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The C2 Domains of Otoferlin, Dysferlin, and Myoferlin Alter the Packing of Lipid Bilayers

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Abstract

Ferlins are large multi-C2 domain membrane proteins involved in membrane fusion and fission events. In this study we investigate the effects binding of the C2 domains of otoferlin, dysferlin and myoferlin have upon the structure of lipid bilayers. Fluorescence measurements indicate that multi-C2 domain constructs of myoferlin, dysferlin and otoferlin change the lipid packing of both small unilamellar vesicles and giant plasma membrane vesicles. The activities of these proteins were enhanced in the presence of calcium, and required negatively charged lipids like phosphatidylserine or phosphatidylglycerol for activity. Experiments on individual domains uncovered functional differences between the C2A domain of otoferlin as compared to dysferlin and myoferlin, and truncation studies suggest that the effects of each subsequent C2 domain on lipid ordering appear additive. Finally, we demonstrate that the activities of these proteins on membranes are insensitive to high salt concentrations, suggesting a non-electrostatic component to the interaction between ferlin C2 domains and lipid bilayers. Together, the data indicate that dysferlin, otoferlin, and myoferlin do not merely passively adsorb to membranes, but actively sculpt lipid bilayers, which would result in highly curved or distorted membrane regions that could facilitate membrane fusion, fission, or recruitment of other membrane trafficking proteins.

Keywords

otoferlin; dysferlin; myoferlin; laurdan; C2 domain

Introduction

Ferlins are a family of eukaryotic membrane proteins that mediate membrane trafficking events[1]. The first ferlin gene characterized was expressed in C. elegans, and appears to mediate membrane fusion between intracellular organelles and the plasma membrane of sperm cells[2]. Similarly, infertility is also associated with defects in the Drosophila ferlin known as misfire[3]. In humans, there are six ferlin genes denoted dysferlin, otoferlin, myoferlin, Fer1L-4, Fer1L-5, and Fer1L-6. While Fer1L-4 and Fer1L-6 remain uncharacterized, the other ferlins have been linked to exocytotic and endocytotic events at the plasma membrane, and several have garnered interest due to their association with human diseases[1]. For example, mutations in otoferlin disrupt neurotransmitter release from cochlear and vestibular hair cells, resulting in profound deafness, while mutations in dysferlin hamper vesicle fusion and the ability of muscle to reseal sarcolemma lesions, resulting in limb-girdle muscular dystrophy and Miyoshi myopathy[4–8]. Studies on myoferlin

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have established a role for the protein in cancer cell invasiveness and muscle development[9,10].

All members of the mammalian ferlin family share a similar structure, consisting of 5–7 C2 domains and a single pass transmembrane region (Fig. 1). Proteins with more than 2 tandem C2 domains are rare, and it is currently unclear as to whether these domains are redundant or synergistic in their behavior. A common feature of the C2 domain is the ability to bind lipids, which is often calcium dependent[11]. In response to elevated calcium concentrations, these domains target a protein to a particular membrane compartment based upon preference for an organelle specific lipid headgroup[12]. After binding, some C2 domains actively cluster lipids or bend the membrane, actively perturbing membrane structure so as to help facilitate cellular processes[13,14]. While studies on the C2 domains of otoferlin, myoferlin, and dysferlin have established that they harbor membrane binding activity, no study has addressed whether these proteins can actively alter lipid membrane structure after binding[15–17].

In this study, we employ fluorescence techniques to monitor interactions between recombinant ferlin proteins and vesicles to address the question as to whether ferlin C2 domains affect membrane structure. Using the polarity-sensitive fluorophore laurdan, we find that the cytosolic region of otoferlin alters the lipid packing within vesicles in a calcium enhanced manner, and that this activity is harbored within multiple domains at both the N- and C-terminus of the protein[18–19]. We also demonstrate that this activity is conserved in myoferlin and dysferlin, and is dependent on the presence of negatively charged lipids like phosphatidylserine or phosphatidylglcerol. Upon examination of individual domains, we find that C2B and C2C of all three proteins behave similarly, but that the C2A of otoferlin differs from the equivalent domains in dysferlin and myoferlin. Studies on truncated constructs suggest that the domains have an additive effect on lipid membranes, with shorter constructs lacking the potency of larger multi-domain fragments. Finally, measurements conducted at different ionic strengths suggest hydrophobic interactions as a major component of the observed activities of the ferlin C2 domains on membranes.

