the C2 domain relative to the rest of the protein. For instance, the Type I topology of phospholipase C-δ1 (PLCδ1)'s C2 domain puts it on the same side as the active site of the enzyme's catalytic domain (Essen et al 1996). Crystal structure analysis of PLCδ1's domains suggest that the phospholipid-bound C2 domain fixes the catalytic domain in the proper orientation to the membrane, so that it may catalyze the hydrolysis of the phospholipid in order to generate lipid mediators for signaling pathways (Essen et al 1996).

A classical ability of C2 domains is the binding of calcium ions. Loops formed between the β-sheets contain up to five conserved aspartic and glutamic acid

residues that are able to coordinate multiple calcium ions (see Figure 1.2C). Type I C2 domains classically contain all five conserved residues (Nalefski & Falke 1996). Many Type II C2 domains lack one or more of these conserved amino acids, and instead have other residues in the loops that are capable of coordinating calcium ions (such as serine, glutamine, asparagine or threonine) (Nalefski & Falke 1996).

Bound calcium ions create a positive environment that allows the C2 domains to interact with negatively-charged membranes. The majority of Type I C2 domains are calcium-dependent lipid binding domains, such as synaptotagmins and classical protein kinase C (PKC). Type II C2 domains can bind lipids in a calcium-dependent or calcium-independent manner. PKCδ and PKCε (two novel PKCs), PLCδ1, Type I inositol polyphosphate 4-phosphatase (IP4P) and the lipid phosphatase and tensin homologue (PTEN) all contain C2 domains that do not require calcium to bind lipids (Corbalan-Garcia et al 2003; Georgescu et al 2000; Lee et al 1999; Ochoa et al 2001; Pappa et al 1998; Shearn & Norris 2007). The C2 domains of PKCδ and PKCε, for instance, contain major structural and sequence differences that result in several non-functional calcium-binding loops (Ochoa et al 2001; Pappa et al 1998). Instead, diverse hydrophobic and electrostatic forces enable these novel PKCs to interact with membranes (Ochoa et al 2001; Pappa et al 1998).

1.3.1 Predicted topologies of dysferlin's C2 domains

Given that the crystal structures of dysferlin's C2 domains have yet to be determined, we used multiple sequence alignment analysis with known Type I and Type II C2 domains to predict the topologies of dysferlin's C2 domains. This study predicted that dysferlin's C2C, C2D, C2F and C2G domains have Type I topologies, whereas the C2A, C2B and C2E domains have Type II topologies (Therrien et al 2006). Additionally, dysferlin's C2A, C2C, C2D, C2F and C2G

domains were predicted to be able to coordinate calcium ions, whereas C2B and C2E were not (Therrien et al 2006).

1.3.2 Membrane interactions of C2 domains

Most C2 domains are able to bind a wide range of phospholipids that constitute membranes. Some C2 domains bind zwitterionic phospholipids (i.e. phospholipids with a neutral charge), such as phosphatidylcholine (PC). Cytosolic phospholipase A2 (cPLA2) preferentially binds PC, which is a component of all membranes (Krauss & Haucke 2007), in a calcium-dependent manner (Clark et al 1991). This interaction promotes cPLA2's translocation from the cytosol to the membrane where it generates lipid mediators from arachidonic acid (Shimizu et al 2006). Many C2 domains preferentially bind negatively-charged phospholipids, such as phosphatidylserine, which comprises the inner leaflet of all membranes (Krauss & Haucke 2007). Synaptotagmin I preferentially binds PS in a calcium-dependent manner to mediate membrane fusion in neuronal synapses.

Many phospholipids differ in their subcellular localization and often allow for the differentiation of intracellular compartments. Phosphatidylinositol 4,5-bisphosphate (PtdIns4,5P_2) is found at the plasma membrane and in the t-tubule system of skeletal muscles (Czech 2000; Milting et al 1994). PtdIns4,5P_2 plays important signaling roles in membrane trafficking, cytoskeletal organization, cellular signaling and protein activation (Czech 2000). Phosphatidylinositol-4-phosphate (PtdIns4P) is concentrated at the trans-Golgi network, on secretory vesicles, the t-tubule system and at terminal cisternae of the sarcoplasmic reticulum of skeletal muscles (Choudhury et al 2005; Milting et al 1994; Sasaki et al 2007). C2 domain-harbouring ASK1-interacting protein-1 (AIP1) uses PtdIns4P to mediate Toll-like receptor 4 signaling in the innate immune response of mammals (Wan et al 2009). Phosphatidylinositol-3-phosphate (PtdIns3P) and phosphatidylinositol 3,5-bisphosphate (PtdIns3,5P_2) are concentrated predominantly on early and late endocytic membranes, respectively, and are

involved in endosomal trafficking (De Matteis & Godi 2004; Krauss & Haucke 2007). Phosphatidylinositol-5-phosphate (PtdIns5P) is less characterized, but was proposed to be involved in trafficking from late endosomes to the plasma membrane (Lecompte et al 2008).

1.3.3 Membrane interactions of synaptotagmin I

A C2 domain-containing protein that is well-studied for its membrane binding and membrane fusion functions is synaptotagmin I (SytI). SytI is a calcium sensor for calcium-dependent synaptic vesicle exocytosis in the synaptic terminals of neurons (Brose et al 1992; Davletov & Sudhof 1994; Geppert et al 1994). This protein contains an N-terminal transmembrane domain and two C2 domains, named C2A and C2B, which are both calcium-dependent lipid binding domains. SytI is bound to synaptic vesicles filled with neurotransmitters, which are kept in a pool of readily-releasable vesicles close to the plasma membrane of the synaptic terminal (McNeil & Wu 2009).

During neurotransmission, an action potential propagated down the axon to the synaptic terminal will open calcium channels near the pool of SytI-bound vesicles. The resulting influx of calcium ions binds to the residues on the first and third loops of synaptotagmin's C2A and C2B domains, thus activating it. The calcium-bound tips of these loops rapidly and simultaneously penetrate partially into PS-harbouring lipid membranes (Bai et al 2000; Chapman 2008; Hui et al 2006; Paddock et al 2008). This penetration causes indentations or buckling of the inner leaflet of the lipid bilayer (Hui et al 2009; Shahin et al 2008), bringing the two membranes into closer proximity of each other and lowering the energy threshold for membrane fusion (Martens et al 2007).

Meanwhile, synaptobrevin, syntaxin and SNAP-25—located on the vesicle or on the plasma membrane—form a tightly-coiled four-helix bundle called the Soluble NSF attachment protein receptor (SNARE) complex (Sutton et al 1998). The

formation of the SNARE complex is thought to drive the fusion process (Martens & McMahon 2008). Together, the SNAREs and SytI mediate membrane fusion of the synaptic vesicle with the synaptic terminal membrane, thus releasing the neurotransmitter contents of the synaptic vesicle into the synaptic cleft.

1.3.4 Protein interactions of C2 domains

In addition to phospholipid interactions, C2 domains are also able to homodimerize with each other (Lu et al 2006) and to heterodimerize with other protein domains, such as Src homology 2 and 3 domains; phosphotyrosine binding domains and Pleckstrin homology domains (Rizo & Sudhof 1998). For example, the C2B domain of SytI was shown to bind to adaptor protein-2 (Grass et al 2004; Zhang et al 1994), calcium channels (Sheng et al 1997) and SNAP-25 (Gaffaney et al 2008) in neurons. Binding of the endosomal trafficking Eps15-homology domain protein 2 (EHD2) to myoferlin's second C2 domain (C2B) is essential for myoblast fusion (Doherty et al 2008).

1.4 Membrane repair in skeletal muscles

There is much evidence pointing to dysferlin's role in membrane fusion:

1. The homology between dysferlin and its ortholog Fer-1 from *C. elegans*, a membrane fusion protein implicated in sperm maturation.
2. The functional homologies between dysferlin and synaptotagmin I's C2A domains, the latter being a calcium-sensing membrane fusion protein in neural synapses.
3. Otoferlin, a homologue of dysferlin, mediates calcium-dependent membrane fusion in the synapses of hair cells.

In skeletal muscles, membrane fusion plays a key role in the repair of damaged plasma membrane.

Skeletal muscles are crucially important for generating the force required for movement. Skeletal muscles are crucially important for generating the force required for movement. Voluntary muscle contractions are generally classified into four categories: *concentric contractions* that cause the muscle to shorten, *eccentric contractions* that cause the muscle to lengthen, *isometric contractions* whereby the muscle retains its length, and *isotonic contractions* in which the muscle lengthens but the tension remains unchanged. Sarcolemmal injury occurs most frequently with eccentric contractions, which are physiologically common movements since much of a muscle's everyday activity occurs when the muscle is lengthening (Goslow et al 1973; Hoffer et al 1989)

The membrane disruptions caused by these injuries allow the movement of ions and molecules into and out of (influx and efflux, respectively) the muscle cells. For instance, the muscle-specific creatine kinase protein will efflux from the cell and calcium will influx into the cells. Since the extracellular to intracellular ratio of calcium is large (10,000-fold) (McNeil 2009), the calcium influx down this steep concentration gradient would result in a rapid (Steinhardt et al 1994) and significant increase in the local intracellular calcium concentration near the site of injury. If the influx of calcium is left unabated, it will lead to cytotoxicity (Verkhratsky 2007) and muscle cell degeneration. Therefore, muscle membrane disruptions must be repaired swiftly and efficiently to ensure cell survival.

There are two models of how muscle cells repair plasma membrane disruptions. The first is the tension reduction hypothesis that is founded on the concepts of line tension and membrane tension. When the plasma membrane is disrupted, the hydrophobic tails of the phospholipids are exposed to water, which is an unfavourable, high-energy state. This creates line tension, which favours the automatic lipid flow (resealing) over the disruption site. However, the plasma membrane is attached to the underlying cortical cytoskeleton, which creates membrane tension that opposes resealing. The tension reduction hypothesis

postulates that a cell can reduce the membrane tension to the point that line tension is sufficient to promote resealing (McNeil & Steinhardt 2003).

The second model is the patch membrane repair process, which postulates that membrane disruptions trigger an accumulation of vesicles to the site of injury, which fuse to each other and to the plasma membrane in order to form a membrane patch across the injury site. This process is highly dependent on calcium; muscle fiber wounding studies revealed that if the extracellular calcium is chelated by EDTA, the membrane disruption does not get repaired (Bansal et al 2003). This process is also extremely rapid, occurring within seconds of membrane wounding.

1.4.1 Dysferlin is essential for skeletal muscle membrane repair

Studies using dysferlin-deficient mouse models have confirmed that dysferlin is indispensable to sarcolemmal patch membrane repair. Immunostaining in wildtype skeletal muscle revealed an accumulation of dysferlin at the site of injury (Bansal et al 2003). Wounding assays performed on dysferlin-deficient skeletal muscle fibers in the presence of an impermeant fluorescent dye, FM1-43, revealed that muscle disruptions are not repaired efficiently (Bansal et al 2003). Electron microscopy analysis of these dysferlin-deficient muscle fibers revealed an accumulation of vesicles at the injury site, but no membrane fusion (Bansal et al 2003). Electron microscopy of skeletal muscle biopsies from patients with dysferlinopathies revealed disrupted plasma membrane, thickening of the basal lamina and vesicle accumulations underneath membrane disruptions (Selcen et al 2001).