Experimental Procedures

Materials

Lipids were obtained from Avanti polar Lipids (Alabaster, AL). Affinity media Ni2+-Sepharose high performance beads and amylose resin were obtained from GE-Amersham Biosciences (Pittsburgh, PA). Common reagents and primers were purchased from Sigma (St. Louis, MO).

Protein expression and purification

All primers were purchased from Sigma Aldrich. All constructs were verified by sequencing by the Center for Genome Research and Biocomputing core facility at Oregon State University. pcDNA4/TO/mGFP-dysferlin-myc-his (gift from K. Bushby Newcastle, U.K) pcDNA 3.1 myoferlin-HA (gift from W. Sessa Yale School of Medicine), and pcDNA3.1 otoferlin plasmid (gift from C. Petit, Institut Pasteur et Université Pierre et Marie Curie, France) were used as templates for amplification of Homo sapiens dysferlin (GenBank: AF075575.1), Homo sapiens myoferlin (AF075575.1), and Mus Musculus otoferlin (GenBank: AY586513.1), respectively. Forward and reverse primers were designed to amplify the coding sequences with 5′ and 3′ sites for LIC insertion into SspI/T4-treated pMCS69. Primers for the C2 domains of otoferlin, dysferlin and myoferlin were based upon the predicted domain boundaries reported previously[20]. For multi-domain constructs, the primers were designed for the following amino acids; dysferlin-C2ABC 1-574aa otoferlin-

C2ABC 1-553aa, otoferlin-C2DEF 960-1885aa, otoferlin C2ABCDEF 1-1885aa, myoferlin C2ABC 1-530aa. Dysferlin C2A D16A was constructed using Stratagene Quickchange site-directed mutagenesis kit with pMCSG9/6His-MBP-dysferlin C2A plasmid template. Resulting constructs were expressed as a fusion with 6his-MBP (maltose binding protein). Multi-domain constructs were transformed into Rosetta BL2, while single C2 domain constructs we transformed into BL21. Expressed 6his- tagged constructs were affinity-purified using nickel IMAC resin followed by buffer exchange into 20mM Tris-HCl, pH 7.5, 100 mM NaCl. The otoferlin construct consisting of all six C2 domains required an additional affinity purification step using amylose resin after nickel IMAC purification. A TEV protease site allowed for cleavage of the MBP purification tag when desired, however studies on the protein with and without the MBP tag indicated that the MBP did not affect the results. We note that a shorter dysferlin C2ABC construct with amino acids 1 – 528 resulted in degradation products during purification, necessitating the longer 1 – 574 construct.

Small unilamellar vesicle preparation

Vesicles were prepared as described previously[13,15]. Briefly, chloroform dissolved lipids were mixed in the desired ratio and dried under vacuum until the solvent was removed. The dried lipids were then rehydrated in buffer to a concentration of 1mM and extruded using a membrane with a 50 nm cutoff. Extruder, syringes, and membranes were purchased from Avanti Polar Lipids.

Giant plasma membrane vesicle preparation

Vesicles were prepared as described previously using HEK293 cells[21]. Briefly, HEK293 cells cultured in DMEM media were washed with PBS and subsequently incubated with 2 mM DTT and 25 mM formaldehyde and small (micromolar) amounts of laurdan at 37 °C for 60 minutes. The resulting vesicles were imaged under bright field using a epi-fluorescent microscope to verify the formation of vesicles.