1.4.2 Membrane-repair machinery and dysferlin-interacting proteins

Dysferlin may have been the first protein revealed to be involved in the skeletal muscle membrane patch repair process, but it is not the only one. Other proteins

have been discovered to be dysferlin-interacting proteins as well, namely annexins A1 and A2 (Lennon et al 2003), affixin (β-parvin) (Matsuda et al 2005), AHNAK (Huang et al 2007), calpain-3 (Anderson et al 2000; Fanin et al 2001), caveolin-3 (Matsuda et al 2001) and MG53 (Cai et al 2009b). These proteins have been shown to be involved in membrane repair, membrane fusion, cytoskeletal remodeling and vesicle trafficking. Several of these proteins have been found to have altered protein expression in dysferlinopathic patients (Anderson et al 2000; Cagliani et al 2005; Fanin et al 2001). Additionally, mutations in calpain-3 and caveolin-3 lead to their own types of limb-girdle muscle dystrophy, namely LGMD1C and LGMD2A, respectively.

The emerging model for the patch membrane repair machinery (McNeil 2009) theorizes that the influx of extracellular calcium, as well as extracellular oxidants (Cai et al 2009a), activates dysferlin (Bansal et al 2003), annexins A1 and A2 (McNeil et al 2006), and MG53 (Cai et al 2009a). The calcium-activated phospholipid-binding annexins promote membrane aggregation of cytoplasmic vesicles (Babiychuk & Draeger 2000; Gerke & Moss 2002; Lambert et al 1997). The oxidant-activated mitsugumin 53 (MG53), a tripartite motif family 72 (TRIM72) protein, is a phosphatidylserine-binding protein that contributes to vesicle trafficking (Weisleder et al 2009). Oxidized MG53 is believed to covalently cross-link the membranes through its oxidizable SH domains (Cai et al 2009a). This cross-linking therefore "nucleates" or docks the membranes so that they can be fused to one another (Cai et al 2009a; McNeil 2009). Membrane fusion is believed to be mediated by dysferlin (Bansal et al 2003) and possibly annexins (McNeil et al 2006). It is reported that a complex is formed between dysferlin, MG53 and caveolin-3 that regulates membrane repair (Cai et al 2009b; Weisleder et al 2009). Caveolin-3 is believed to be important for proper localization of the complex at the plasma membrane (Cai et al 2009b).

The plasma membrane of skeletal muscles is the thin outermost layer of the sarcolemma. Beneath the plasma membrane is the cortical cytoskeleton, which is

a complex network of cytoskeletal and sarcolemmal proteins, actin and microtubules. Calpain-3 is a muscle-specific calcium-dependent cysteine protease that is believed to disassemble the cortical cytoskeleton at the disruption site (McNeil 2009; Mellgren et al 2007), thus facilitating patch membrane fusion to the damaged plasma membrane. Calpains have been shown to be required for calcium-mediated membrane repair in many cultured cell types (Godell et al 1997; Mellgren et al 2007), although one study showed that calpain-3 was not involved in calcium-mediated membrane repair in calpain-deficient mouse skeletal myotubes (Mellgren et al 2009). Instead, the authors proposed that calpain-3 may be involved in cytoskeletal remodelling and reorganisation in the first 24 hours after injury, rather than in the immediate membrane repair process itself. Calpain-3 was also shown to cleave annexins (Lennon et al 2003) and AHNAK (Huang et al 2008), the latter being an actin-binding protein proposed to be important for membrane fusion events during muscle regeneration (Huang et al 2007). AHNAK is found in the lumen of enlargeosomes (Benaud et al 2004), which are cytoplasmic vesicles that undergo rapid, calcium-dependent endocytosis and exocytosis. Affixin (β-parvin) links the cytoskeleton (α-actinin) with integrin proteins (Sepulveda & Wu 2006) and may play a role in the membrane repair process (Matsuda et al 2005) or in post-injury cytoskeletal restructuring.

1.4.3 Vesicular compartments or endomembranes used in membrane repair

The identity of the vesicular membranes, or endomembranes, used for muscle membrane repair is still unknown, although various suggestions have been made.

In sea urchin eggs, yolk granules are used in a calcium-dependent membrane repair process (McNeil et al 2000). Yolk granules are acidic compartments containing hydrolytic enzymes (Armant et al 1986; McNeil & Terasaki 2001). Lysosomes in mammalian cells are also acidic compartments containing acid hydrolases, cathepsins and other digestive enzymes. In the presence of high

calcium concentrations, lysosomes can fuse to one another and to the plasma membrane (Bakker et al 1997; Mayorga et al 1994; Rodriguez et al 1997). Membrane repair studies on rat kidney fibroblasts and skin fibroblasts demonstrated that calcium-dependent lysosomal exocytosis was mediated by lysosomal synaptotagmin VII (Reddy et al 2001). Successfully repaired myotube sarcolemma revealed surface expression of Lamp-1, which is found on the inner membrane of lysosomes (Lennon et al 2003; Reddy et al 2001). A note of concern is that using lysosomes as a regular source of endomembranes would cause the release of the lysosomal contents into the extracellular milieu of muscle cells, which would be expected to have deleterious effects on surrounding healthy muscle fibers. However, McNeil points out that exposure to the extracellular milieu (pH ~7.4) would decrease the hydrolytic activity of the lysosomal enzymes, which operate optimally in an acidic environment (pH~3.5) (McNeil 2002). Secondly, many cells, such as muscle cells and hepatocytes, have cation-dependent mannose-6-phosphate receptors that can scavenge lysosomal enzymes by endocytic uptake (McNeil 2002). Nevertheless, the involvement of lysosomes in membrane repair is still a contested topic (Cerny et al 2004; Huynh & Andrews 2005).

Another proposed source of endomembranes is enlargeosomes (Benaud et al 2004; Cocucci et al 2004; Lennon et al 2003). These cytoplasmic vesicles undergo rapid, calcium-dependent endocytosis and exocytosis, and contain dysferlin-interacting AHNAK (Cocucci et al 2004) and annexins (Benaud et al 2004). However, nothing further on this subject has been published, and it is speculated that the interaction between dysferlin and AHNAK may be related more to muscle regeneration than to muscle membrane repair (Han & Campbell 2007).

The source of the endomembranes used in membrane repair of skeletal muscles is still under investigation.

1.5 Hypothesis and objectives

To better understand dysferlin and its functions, continued studies into dysferlin's structure and interacting proteins are essential. Notably, dysferlin's seven C2 domains are significantly under-characterized. We hypothesize that dysferlin's multiple C2 domains are likely candidates for mediating dysferlin's binding interactions, particularly with phospholipids and various protein motifs. Dysferlin's C2 domains have not been studied for either phospholipid binding interactions (except for dysferlin's C2A domain (Davis et al 2002)) or protein binding interactions. To this end, we sought to identify novel binding molecules for dysferlin's C2 domains, notably with phospholipids and novel binding proteins.

1.5.1 Dysferlin and phospholipids

We sought to identify novel phospholipid binding specificities for dysferlin's C2 domains. Since dysferlin is believed to be involved in the fusion of intracellular vesicles with the plasma membrane, dysferlin would be expected to interact with the major components of the membrane: phospholipids. It is highly probable that dysferlin's C2 domains could be calcium-dependent or -independent phospholipid binding domains.

To date, only dysferlin's C2A domain has been studied for calcium-dependent lipid binding. Dysferlin's C2A domain exhibited calcium-dependent binding to liposomes composed of 50% phosphatidylserine (Davis et al 2002). Myoferlin's C2A domain was also shown to bind to liposomes composed of 50% phosphatidylserine in a calcium-dependent manner, whereas the other myoferlin C2 domains failed to exhibit any binding activity (Davis et al 2002). Otoferlin's C2D domain was shown to bind calcium by fluorescence emission spectroscopy, but no lipid binding studies were reported (Roux et al 2006).

Hence, only dysferlin's C2A domain was investigated for binding to a single phospholipid, PS. The lipid binding specificities of the other dysferlin C2 domains have not been studied. To this end, we sought to characterize lipid specificities and calcium dependency of all seven dysferlin C2 domains.

1.5.2 Dysferlin and alpha-tubulin

To identify novel protein binding partners for dysferlin, we performed a liquid chromatography-mass spectrometry (LC-MS/MS) analysis of proteins that co-immunoprecipitated with dysferlin in skeletal muscle. Through these studies, we identified proteins involved in a variety of cellular functions, including cellular trafficking, recycling, ubiquitination and cytoskeletal organization (Azakir et al 2010). How dysferlin is trafficked within the muscle cell, and how dysferlin-harbouring vesicles are trafficked to the plasma membrane, remain unsolved issues. Reasonably, such movement would occur along the 'highways' of the cell: the microtubules and actin filaments. Alpha-tubulin, one of the basic components of microtubules, was indeed one of the proteins identified by our LC-MS/MS analysis. Dysferlin has not yet been reported to interact with alpha-tubulin or with microtubules. To gain insight into dysferlin's trafficking pathways, we chose to begin our studies with one of the basic elements of the microtubule transport network: alpha-tubulin.

Alpha-tubulin is a monomeric component of microtubules. Alpha-tubulin heterodimerizes with beta-tubulin to form protofilaments, which polymerize into microtubule filaments. Microtubules are polarized such that their minus end is oriented towards their site of origin near the nucleus (called the microtubule organization centre or MTOC) and their plus end projects to the periphery of the cell. The orientation of microtubules differs in undifferentiated and differentiated muscle cells. In myoblasts, microtubules originate from an MTOC located near the nucleus. The microtubules project outwards towards the periphery of the cell. In myotubes, there is no single MTOC. Instead, microtubules originate from

multiple perinuclear locations and project towards the periphery of the cell by assuming a longitudinal configuration (Prins et al 2009).

Owing to the polarization of microtubules, motor proteins use them to travel and transport proteins, organelles and vesicles towards the periphery (anterograde transport) or towards the nucleus (retrograde transport). Microtubules are also crucial for determining and maintaining the cellular morphology and structural integrity of cells.

To confirm the interaction between dysferlin and alpha-tubulin, we used several protein binding assays (Azakir et al 2010). We then wanted to determine if dysferlin's interaction with alpha-tubulin involved dysferlin's C2 domains, since C2 domains are documented protein-protein binding motifs.

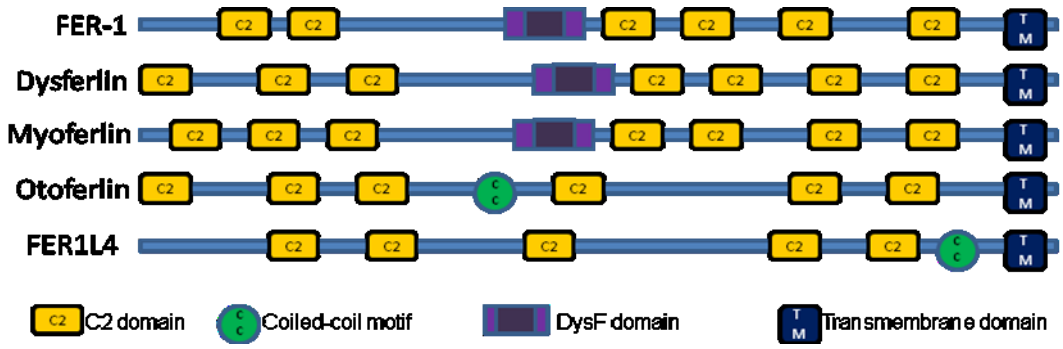

Figure 1.1: Protein motif organization of the ferlin family of proteins

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Shown here are *C. elegans* Fer-1 protein and four of the six known mammalian ferlin family members. They all contain multiple C2 domains and a single C-terminal transmembrane domain. Dysferlin, myoferlin and Fer-1 all contain two DysF domains, one being nested within the other. A coiled-coil motif is common to otoferlin and FER1L4.