Protein fluorescence measurements

Steady state fluorescence measurements were conducted using a QM-40 with Glan Thompson polarizers (Photon Technology International, Birmingham, NJ) at 37 °C. All fluorescence measurements were conducted in samples containing 20mM Tris-HCl or HEPES buffer, pH 7.5, with 150 mM NaCl. Excitation at 350 nm was used for laurdan, and the generalized polarization value was calculated using the following equation

$$\mathrm{GP}=(\mathrm{I430}-\mathrm{I480})/(\mathrm{I430}+\mathrm{I480})$$

where I430 and I480 are the emission intensities at 430 and 480 nm. Anisotropy measurements were calculated using the following equation

$$\mathrm{r}=(\mathrm{IVV}-\mathrm{IVH})/(\mathrm{IVV}+2\mathrm{IVH})$$

where IVV and IVH correspond to the parallel and perpendicular emission intensities. Reported values represent the mean ± S.D. for three or more samples. Each sample was measured multiple times to ensure that the system was not changing over time.

Results

Otoferlin alters the lipid order and fluidity of small unilamellar vesicles

Otoferlin is directly involved in exocytosis and neurotransmitter release through an as-yet unclear set of mechanisms[5,6,15,22,23]. Synaptotagmin, a C2 domain protein also involved in neurotransmitter release actively manipulates membrane structure to achieve exocytosis[13]. It is possible that otoferlin operates through similar mechanisms. However, while co-floatation assays have demonstrated the membrane binding ability of otoferlin, no study has tested whether binding of otoferlin has a downstream effect on membrane structure[6,15]. To determine if membrane bound ferlin proteins change the structure of lipid bilayers, small unilamellar vesicles (SUV) composed of 25% 1-palmitoyl-2-oleoyl-sn-glycero-3-phospho-L-serine (POPS), 74% 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine (POPC), and ~ 1% laurdan were mixed with recombinant otoferlin constructs and monitored using a steady state fluorometer. Laurdan is a small, solvatochromic fluorescent membrane probe with an emission maxima that is sensitive to the ordering of lipids in bilayers[18,19,] (Fig. 2a). When inserted into membranes, the laurdan fluorescent moiety is located at the level of the phospholipid glycerol backbone, and the emission spectrum is highly sensitive to hydration of this region of the bilayer. When immersed in a highly ordered lipid bilayer, the laurdan emission spectra possesses a maxima at 430 nm, while in more disordered (greater degree of hydration) bilayers the maxima occurs at longer wavelengths, typically in the range of 480 nm. The normalized intensity ratio at these two wavelengths is often referred to as the General Polarization (GP) value, and ranges from −1 to + 1, representing the most disordered and most ordered states respectively. GP values are not biased by phospholipid headgroup charge, nor are they affected by protein penetration into lipid bilayers[18,19,24]. Further, laurdan does not associate preferentially with any specific lipids[18,19]. These characteristics make it an ideal probe of the global properties of lipid bilayer organization. Unlike electron microscopy imaging of vesicles, which can suffer from artifacts due to dehydration and heavy metal staining, measurements using laurdan can be performed in solution.

As illustrated schematically in Fig. 2b, and summarized in Fig. 2c,d, addition of 10 μM of maltose binding protein (MBP) did not elicit any shift in the laurdan spectra while 2 μM of a recombinant otoferlin construct composed of the entire cytoplasmic region of the protein (C2ABCDEF) blueshifted the emission spectra, suggesting that the effects of otoferlin are protein specific. The magnitude of the observed shift was calcium enhanced, although in the absence of calcium an attenuated shift in the spectra was observed, suggesting some calcium independent activity (Fig. 2d). To test whether the observed changes in the spectra could arise due to SUV aggregation by otoferlin, we monitored the change in GP upon addition of 5 μM avidin to SUV harboring 1% 1,2-dioleoyl-sn-glycero-3-phosphoethanolamine-N-biotinyl (biotin-PE). No significant change in GP was observed due to biotin-avidin induced aggregation of vesicles (Fig. 2d). To further characterize the role of calcium in enhancing the effects of otoferlin on SUV, we conducted calcium and magnesium titrations (Fig. 2e). Calcium enhanced the effect of otoferlin on SUV in a dose dependent manner with a Kd of 9.3 ±0.3 μM. Under the range of concentrations tested (0 – 80 μM), magnesium did not elicit a large change in the GP, indicating that the effects of calcium on otoferlin activity is divalent cation specific. Based upon the MBP and biotin-avidin control studies, we conclude that otoferlin specifically interacts with SUV and directly perturbs the structural state of the lipid bilayers.