This figure was designed from data provided in (1) Washington and Ward. *J Cell Sci* **119**, 2552-2562 (2006) and (2) Smith, Gurrola, Kelley. GeneReviews at GeneTests: Medical Genetics Information Resource (database online). Copyright, University of Washington, Seattle. February 2008. Available at http://www.genetests.org. Accessed 3 January 2010.

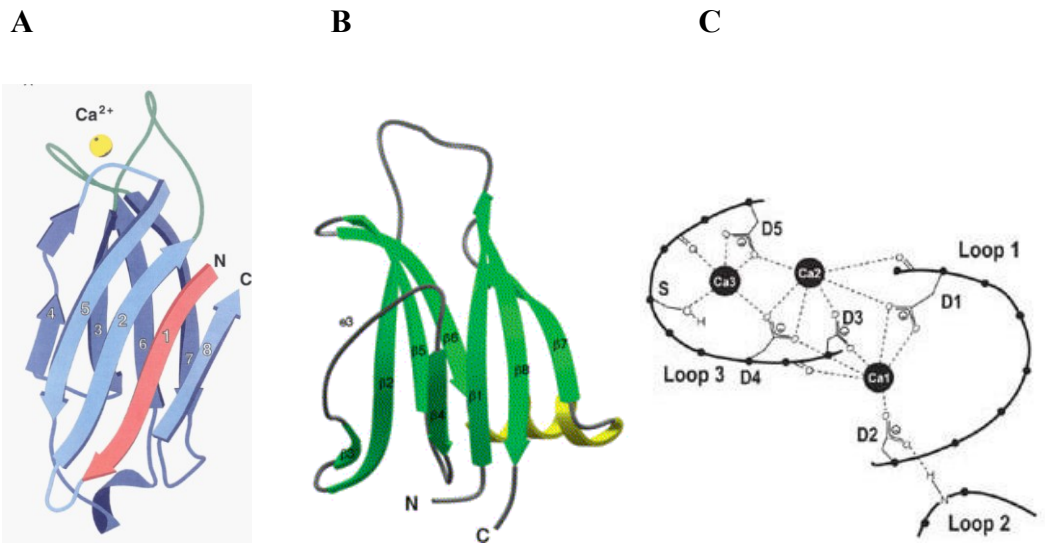


Figure 1.2: The topologies and calcium-coordinating features of C2 domains

(A-B) Ribbon diagrams illustrating how C2 domains adopt a β-sandwich motif consisting of eight anti-parallel β-sheets. C2 domains can adopt one of two topologies: Type I or Type II. (A) Type I C2 domains have their N and C termini at the top of β-sheets 1 and 8, as shown for synaptotagmin I [i]. (B) Type II C2 domains have their N and C termini at the bottom of β-sheets 1 and 8, as shown for protein kinase C-δ (PKCδ) [ii]. (C) Loops formed between the β-sheets contain acidic residues that bind calcium ions. Synaptotagmin I's N-terminal C2A domain has five glutamic acid residues in loops 1 and 3 that coordinate up to three calcium ions [iii].

[i] Reprinted from Protein Science, volume 5, Nalefski and Falke, C2 domain calcium-binding motif: Structural and functional diversity, pages 2375-2390, Copyright 1996, with permission from John Wiley and Sons.

[ii] Reprinted from Structure, volume 15, Pappa, Murray-Rust, Dekker, Parker and McDonald, Crystal structure of the C2 domain from protein kinase C-delta, pages 885-894, Copyright 1998, with permission from Elsevier.

[iii] Reprinted with permission from Macmillan Publishers Ltd: Nature Structural & Molecular Biology, volume 11, Dai, Shin, Machius, Tomchick, Sudhof and Rizo, Structural Basis for the evolutionary inactivation of Ca2+ binding to synaptotagmin 4, pages 844-849, Copyright 2004.

2 Materials and Methods

2.1 Antibodies

Polyclonal goat anti-GST antibodies (catalogue number 460) were purchased from GE Healthcare. Monoclonal mouse anti-alpha-tubulin antibodies were purchased from Zymed Laboratories, Inc (32-2500). Rabbit anti-goat IgG-horseradish peroxidase conjugated (HRP) antibodies (81-1620) were purchased from Zymed Laboratories, Inc. Sheep anti-mouse IgG-HRP conjugated antibodies (NA931V) were purchased from GE Healthcare.

2.2 C2 domain constructs

The predicted boundaries of each of dysferlin's C2 domains were determined by sequence alignment as previously described (Therrien et al 2009; Therrien et al 2006). The sequences for dysferlin's C2 domains were amplified by PCR using the human dysferlin cDNA, cloned into the DFL-2 plasmid (kindly provided by K Bushby, Newcastle, U.K.) and Klentaq polymerase (Sigma). The C2A domain for human synaptotagmin I (GenBank entry NM_005639.1) was amplified from a commercially available cDNA clone (Origene). *Eco*RI and *NotI* sites were included in the primer sequences to subclone the fragments in frame with glutathione S-transferase (GST) in the multiple cloning site of pGEX4T1 (GE Healthcare) to generate eight GST fusion proteins (see Figure 2.1). All recombinant constructs were verified by DNA sequencing.

2.3 Expression and purification of dysferlin C2 domains

All of the C2 domain clones were expressed in the *Escherichia coli* BL-21 expression system. A single colony grown on Luria Broth (LB) (BioShop) + 100μg/ml Ampicillin (Sigma) agar (Bioshop) plates was selected and incubated overnight in 2xYT (BioShop) + 100μg/ml Ampicillin at 37^{o}C, with vigorous

shaking (225rpm). The culture was diluted (1:50) in 50ml (GST and C2A) or 500ml (other C2 domains) of 2xYT + 100µg/ml Ampicillin and incubated at 37^{o}C, 225rpm, until the optical density (OD) at 600nm reached ~0.5, as measured by spectrophotometry. The expression of the fusion proteins was promptly induced with isopropyl β-D-1-thiogalactopyranoside (IPTG) (Sigma) at a final concentration of 0.5mM, followed by 2h incubation at 24^{o}C, 225rpm to reduce the aggregation of recombinant proteins into inclusion bodies. The bacterial cells were centrifuged at 6000rpm, 4^{o}C for 15min. The bacterial pellet was collected and was used immediately or was kept frozen at -80^{o}C until needed.

Upon thawing, the bacterial pellet was resuspended in ice-cold lysis buffer [20mM Tris-HCl (BioShop), pH 7.5, 150mM NaCl (BioShop), 1mg/ml lysozyme (Sigma), 1µg/ml DNase I (Sigma), 1x protease inhibitors (Roche, Complete cocktail)]. The solution was incubated on ice 10min then further lysed by three rounds of sonication (15sec each) at an intensity of 4 (Vibrocell, Sonics), with icing between bursts. Triton X-100 was added to a final concentration of 1% and the lysates were incubated for 30min at 4^{o}C on a rotating platform. The lysates were centrifuged at 13000rpm, 4^{o}C for 15min. The supernatant was collected and incubated with 50% glutathione Sepharose 4B bead slurry (GE Healthcare) at 4^{o}C for 2h or overnight. Prior to use, the beads were washed three times with PBS, pH 7.4, to remove residual ethanol from the storage solution. Following binding incubation, the beads were washed three times with ice-cold wash buffer [20mM Tris-HCl, pH 7.5, 150mM NaCl for the lipid experiments; or phosphate buffered saline (PBS), pH 7.4, for the GST pulldown experiments].

For the GST pulldown experiments, proteins remained immobilized on the beads, and were stored at 4^{o}C until needed. For the lipid experiments, GST-C2 domain fusion proteins were eluted from the beads at room temperature using 10mM reduced glutathione (GE Healthcare) in 50mM Tris-HCl, pH 8.0. These eluants were stored at 4^{o}C. Protein purity and size were verified by SDS-PAGE and staining with SimplyBlue SafeStain (Invitrogen). The concentrations of eluted

proteins were measured by Bradford protein assay (BioRad). The concentrations of immobilized proteins were determined by first running standard amounts of purified bovine serum albumin (BSA) (Sigma) (1, 2, 5, 10μg) and known volumes of the immobilized protein on SDS-PAGE gels, staining with SimplyBlue SafeStain and scanning the gels with an Epson Perfection 4490 Photo Scanner. The scanned gels were analyzed by densiometric analysis using Adobe Photoshop CS (version 8.0). Proteins were used within two weeks.

2.4 Protein-lipid overlay experiments

Lipid binding specificity was assessed with protein-lipid overlay assays using commercial lipid strips (Echelon Biosciences) or custom lipid strips. Commercial lipid strips contained 100pmol of more than a dozen biologically relevant lipids that are found in mammalian membranes. Custom lipid strips were prepared in our laboratory using increasing amounts of lipids to enhance the sensitivity of the binding interactions.

Procedures for the design of the custom lipid strips were adapted from those of Takahashi et al (Takahashi et al 2006) and Dowler et al (Dowler et al 2002). Serial dilutions of chloroform/methanol/water (1:2:0.8) solutions containing phosphatidylserine (PS), phosphatidylinositol-4-phosphate (PIP) and phosphatidylinositol 4,5-bisphosphate (PIP2) (Avanti Polar Lipids Inc.) were prepared. One microliter of each solution was spotted in an increasing array onto pure 0.45μm Trans-Blot nitrocellulose membranes (BioRad) and air-dried.

Lipid strips were blocked for 1h with 3% fatty acid-free BSA (Sigma) in Tris-buffered saline + Tween-20 (0.05%) (TBST) buffer for 1h at room temperature. Membranes were then incubated with 0.5 μg/mL soluble GST-C2 domain fusion proteins in 3% BSA-TBST for 16 h at 4°C.

0.5µg/ml of soluble SytIC2A was used as a positive and negative control for calcium-dependent lipid binding C2 domain binding in PS, PIP and PIP2 binding assays. 0.5µg of PIP2 Grip (Echelon) was also used a positive control for PIP2 binding. PIP2 Grip (GRIP2) is made from the N-terminal GST-tagged recombinant PLCδ1 Pleckstrin homology (PH) domain.

Following primary antibody incubation, membranes were washed three times in 3% BSA-TBST for 10 min, followed by incubation with a 1:5000 dilution of anti-GST antibodies (GE Healthcare) in 3% BSA-TBST for 1h at room temperature. The membranes were washed ten times with 3% BSA-TBST for 5min over a period of 50min. Membranes were then incubated with a 1:5000 dilution of anti-goat IgG-HRP conjugate (Zymed) in 3% BSA-TBST for 1h at room temperature, and subsequently washed twelve times with TBST for 5min over a period of 1 h. The protein-lipid interactions were detected by enhanced chemiluminescence (Amersham). Experiments were performed in triplicate.

2.5 Liposome centrifugation assay

Because protein-lipid overlay assays are qualitative in nature, liposome centrifugation assays were performed to quantitate the PS binding ability of dysferlin's C2 domains. Since a liposome is essentially a spherical lipid sheet, liposomes represent a more biologically relevant system for assessing protein-phospholipid binding abilities than protein-lipid overlays assays, which use dried blotted lipids.

Liposome centrifugation assays using large unilamellar vesicles (LUV or liposomes) were performed according to reported methods (Fernandez et al 2001; Min et al 2007; Pepio & Sossin 2001; Shin et al 2002). Briefly, chloroform solutions composed of 35% phosphatidylcholine (PC) and 65% PS, 80% PC and 20% PIP, or 80% PC and 20% PIP2 were mixed and dried under a stream of nitrogen gas to evaporate the chloroform. The dried lipid films were resuspended

in liposome binding buffer [50mM HEPES-NaOH, pH 6.8, 100mM NaCl, 4mM Na_2EGTA, pH 8.0] containing 0.5M sucrose, then subjected to five freeze-thaw cycles in dry ice-cooled 100% ethanol and a 37°C water bath. The solution was passed at least ten times through a 0.1 μm filter (Whatman) using a Mini-Extruder instrument (Avanti Polar Lipids Inc.) to produce homogeneously sized 100μm sucrose-loaded liposomes.

For the binding assay, 5μg of soluble GST-C2 domain fusion proteins were mixed with 500μg of liposomes in liposome binding buffer (without sucrose). The total reaction volume of 500ul contained $CaCl_2$ or EGTA, pH 8.0, whose compositions and final concentrations were calculated with WEBMAXCLITE version 1.15 (www.stanford.edu/cpatton/webmax). The mixture was incubated for 30min at room temperature, and then centrifuged at 100000g for 30min in a TLA-100 ultracentrifuge (Beckman Instruments). Liposomes and any associated proteins were pelleted and any unbound proteins remained in the supernatant. Supernatant and pellet fractions were collected and analyzed by SDS-PAGE. Protein bands were stained with SimplyBlue SafeStain for 1h and washed in water to reduce the background. Gels were scanned with an Epson Perfection 4490 Photo Scanner, and densitometric analysis of the protein bands was performed with NIH ImageJ version 1.41. The percentages of lipid bound protein were calculated with the equation:

$$\% \text{ of lipid-bound protein} = \frac{\text{pellet fraction}}{(\text{pellet fraction} + \text{soluble fraction})} \times 100$$

For the calcium titration experiments, the percentage of lipid bound protein was calculated with the equation:

$$\% \text{ of lipid-bound protein} = \frac{\text{pellet fraction}}{\text{total protein input}} \times 100$$

The terminology of 'apparent calcium affinity' describes the lowest calcium concentration at which strong lipid binding is detectable, which is distinguishable from nonspecific binding, as previously described (Min et al 2007; Shin et al 2005; Shin et al 2002). Experiments were performed in triplicate.

2.6 GST pulldown assays for alpha-tubulin

Mouse myoblast lysates were prepared as follows. C2C12 myoblasts were grown in Dulbecco's Modified Eagle Medium (DMEM) + 10% fetal bovine serum (FBS) at 37^{o}C in 5% CO_2. Cells were collected in ice-cold cell lysis buffer [10mM HEPES, pH 7.4, 33mM NaCl, protease inhibitors: 10μM aprotinin (Sigma), 10μM leupeptin (Sigma), 1mM benzamidine (Sigma), 1mM PMSF (Sigma), 1μM E-64 (Sigma)], and further lysed by sonication with three bursts of 3sec each. Triton X-100 was added to a final concentration of 1% and the lysates were incubated for 30min at 4^{o}C on a rotating platform. The lysates were centrifuged at 15000rpm, 4^{o}C for 15min. The supernatant was collected, the protein concentration was determined by Bradford protein assay, and aliquots were frozen at –80^{o}C until needed.

For the GST pulldown assay, 10μg of immobilized GST-C2 domain fusion proteins were added to 1mg of C2C12 myoblast lysate diluted to a final reaction volume of 1ml. The reaction volume contained either a final concentration of 1mM calcium or 1mM EGTA to determine any calcium-dependency of the binding interaction. The mixture was incubated overnight at 4^{o}C on an end-over-end rocking platform. Then, the beads were washed three times in ice-cold wash buffer [10mM HEPES, pH 7.4, 33mM NaCl, 1% Triton X-100]. Associated proteins were separated by SDS-PAGE, transferred onto pure nitrocellulose membrane, and Western blotted with anti-alpha-tubulin antibodies. Experiments were performed in triplicate.

2.7 Western blot for GST pulldown experiments

Membranes were blocked for 1h in PBS-T (0.1% Tween-20) plus 5% dry non-fat milk (Carnation) at room temperature. Membranes were incubated in PBS-T + 5% milk with mouse anti-tubulin antibodies (1:1000) overnight at 4^{o}C. Membranes were then washed three times in PBS-T + 5% milk at room temperature for 15-20min. Membranes were incubated with anti-mouse IgG-HRP conjugated antibody (1:1000) in PBS-T + 5% milk for 1h at room temperature. Membranes were washed three times in PBS for 10-15min, and then detected by enhanced chemiluminescence.

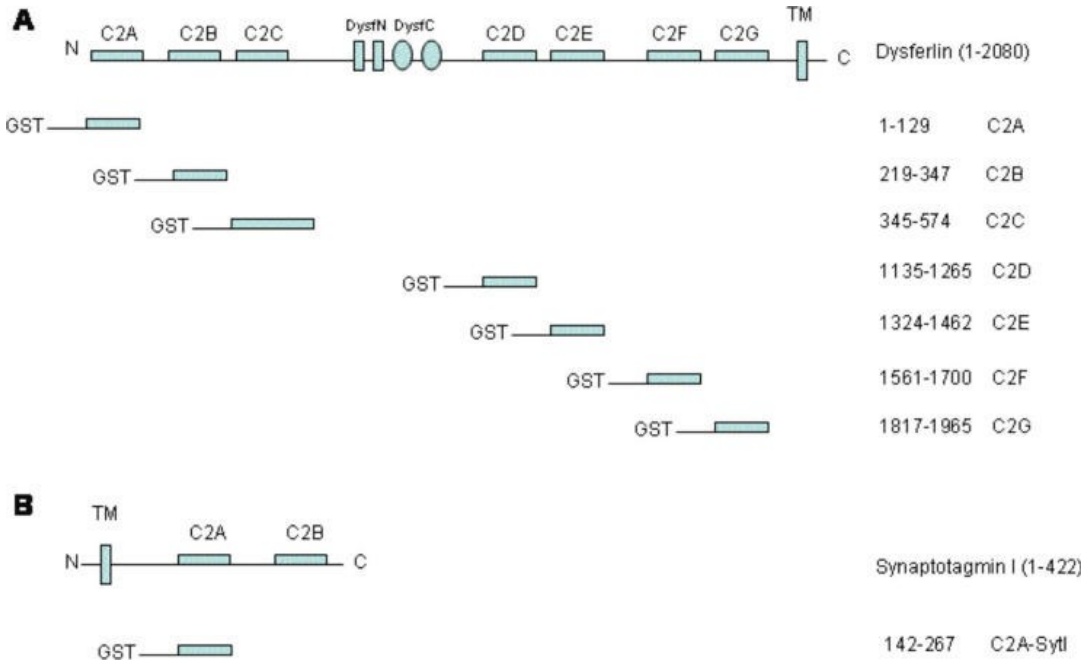


Figure 2.1: Schematic representation of the GST-C2 domain fusion proteins

(A) Each of dysferlin's seven C2 domains was cloned from the full-length dysferlin. The number of residues used to demarcate each C2 domain is indicated on the right. The pGEX4T1 vector was used to fuse a GST moiety to the N-terminus of each C2 domain. (B) The same procedure was used to generate the GST-SytIC2A domain fusion protein.

3 Results

3.1 Screening for novel phospholipid binding interactions

The only phospholipid investigated for interactions with dysferlin's C2A domain reported to date was phosphatidylserine (PS), therefore we undertook a phospholipid binding study to screen for additional lipid binding specificities associated with each of dysferlin's C2 domains. Protein lipid overlay assays were performed by incubating glutathione S-transferase (GST)-C2 domain fusion proteins for each of dysferlin's C2 domains (DysfC2A through G) and synaptotagmin I's first C2 domain (SytIC2A) on commercial lipid strips in the presence (1mM $CaCl_2$) or absence (1mM EGTA) of calcium.

Western blotting with anti-GST antibodies revealed that GST-SytIC2A and GST-DysfC2A bind to phosphatidylserine (PS), phosphatidylinositol-4-phosphate (PtdIns4P or PIP) and phosphatidylinositol-5-phosphate (PtdIns5P) in a calcium-dependent fashion (Figure 3.1). GST-DysfC2A also bound weakly to phosphatidylinositol-3-phosphate (PtdIns3P) and phosphatidylinositol 3,5-bisphosphate (PtdIns3,5P_2). All of dysferlin's other GST-C2 domains exhibited no detectable binding. GST-DysfC2F and C2G exhibited weaker calcium-independent binding to PS and phosphoinositide monophosphates (PtdIns3P, PtdIns5P). Importantly, GST alone did not bind to any of the phospholipids in either the presence or absence of calcium.

We chose to characterize the phospholipid binding abilities of dysferlin's C2 domains to three phospholipids. Phosphatidylserine (PS) was selected because it is a component of all membranes. Phosphatidylinositol-4-phosphate (PIP) and phosphatidylinositol 4,5-bisphosphate (PtdIns4,5P2 or PIP2) were selected because their compartmental localizations overlapped with dysferlin's reported localization.

3.2 All of dysferlin's C2 domains bind to phosphatidylserine

To investigate the PS-binding properties of dysferlin's seven C2 domains, we performed protein-lipid overlay assays using custom lipid strips that were blotted with increasing lipid concentrations so as to enhance the sensitivity of this binding assay. GST-C2 domain fusion proteins for each of dysferlin's C2 domains (DysfC2A through G) and synaptotagmin I's first C2 domain (SytIC2A) were incubated on PS-spotted lipid membranes in the presence (1mM $CaCl_2$) or absence (1mM EGTA) of calcium.

Western blotting with anti-GST antibodies revealed that all of dysferlin's C2 domains were able to bind PS (Figure 3.2). Only dysferlin's C2A domain exhibited calcium-dependent binding to PS. Dysferlin's other C2 domains displayed weaker calcium-independent binding to PS. Both GST-DysfC2A and GST-SytIC2A evidenced strong PS binding, and this interaction was calcium dependent.

To quantify this phospholipid binding interaction, we performed liposome centrifugation assays. Each of the GST-C2 domain fusion proteins was incubated with PS/PC-containing liposomes in the presence (1mM $CaCl_2$) or absence (1mM EGTA) of calcium.

The results showed that 40% of GST-DysfC2A bound in a calcium-dependent manner to the PS/PC liposomes (Figure 3.3). This interaction was reduced two-fold in the absence of calcium. GST-SytIC2A exhibited similar calcium-dependent PS-binding behavior: over 50% of GST-SytIC2A protein bound to the PS/PC liposomes in the presence of calcium. When calcium was depleted, only 15% of GST-SytIC2A bound to the PS/PC liposomes.

All of dysferlin's other C2 domains exhibited weaker phospholipid binding abilities: 5-20% of each GST-C2 domain fusion protein (C2B to C2G) was detected in the liposome-harbouring pellet (Figure 3.3). The PS/PC-binding interactions of these GST-C2 domains were calcium-independent. GST alone did not bind to the PS/PC liposomes.