We next determined which regions of otoferlin harbor this functional activity by testing constructs composed of either the first three C2 domains (C2ABC) or the last three C2 domains (C2DEF) (Fig. 3a,b). When tested, both the C2ABC and C2DEF protein fragments blueshifted the spectra in a dose dependent manner, with the C2DEF construct appearing

more potent within the concentration range tested (0–4 μM). Thus, domains within both the N- and C-terminus of the protein alter the lipid packing of synthetic SUV.

It is generally believed that laurdan is not sensitive to peripheral membrane binding to lipid headgroups, but rather to the physical state of the membrane[18,19,24]. As an additional test of whether the changes in laurdan spectra are due to alterations in lipid bilayer structure, we measured the anisotropy of the laurdan probe in the presence and absence of the otoferlin proteins. Whereas the laurdan GP is a reflection of the order of the bilayer, anisotropy values reflect the fluidity of the bilayer[25]. For both constructs, an increase in the fluorescence anisotropy upon addition of 5μM protein was observed, indicating that the rotation of the laurdan probe was slowed (Fig. 3c). In accordance with the observed GP values for C2ABC and C2DEF, the change in anisotropy was greater for the C2DEF region compared to the C2ABC construct. By contrast, no change in anisotropy was observed when either 5μM bovine serum albumin or 5 μM MBP were tested. The decreased rotation of the laurdan probe upon protein binding to SUV supports the conclusion that the proteins directly alter the physical properties of the lipid bilayer including the fluidity.

The experiments in Fig. 2 and 3a–c were conducted with SUV composed of POPC and POPS. However, the compositions of cellular membranes are significantly more diverse. To ascertain whether the effects of the protein constructs are relevant in more complex systems, laurdan was added to giant plasma membrane vesicles (GPMV) derived from HEK293 cells and mixed with each of the multi-domain constructs. GPMV are large micrometer sized unilamellar vesicles chemically blebbed from cells, and possess many of the endogenous lipids found in the plasma membrane, although it has been demonstrated that blebbing induces mixing of the inner and outer leaflet lipids of the plasma membrane bilayer[21,26]. Visual inspection of the GPMV spectra in the absence of otoferlin show a peak in the 440–450nm range, suggesting that unlike the SUV, the GPMV membranes possess a greater degree of lipid packing, most likely due to the ordering effect of cholesterol (Fig. 3d). However, addition of 5 μM otoferlin C2ABC or C2DEF further shifted the emission maxima to shorter wavelengths, (Fig. 3d). Addition of 10 μM MBP did not shift the laurdan emission profile, and served as a negative control. These results are in qualitative agreement with the conclusions based upon measurement with SUV, and demonstrate that even in a more complex “cell-like” lipid mixture, the lipid ordering activities of otoferlin are still observed.

Conservation of activity among otoferlin, dysferlin and myoferlin C2 domains

Otoferlin is one member of the ferlin family, and we next sought to determine if the results obtained with otoferlin are generalizable to other members. We therefore extended our laurdan measurements to include dysferlin and myoferlin. Specifically, we restricted our studies to the comparison of the N-terminus C2ABC region of these proteins, as a recent study found greater sequence divergence within this region as compared to the C-terminus of the proteins[20]. When tested, 5 μM of constructs composed of the first three C2 domains (C2ABC) of both myoferlin and dysferlin blueshifted the spectra of laurdan labeled SUV in a calcium enhanced manner similar to that of otoferlin C2ABC (Fig. 4). Thus, the membrane altering activity observed with otoferlin appears to be a general property common to members of the ferlin family.