3.3 Dysferlin's C2A domain binds phosphatidylinositol-4-phosphate and phosphatidylinositol 4,5-bisphosphate

Protein-lipid overlay assays using custom lipid strips were performed to assess whether dysferlin's seven C2 domains bind to PIP and PIP2. GST-C2 domain fusion proteins for each of dysferlin's C2 domains (DysfC2A through G) and synaptotagmin I's first C2 domain (SytIC2A) were incubated on PIP- or PIP2-spotted custom lipid strips in the presence (1mM $CaCl_2$) or absence (1mM EGTA) of calcium.

Western blotting with anti-GST antibodies revealed that only GST-DysfC2A was able to bind to PIP and PIP2, and that this interaction was calcium-dependent (Figure 3.4). Dysferlin's other C2 domains and GST alone showed no significant interactions with PIP or PIP2 in either the presence or absence of calcium.

3.4 The interactions between dysferlin's C2A domain and phosphatidylserine, phosphatidylinositol-4-phosphate, phosphatidylinositol 4,5-bisphosphate are calcium-dependent

To further characterize the calcium-dependent behaviour of dysferlin's C2A domain, calcium-titration experiments were designed using both protein-lipid overlay assays and liposome centrifugation assays.

For the protein-lipid overlay assays, dysferlin's C2A domain (GST-DysfC2A) and synaptotagmin I's first C2 domain (GST-SytIC2A) were incubated on custom

lipid strips containing increasing amounts of all three phospholipids (PS, PIP and PIP2) in either the absence of calcium (1mM EGTA) or in the presence of increasing amounts of calcium (0.1μM, 1μM, 0.1mM, 0.5mM and 1mM).

The results of the protein-lipid overlay assays showed that GST-DysfC2A bound most strongly to PS, even binding weakly at low (1μM) calcium concentrations (Figure 3.5). This interaction was strongly calcium dependent, since no binding was observed when calcium was depleted with 1mM EGTA. Additionally, the intensity of protein binding to 125pmol and 250pmoles of PS became stronger with increasing calcium concentrations. GST-SytIC2A showed similar results in this assay.

GST-DysfC2A binding to PIP and PIP2 was also calcium-dependent, since no binding to these lipids was observed when calcium was depleted (Figure 3.5). GST-DysfC2A binding to PIP and PIP2 was much weaker in comparison to PS binding: binding was observed at higher amounts of these lipids (1000 pmoles) and also required higher calcium concentrations (100μM). In comparison to GST-SytIC2A, GST-DysfC2A bound less strongly to similar amounts of PIP and PIP2 at the same calcium concentrations.

To assess the apparent calcium affinity of dysferlin's C2A domain to each of the phospholipids (PS, PIP and PIP2), calcium titration experiments were performed using liposome centrifugation assays. Dysferlin's C2A domain (GST-DysfC2A) and synaptotagmin I's C2A domain (GST-SytIC2A) were incubated with PS/PC or PIP/PC or PIP2/PC liposomes in the absence of calcium (1mM EGTA) or in the presence of increasing amounts of calcium (1μM, 10μM, 50μM, 0.1mM and 1mM).

GST-DysfC2A demonstrated calcium-dependent binding to all three lipids (Figure 3.6), in agreement with the protein lipid overlay assay results (compare with Figures 3.2, 3.4 and 3.5). GST-DysfC2A bound to PS and to PIP with

apparent calcium affinities of 10μM, whereas for GST-SytIC2A the apparent calcium affinities for PS and PIP were 10μM and 50μM, respectively (Figure 3.6). GST-DysfC2A bound to PIP2 with an apparent calcium affinity of 1μM, whereas GST-SytIC2A bound with an apparent calcium affinity between 10μM and 50μM.

3.5 Alpha-tubulin interacts with dysferlin's C2A and C2B domains in a calcium-independent manner

To identify novel protein binding partners for dysferlin, our lab had performed a liquid chromatography-mass spectrometry (LC-MS/MS) analysis of proteins that co-immunoprecipitated with dysferlin in skeletal muscle. Alpha-tubulin was identified and confirmed by co-immunoprecipitation and GST pulldown (unpublished data) as a novel dysferlin-interacting protein. Since C2 domains are known to be involved in protein-protein interactions, we sought to determine if the interaction between dysferlin and alpha-tubulin involved dysferlin's C2 domains.

GST pulldown assays were performed by incubating immobilized GST-C2 domain fusion proteins with C2C12 myoblast extracts. Western blotting with anti-alpha-tubulin antibodies revealed that only dysferlin's C2A and C2B domains interacted with alpha-tubulin (Figure 3.7A). The other dysferlin C2 domains (C2C to C2G), as well as GST alone, did not interact with alpha-tubulin.

To determine if there was any calcium-dependency to the alpha-tubulin interaction, GST pulldown assays were performed in the presence (1mM $CaCl_2$) or absence (1mM EGTA) of calcium (Figure 3.7B). The results revealed that DysfC2A and DysfC2B were the only dysferlin C2 domains to bind to alpha-tubulin. This interaction was not significantly altered by the addition or depletion of 1mM calcium, thus suggesting that the interaction is calcium-independent. The other GST-C2 domains did not demonstrate any binding to alpha-tubulin in either the presence or absence of 1mM calcium.

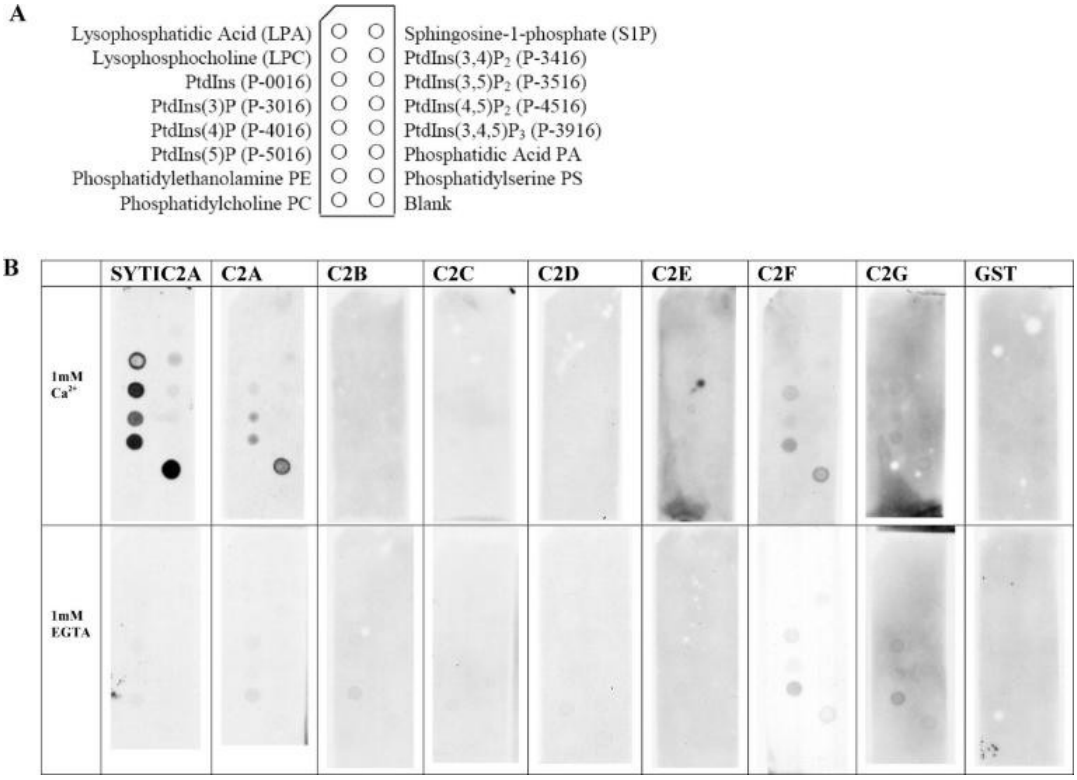
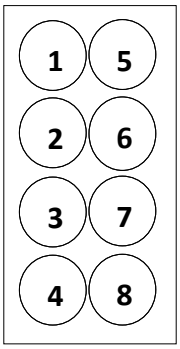


Figure 3.1: Screen of lipid binding specificities for dysferlin's C2 domains.

(A) The legend of phospholipids found on each lipid strip. (B) Dysferlin and synaptotagmin I GST-C2 domain fusion proteins were incubated with Echelon lipid strips in the presence of 1mM calcium (top) or 1mM EGTA (bottom). Binding was detected by Western blotting with anti-GST antibodies.

A



pmoles

1=31000	5= 1940
2=15500	6= 970
3= 7750	7= 310
4= 3860	8= 31

B

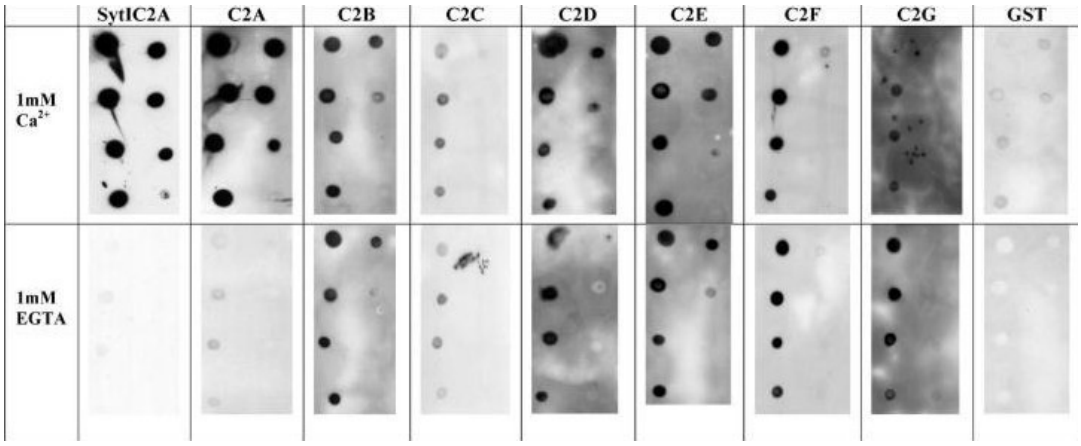


Figure 3.2: Calcium-dependent binding to phosphatidylserine (PS) by dysferlin C2 domains determined by protein-lipid overlay (PLO) assay

(A) The legend of the amount of PS spotted on each custom lipid strip. (B) Dysferlin and synaptotagmin I GST-C2 domain fusion proteins were incubated with the custom lipid blots in the presence of 1mM calcium (top) or 1mM EGTA (bottom). Binding was detected by Western blotting with anti-GST antibodies.

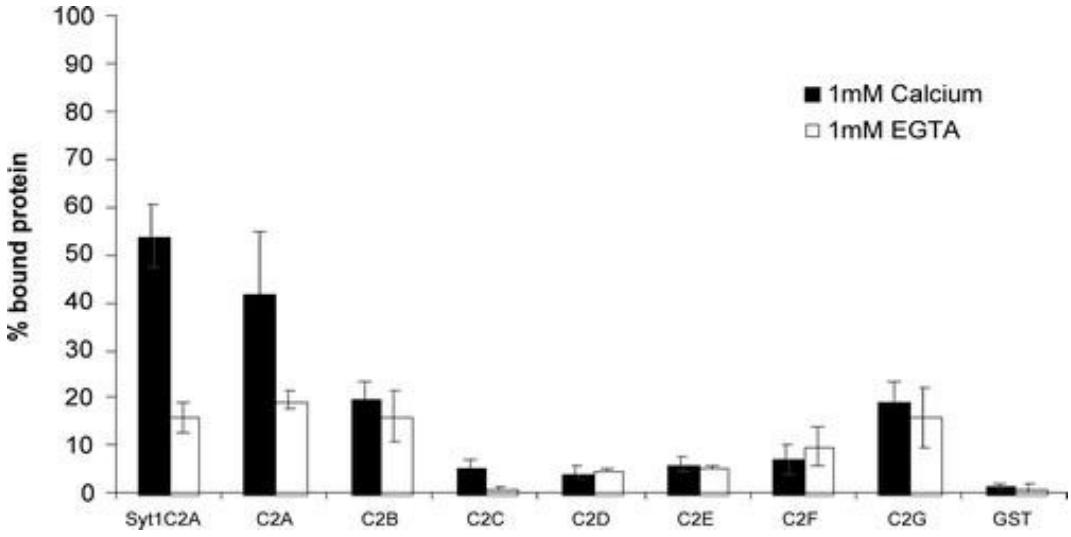
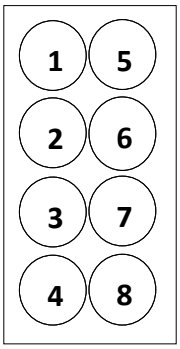


Figure 3.3: Calcium-dependent phosphatidylserine (PS) binding by dysferlin's C2 domains measured by liposome centrifugation assay.

GST-C2 domain fusion proteins were incubated with PS/PC liposomes in the presence of 1mM $CaCl_2$ or 1mM EGTA. The samples were ultracentrifuged and analyzed by SDS-PAGE and the percentage of lipid-bound protein was determined by densiometry (ImageJ). Mean values and error bars were determined from triplicate experiments.

A



	pmoles PIP	pmoles PIP2
1	10000	5000
2	5000	2500
3	2500	1250
4	1250	625
5	625	312
6	312	150
7	100	50
8	10	5

B

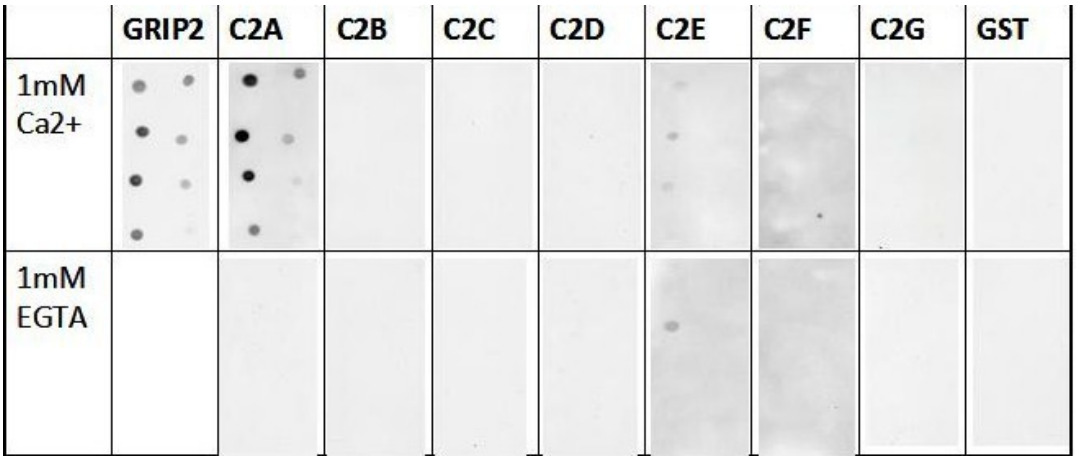


Figure 3.4: Calcium-dependent binding to PIP and PIP2 by dysferlin C2 domains determined by protein-lipid overlay (PLO) assay

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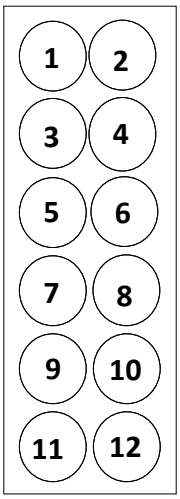
(A) The legend of the amount of PIP or PIP2 spotted on each nitrocellulose membrane. (B) Dysferlin GST-C2 domain fusion proteins and GRIP2 were incubated with PIP2-spotted lipid blots in the presence of 1mM calcium (top) or 1mM EGTA (bottom). Binding was detected by Western blotting with anti-GST antibodies. Results were the same for binding to PIP-spotted lipid strips.

GRIP2 = N-terminal GST-tagged recombinant PLCδ1 Pleckstrin homology (PH) domain (i.e. PIP2 binding domain from PLCδ1)

A



	pmoles	Lipid
1=	1000	PS
2=	500	PS
3=	250	PS
4=	125	PS
5=	1000	PIP
6=	500	PIP
7=	250	PIP
8=	125	PIP
9=	2000	PIP2
10=	500	PIP2
11=	250	PIP2
12=	125	PIP2

B

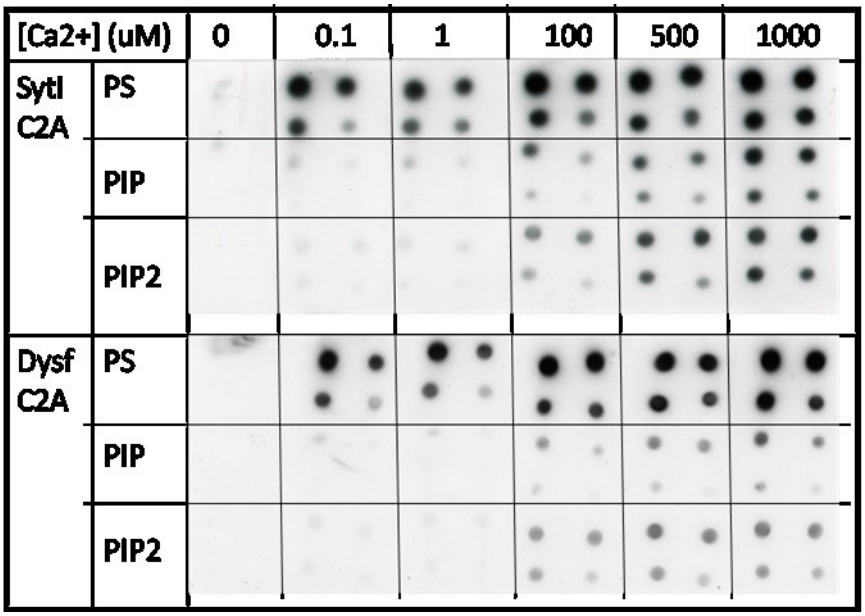


Figure 3.5: Calcium titration of phospholipid binding of dysferlin's C2A domain measured by protein-lipid overlay assay**.**

(A) Each custom lipid strip contained all three phospholipids (PS on the top, PIP in the middle and PIP2 on the bottom), spotted with increasing concentrations of each lipid (125, 250, 500, 1000 or 2000pmol). (B) Dysferlin and synaptotagmin I GST-C2A fusion proteins were incubated with these custom lipid strips in the presence of increasing calcium concentrations. Binding was detected by Western blotting with anti-GST antibodies.

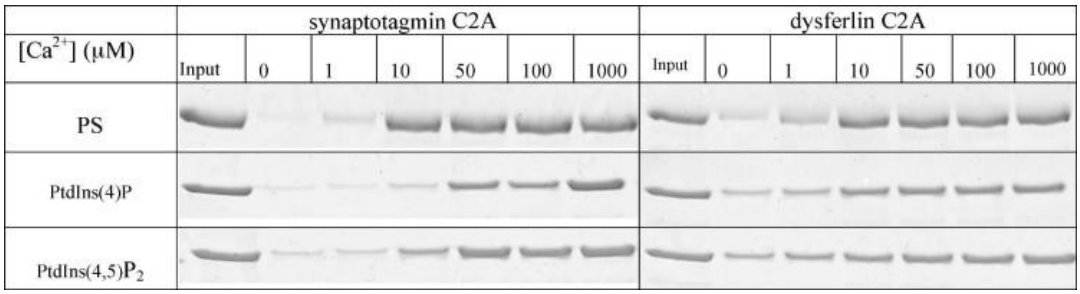


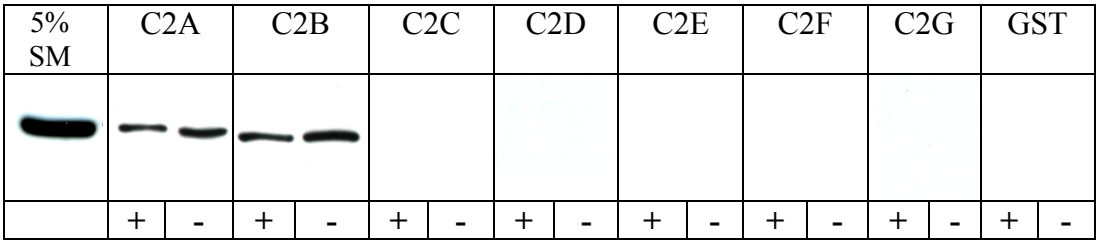
Figure 3.6: Calcium titration of phospholipid binding of dysferlin's C2A domain measured by liposome centrifugation assay.

Dysferlin and synaptotagmin I GST-C2A domain fusion proteins were incubated with liposomes (PS/PC or PIP/PC or PIP2/PC) in the presence of increasing calcium concentrations. The samples were ultracentrifuged, analyzed by SDS-PAGE and ImageJ densiometry.

A

IB: alpha-tubulin

B



IB: alpha-tubulin

Figure 3.7: Alpha-tubulin interacts with dysferlin's C2A and C2B domains in a calcium-independent manner

(A) Immobilized dysferlin GST-C2 domain fusion proteins were incubated with C2C12 myoblast lysates. Bound proteins were separated by SDS-PAGE and immunoblotted with anti-alpha-tubulin antibodies.

(B) Immobilized dysferlin GST-C2 domain fusion proteins were incubated with C2C12 myoblast lysate in the presence of 1mM CaCl2 (+) or 1mM EGTA (-). Bound proteins were separated by SDS-PAGE and immunoblotted with anti-alpha-tubulin antibodies.

IB: immunoblot.

4 Discussion

Dysferlin is a multi-C2 domain protein implicated in skeletal muscle membrane repair but its mechanism is poorly understood. Further characterization of dysferlin's multiple domains is requisite. This study sought to characterize the binding specificities of dysferlin's seven C2 domains to three phospholipids that compose mammalian lipid membranes, as well as to alpha-tubulin, a protein that was identified by liquid chromatography-mass spectrometry as a novel dysferlin interacting protein.

4.1 Characterization of novel phospholipid binding interactions with dysferlin's C2 domains: possible implications in membrane repair and fusion

Lipid-protein overlay assays and liposome centrifugation assays revealed that dysferlin's C2A domain bound to phosphatidylserine (PS), phosphatidylinositol-4-phosphate (PIP) and phosphatidylinositol 4,5-bisphosphate (PIP2) in a calcium-dependent manner. Dysferlin's other C2 domains each bound PS in a much weaker and calcium-independent manner, but did not bind PIP or PIP2 in either the presence or absence of calcium.

4.1.1 C2 topology and calcium dependency: our predictions and observations

From their predicted C2 domain topology and number of conserved calcium-coordinating acidic residues, we hypothesized that dysferlin's C2A, C2C, C2D, C2F and C2G domains would demonstrate calcium-dependent lipid binding properties as they contained at least four of the five conserved residues necessary to coordinate multiple calcium ions. We also hypothesized that dysferlin's C2B and C2E domains would demonstrate calcium-independent lipid binding as they contained two or three of the conserved acidic residues and thus were not

expected to coordinate multiple calcium ions. In this study, we showed that C2A, C2B and C2E did meet our expectations as they bound PS in a calcium-dependent (C2A) or independent manner (C2B and C2E). However, the other C2 domains bound PS in a calcium-independent manner, suggesting that sequence analysis alone cannot reliably predict calcium dependency of C2 domains.

There exist many examples of an indirect relationship between number of calcium-coordinating acidic residues and calcium dependency. Even though the C2B domain of all three isoforms of Doc2 (α, β and γ) contains all five conserved acidic residues, it demonstrated calcium-independent phospholipid binding activity (Fukuda & Mikoshiba 2000; Kojima et al 1996; Orita et al 1995). Likewise, the C2A domain of synaptotagmin-like protein 3 (Slp3) contains only one conserved acidic residue and would not be expected to coordinate calcium ions, and yet it shows calcium-dependent lipid binding properties (Fukuda & Mikoshiba 2000). Slp3 achieves this by using a polybasic sequence in the β4 strand in addition to its single conserved acidic residue to achieve calcium-dependent liposome binding activity (Fukuda & Mikoshiba 2000).

Moreover, not all C2 domains are reported to bind phospholipids or calcium. The C2A and C2B domains of rat synaptotagmin IV contain the necessary acidic residues for calcium-dependent lipid binding, however both domains fail to bind calcium ions or phospholipids. Analysis of the crystal structure of rat synaptotagmin's C2B domain revealed that altered β-strand orientation prevents the formation of full calcium-coordinating sites (Dai et al 2004). Nevertheless, this mutation was not observed in fly synaptotagmin IV, and this orthologue's C2 domains exhibited calcium-dependent phospholipid binding (Dai et al 2004). Evolutionally conserved Multiple C2 domain proteins with two transmembrane regions (MCTPs) also contain all necessary residues for calcium-dependent phospholipid binding activity. Although they are able to bind calcium ions, MCTPs do not bind phospholipids in either the presence or absence of calcium (Shin et al 2002). Double C2 domain-γ (Doc2γ) and Tandem C2 domain in

Nucleus (Tac2-N) proteins lack most or all, respectively, of the conserved acidic residues and are unable to bind calcium ions (Fukuda & Mikoshiba 2000; 2001). Instead, their C2A and C2B domains, respectively, contain a polybasic sequence that functions as a nuclear-localization sequence (Fukuda & Mikoshiba 2001; Fukuda et al 2001). Future calcium-binding studies, deletion and mutation assays with dysferlin's C2 domains can assess whether its domains too can bind calcium without binding phospholipids, and if they may have functions other than membrane binding.

It must be noted that some of the GST-C2 domain fusion proteins may not have been folded properly, thus explaining the lack of binding to PIP and PIP2. However, we showed that dysferlin's seven GST-C2 domains bound PS in the protein-lipid overlay assays, suggesting that the fusion proteins were functional and hence properly folded. A circular dichroism (CD) spectrum analysis could be used to determine the protein's secondary structures, and reveal whether the protein maintains in its native state. However, we were unable to purify the protein sufficiently for this technique.

It is also possible that, individually, dysferlin's C2B to C2G domains are inefficient phospholipid binding modules and that they require additional interactions or modifications in order to achieve phospholipid binding. In Aplysia neurons, the calcium-independent novel protein kinase C Apl II (nPKC Apl II) requires autophosphorylation of serine 36 in the first loop of the C2 domain to regulate its membrane binding (Pepio & Sossin 2001). In another study, synaptotagmin I's phospholipid binding affinity was shown to depend on the presence of adjacent C2 domains: in proximity to the activated C2A domain of synaptotagmin, the C2B domain binds to phosphatidylserine-containing membranes with higher affinity (Bai et al 2002; Chapman 2002).

4.1.2 Implications of phospholipid binding specificities in vesicle aggregation during membrane repair

The significance of these novel phospholipid interactions with dysferlin's C2 domains is not fully understood at this time, but they may be important for dysferlin's role in skeletal muscle membrane repair. When the plasma membrane of muscle cells is damaged, dysferlin mediates the fusion of endomembranes to the plasma membrane thus forming a membrane patch across the disruption site. The results of our study suggest that in the event of muscle membrane injury, the influx of extracellular calcium would activate the C2A domain of sarcolemmal dysferlin, thus allowing it to interact with PS and PIP2 found in the plasma membrane. It could also bind to PS and PIP found on intracellular vesicles, such as secretory vesicles. These interactions could serve to bring the membranes of the vesicles and plasma membrane into close enough proximity to overcome the energy barrier so that dysferlin could mediate their fusion. To test this hypothesis, membrane repair assays could be performed using a recombinant dysferlin protein lacking its C2A domain. One would expect that such a mutant dysferlin protein would demonstrate an inability or impairment in membrane repair following injury.

Annexin A1 has also been reported to exhibit calcium-dependent phospholipid binding properties during plasma membrane repair (McNeil et al 2006). It is possible that dysferlin's phospholipid binding activities could synergize or work cooperatively with annexin's to mediate intervesicular binding and/or fusion events.

4.1.3 Implications of phospholipid binding specificities in membrane fusion during membrane repair

The ferlin family of proteins is integrally involved in membrane fusion events: Fer-1 mediates membranous organelle fusion in worm spermatocytes, otoferlin

mediates synaptic exocytosis in hair cells; myoferlin mediates myoblast fusion in maturing muscle cells; and dysferlin mediates plasma membrane repair. But the mechanisms of ferlin's membrane fusion functions have not been elucidated. Many C2 domain-containing proteins have been proposed to induce membrane curvature by causing shallow wedge-like insertions into the lipid membrane (Martens & McMahon 2008). Synaptotagmin I has been shown to bind calcium with the first and third loops of its C2 domains, and that the tips of these loops physically penetrate into the lipid bilayer of the synaptic terminal (Chapman 2002; Martens et al 2007). This penetration causes buckling of the lipid bilayer's leaflet, decreasing the energy threshold for membrane fusion (Martens et al 2007). It has also been demonstrated that PIP2 clusters serve to steer membrane penetration of synaptotagmin's C2 domains (Bai et al 2004). We demonstrated that dysferlin's C2A domain binds to PIP2 in a calcium-dependent manner. It is possible that similar plasmalemmal PIP2 clusters could steer dysferlin's C2A domain to bind to—or even penetrate—the lipid membrane in an analogous fashion.

Many membrane fusion events are critically dependent on SNARE proteins (such as syntaxin, SNAP-25 and synaptobrevin) and fusion proteins (notably C2 domain-containing proteins) for overcoming the energy barrier and initiating fast membrane fusion (Lam et al 2008; Martens & McMahon 2008; Pobbati et al 2006). In vertebrate mechanosensory hair cells, otoferlin was discovered to function as the membrane fusion protein and calcium sensor during neurotransmitter release at auditory synapses, after researchers failed to detect synaptotagmin I and II (Safieddine & Wenthold 1999). Like synaptotagmin, otoferlin binds to syntaxin 1A and SNAP-25 (Roux et al 2006) through its C2F domain (Ramakrishnan et al 2009) in a calcium-dependent fashion. Otoferlin's C2D domain also binds syntaxin 1A calcium-dependently, but with much weaker affinity than the C2F domain (Ramakrishnan et al 2009). Otoferlin's C2D domain was shown to be a calcium-binding domain (Roux et al 2006) and was further

shown to bind specifically to a voltage-gated calcium channel ($Ca_v1.3$) in a calcium-dependent manner (Ramakrishnan et al 2009).

Whereas otoferlin and synaptotagmin utilize the SNARE complex to mediate the extremely fast membrane fusion events required for synaptic neurotransmitter release, SNARES have not been shown to be involved in plasma membrane repair (Martens & McMahon 2008) and dysferlin has not yet been reported to interact with SNAREs. Of course, further studies would be needed to confirm that dysferlin does not interact with SNAREs, keeping in mind that the SNARE isoforms in skeletal muscle differ from those in synapses. It is unknown how dysferlin regulates the membrane fusion events observed in skeletal muscle. One possibility is that the dysferlin interactome could form its own tightly-associated complex that would provide the energy needed for membrane fusion. For instance, syntaxin-phospholipid interactions were shown to regulate SNARE-mediated membrane fusion (Lam et al 2008). Notably, syntaxin 1A partially colocalized with PIP2 clusters in the plasma membrane in order to mediate vesicle fusion (Aoyagi et al 2005; Lam et al 2008). These protein-PIP2 interactions are essential for the priming and fusion of vesicles (Bai et al 2004). We showed here that dysferlin's C2A domain binds multiple phospholipids, including PIP2, in a calcium-dependent manner. Further studies could assess the importance of PIP2 clusters for skeletal membrane repair.

Another possibility is based on the observation that dysferlin contains seven C2 domains, compared to otoferlin's six or synaptotagmin I's two. These additional domains could cooperatively potentiate dysferlin's phospholipid or protein binding affinities. We showed in our study that all of dysferlin's C2 domains are able to bind to PS, although dysferlin's C2A domain showed much stronger binding compared to that of the other domains. It is possible that activation of the C2A domain could modify the phospholipid binding affinity of the other C2 domains such that the overall phospholipid binding affinity of the dysferlin protein is increased. Such a situation was suggested for otoferlin's C2F domain.

Optimal binding between otoferlin's C2F domain and a SNARE protein, syntaxin 1A, was observed at 61μM of calcium and binding affinity decreased at higher calcium concentrations (Ramakrishnan et al 2009), whereas a construct containing a complete stretch of otoferlin's C2D-E-F domains demonstrated steadily increasing binding to syntaxin A1 for rising calcium concentrations of up to 1mM (Roux et al 2006), conceivably because both C2D and C2F bind synataxin 1A but with different affinities. This suggests that cooperativity between C2 domains could alter calcium sensitivity and protein binding. To investigate the possibility of cooperativity between dysferlin's C2 domains, we can compare the phospholipid binding affinities and kinetics of single-C2 domain and multiple C2 domains. Although we produced a GST-fusion protein construct containing the first three C2 domains of dysferlin (C2A-B-C), the construct was poorly soluble and exhibited a high degree of degradation.

Finally, it must be noted that dysferlin differs from otoferlin by the presence of an additional domain: the DysF domain. The DysF domain in dysferlin exists as a unique internal duplication, such that the inner DysF domain is flanked by the N-terminus and C-terminus of the outer DysF domain (Patel et al 2008). The solution structure of the inner DysF domain for myoferlin has been determined, and is speculated to be important for the protein's structural stability; an incorrectly folded DysF domain could lead to degradation of the entire protein (Patel et al 2008). It is also speculated that this domain may be involved in protein binding interactions, for instance with caveolin-3 through the tryptophan-rich binding motif [109]. Other protein interactions, and their significance in membrane fusion and repair, are still to be resolved.

4.2 Potential endomembrane sources and dysferlin's intracellular storage site

Immunostaining for dysferlin showed that dysferlin is found at the sarcolemma, in the t-tubule system and intracellularly in vesicles. However, the identity of these

dysferlin-containing vesicles is still unknown. Additionally, the identity of the endomembranes used for muscle membrane repair is still unknown, although various suggestions have been made, including lysosomes and enlargeosomes.

Our results demonstrated that dysferlin's C2A domain bound to PIP-containing liposomes. This suggests that dysferlin could be localized to the membrane of secretory vesicles, which contain PIP in their lipid membranes. This idea would fit in with the membrane repair process, since dysferlin found on secretory vesicles located close to a disruption site would get activated (through its C2A domain) by the influx of calcium, and would be recruited to the disruption site where dysferlin could interact with PIP2 in the plasma membrane. There would also be intervesicular interactions between dysferlin, PS and PIP on the secretory vesicles, serving to bring the vesicles into close proximity so that they could be fused together.

Since PIP is found predominantly on secretory vesicles and in the trans-Golgi network, it suggests that a source of dysferlin-containing endomembranes could be secretory vesicles. Other studies have also suggested Golgi-derived vesicles to be possible sources of endomembranes utilized in membrane repair. Plasma membrane wounding experiments in fibroblasts demonstrated that the calcium influx from the first wound activated protein kinase C, which triggered vesicle formation from the Golgi complex, and that these Golgi-derived vesicles were used to seal a second plasma membrane wound (Togo et al 1999). Examination of electron micrographs of dysferlinopathic patient muscle biopsies revealed subsarcolemmal aggregations of small vesicles, which were proposed but not demonstrated to be probably derived from the Golgi, since the Golgi had empty, swollen cisternae (Cenacchi et al 2005). To determine the involvement of PIP-harbouring vesicles in membrane repair, we could perform membrane repair assays using brefeldin A, which interferes with retrograde transport from the Golgi to the endoplasmic reticulum, or phenylarsine oxide (PAO), which inhibits

phosphorylation by phosphoinositol 4-kinase (PI4K) and prevents vesicle transport from the Golgi to the plasma membrane.

Our phospholipid screening experiments suggested that dysferlin's C2A domain binds to phosphatidylinositiol-3-phosphate (PtdIns3P), which is a lipid found predominantly on endocytic membranes. Although we did not characterize this phospholipid interaction in this study, it is possible that cytoplasmic dysferlin could be localized to endosomes or that endosomes could serve as a source of endomembranes during patch membrane repair. Annexin A2, a documented dysferlin binding partner, has been reported to bind to recycling endosomes and is involved in endosomal transport (Hayes et al 2004; Morel & Gruenberg 2009). Membrane repair assays performed on fibroblasts have shown that an endocytic compartment was used as a source of endomembranes to reseal membrane disruptions (Togo et al 1999).

It is quite possible that multiple sources of endomembranes are used for membrane repair. In sea urchin eggs, two separately recruited pools of vesicles were required for membrane repair, and treatment with stachyose reversibly inhibited membrane resealing, suggesting a role for cortical granules in the membrane repair process in addition to yolk granules (Bi et al 1995; Bi et al 1997). It has also been shown in fibroblasts that calcium-dependent membrane repair of successive wounds to the same injury site involved endomembranes from different sources: brefeldin A, a fungal metabolite that inhibits vesicle formation at the Golgi apparatus, inhibited resealing of the second injury but not the first (Togo et al 1999). This suggested that the source of endomembranes used to repair the second same-site injury was Golgi-derived, whereas the first source was not. Although it has not been confirmed that two membrane sources are also used in membrane repair of muscle fibers—much less that one source is Golgi-derived—our results demonstrating an interaction between dysferlin and PIP (which is found predominantly at the trans-Golgi network and on secretory vesicles) suggest that this possibility may merit further investigation. Considering

that skeletal muscle injury is an extremely common event, and that membrane repair is a recurrent process that most likely demands large resources of endomembranes, the involvement of multiple sources of endomembranes is quite plausible.

Whatever the identity of these endomembranes, it is highly probable that the primary source should be dysferlin-containing vesicles, since dysferlin is such an integral part of the membrane repair process. It is logical to store dysferlin subsarcolemmally in vesicles since cytoplasmic localization of dysferlin would maximize its availability, which is an ideal situation for a prompt repair response.

4.3 Characterization of dysferlin's C2 domain binding interactions with alpha-tubulin: possible implications in dysferlin trafficking and storage

LC-MS/MS analysis of dysferlin interacting proteins identified alpha-tubulin as a potential binding partner for dysferlin. Furthermore, our GST pulldown assays showed that alpha-tubulin interacts with dysferlin's C2A and C2B domains in a calcium-independent manner.

Microtubules, the polymerized form of tubulin, are essential for maintenance of cell morphology, cell polarization, cellular motility and intracellular protein trafficking. The trafficking pathways of dysferlin are under-characterized. Caveolin-3 has been implicated in proper trafficking of dysferlin from the Golgi to the plasma membrane, since dysferlin is mislocalized to the Golgi in muscle biopsies from patients with mutated caveolin-3 (Hernandez-Deviez et al 2006). Caveolin-3 was also shown to retain a plasmalemmal pool of dysferlin by inhibiting its endocytosis through a clathrin-independent pathway (Hernandez-Deviez et al 2008). It is highly probable that dysferlin trafficking would be mediated by microtubule-associated motor proteins, although further studies

would be needed to ascertain the role of dysferlin's interaction with microtubules in this process.

The significance of the interaction between C2 domains and tubulin, specifically, is unknown at this time. Several C2 domain-harbouring proteins have been reported to interact with tubulin and microtubules. In the parasite *Trypanosoma brucei*, the C2 domain-harbouring WCB (whole cell body) protein is essential for cell morphology as it links the microtubule corset to the plasma membrane (Baines & Gull 2008). In plant cells, phospholipase D was also shown to link microtubules to the plasma membrane, and that activation of this enzyme severs this link and triggers microtubule reorganization (Dhonukshe et al 2003; Gardiner et al 2001). Notably, synaptotagmins I and IX in mammalian cells have also been shown to interact with tubulin and microtubules. In rat basophil leukemia (RBL) cells, synaptotagmin IX was shown to co-immunoprecipitate with β-tubulin in a calcium-independent manner (Haberman et al 2003). Each of its C2 domains was able to bind α-tubulin and β-tubulin and although calcium was not required, its presence did increase the binding of α-tubulin or β-tubulin to the C2A domain (Haberman et al 2003). The authors proposed that this interaction could serve to link microtubules and endosomal trafficking from the endosomal recycling compartment to the plasma membrane (Haberman et al 2003). On the other hand, the neuronal calcium-sensing synaptotagmin I was shown to interact directly with the C-terminal region of β-tubulin through the YVK motif found in its two C2 domains (Honda et al 2002), and promoted tubulin polymerization in a calcium-dependent manner. The authors proposed that this latter interaction could permit transient attachment of synaptic vesicles to microtubules in the presence of high calcium concentrations, such as those experienced near the presynaptic membrane during synaptic neurotransmission (Honda et al 2002). The observation that two synaptotagmin paralogues exhibit opposing calcium dependency properties to the same protein (tubulin) suggests that calcium dependency of one protein to another is cell-specific and even function-specific. Therefore, even though dysferlin shares much structural and functional similarities with synaptotagmin I, it is not a

given that dysferlin should demonstrate calcium-dependent tubulin binding, since dysferlin and synaptotagmin I are located in different cell types (muscle versus neuron) and have different functions (membrane repair versus neurotransmission).

It is possible that during membrane injury, dysferlin could mediate transient attachment of vesicles (such as endomembranes destined for patch membrane fusion) to microtubules near the edges of the membrane disruption, in an analogous manner to synaptotagmin I. Conversely, the interaction between microtubules and dysferlin could be important in post-injury events. It has been shown that following membrane disruption in PtK2 cells, there is microtubule rearrangement and lipid trafficking towards the site of injury (Togo 2006). It is possible that dysferlin-harbouring vesicles could be stored subsarcolemmally on microtubules, maintaining their localization by the interaction between the C2 domains and tubulin. Further studies would be necessary to test this hypothesis.

Although we demonstrated that dysferlin's C2A and C2B domains bound to alpha-tubulin, the specificity and affinity of this binding interaction needs to be ascertained, since tubulin contains many acidic residues that can bind non-specifically to negatively-charged regions of the dysferlin protein. This issue could be addressed by using surface plasmon resonance, whereby one can determine the kinetic parameters of the interactions in addition to the association/dissociation affinity constants.

4.4 Therapeutic alternatives for dysferlinopathies

Finding treatments or even a cure for dysferlinopathic patients is an ongoing mission. Ideally, one would strive to use gene replacement therapy to restore the mutated protein in diseased muscles; for instance, by using viral vectors containing the gene of interest to infect diseased muscle cells. This technique is being evaluated in a number of muscular dystrophy animal models, and is in

clinical trials for treatment of Duchenne muscular dystrophy using adeno-associated viral vectors (AAV) harbouring truncated dystrophin (Tang et al 2010).

However, the size of the dysferlin gene surpasses the insert capacity of the AAV vector. By characterizing dysferlin's individual C2 domains, we hope to determine which C2 domains may be redundant, in order to ascertain the minimum domain requirement for a functional truncated dysferlin protein. Hence, we aspire to design a 'mini-dysferlin' gene that will fit into the AAV vector.

Alternative therapies for dysferlinopathies being developed include strengthening the membrane of muscle cells against damage by using Poloxamer 188. Poloxamer 188 is a membrane sealant that can promote the repair of damaged skeletal muscle membrane, as it has been shown to have a protective effect on damaged cardiac muscle (Egan et al 2009; Townsend et al 2010). Another avenue of investigation is mutation targeted therapy using exon skipping oligonucleotides to circumvent mutations in the dysferlin gene. If a known pathogenic mutation is located within a "skippable" exon of the dysferlin gene (i.e. the peptide region that the exon encodes is not essential for the protein activity; such that even if the exon is deleted, the gene retains an open reading frame that will produce a functional protein), an antisense oligonucleotide can be used that hides that exon from being recognized by the splicing protein machinery. This causes the splicing machinery to skip over the exon, thus producing a functional (or partially functional) truncated protein (Wein et al 2010).

5 Conclusion

In conclusion, this study identified and characterized novel binding interactions between dysferlin's C2 domains and three mammalian phospholipids (PS, PIP and PIP2), as well as with alpha-tubulin. The novel calcium-dependent phospholipid binding properties of dysferlin's C2A domain revealed by this study could play

important roles in membrane repair of skeletal muscles. Future studies will determine if these interactions are involved in dysferlin-mediated membrane aggregation or membrane fusion, the mechanisms of which are poorly characterized. The results of the phospholipid screening assay present interesting avenues of investigation into the identity of dysferlin's intracellular storage compartment and the source of endomembranes used during the membrane repair process, both of which remain under debate. The discovery of dysferlin's interaction with alpha-tubulin sets the foundation for studies into dysferlin trafficking pathways, which are poorly-defined. By improving our understanding of dysferlin's multiple interactions we gain further insight into the mechanisms governing dysferlin's role in membrane repair of skeletal muscle. Furthermore, by understanding dysferlin's normal execution, we can understand how mutations in dysferlin could lead to pathology, thus leading to the development of therapeutic alternatives.

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