We next compared the GP values of SUV mixed with 5 μM C2ABC to SUV samples mixed with 5μM C2AB and 5μM C2C. The GP of laurdan SUV in the presence 5 μM otoferlin C2ABC was determined to be only slightly higher than that of samples containing both 5 μM otoferlin C2AB and 5 μM C2C suggesting that a small amount of lipid ordering activity is lost by the separation of C2AB from C2C (Fig. 4a). However, in similar experiments with dysferlin and myoferlin, the GP is shifted similarly in the presence of 5 μM C2ABC

compared to 5 μM C2AB with 5μM C2C (Fig. 4b, c). Overall, it appears that separation of C2C from C2AB results in a reduction in activity roughly equivalent to the activity of isolated C2C, and thus the effects of C2C are additive to those of C2AB.

Having established the activity of multi-domain constructs, we next compared equivalent individual C2 domains across ferlin family members. Starting with C2A, we observed that otoferlin did not exhibit appreciable lipid ordering activity at the three concentrations tested (5, 15, 30 μM), while the C2A domains of dysferlin and myoferlin did alter the laurdan signal in a dose-dependent manner, with dysferlin C2A appearing slightly more potent than myoferlin (Fig. 5a). All C2B and C2C domains of otoferlin, dysferlin, and myoferlin shifted the laurdan spectra of SUV in a dose dependent manner, with the C2C domains appearing more potent than the C2B domains (Fig. 5b, c). Thus, with the exception of otoferlin C2A, all the examined C2 domains shifted the laurdan spectra in a calcium enhanced manner (Fig. 5a–c).

A previous report predicted that a mutation to residue D16 in the C2A domain of dysferlin would result in loss of function, and it is hypothesized that this aspartate is involved in calcium the binding activity of the domain[17]. As an additional test to determine the role of calcium on the lipid ordering activity of the C2 domains, a dysferlin C2A D16A mutation was generated and tested for activity. Relative to wild-type, this mutant displayed significantly attenuated activity regardless of the presence of calcium (Fig. 5d). Further, a aspartate mutation predicted to effect calcium binding for the myoferlin C2A domain was also tested (D71A). Like the dysferlin mutant, the myoferlin C2A mutant did not shift the laurdan spectra regardless of calcium (Fig. 5e).

The interaction between ferlin C2 domains and membranes is composed of both electrostatic and hydrophobic contributions

The C2 domains of many proteins bind membranes with a high degree of lipid specificity. For instance, the C2 domains of Golgi localized proteins typically bind to zwitterionic lipids, while proteins that target the plasma membrane harbor C2 domains that bind acidic lipids[12]. To determine if otoferlin interacts preferentially with specific lipids, we repeated the laurdan measurements with SUV of varying anionic lipid compositions. When tested, the C2ABC region of otoferlin failed to alter the measured GP when mixed with SUV comprised of 0% POPS or 10% POPS (Fig. 6a). However, the construct altered the GP of liposomes composed of 25% POPS and 50%POPS. To ascertain whether sensitivity to acidic lipids is a general property of ferlin C2 domains, we conducted similar GP measurements on SUV composed of 0 or 25% POPS mixed with dysferlin or myoferlin C2ABC. For both proteins, and increase in the GP value was observed for SUV composed of 25% POPS but not 0%. Fig. 6b summarizes the results of these measurements. To explicitly test whether the interactions are electrostatic in origin, or due to highly specific interactions between the POPS headgroup and proteins, we carried out GP measurements on SUV composed of 25% POPG and 75% POPC. Like POPS, POPG is negatively charged, but possesses a phosphoglycerol headgroup instead of a phosphatidylserine headgroup. As shown in Fig. 6c, all three ferlin constructs increased the GP values when mixed with POPG containing SUV, supporting a generalized electrostatic interaction. Based upon these results, we conclude that the activities of the ferlin constructs require negatively charged lipids.

A previous report on the C2 domains of synaptotagmin I and VII determined that the mechanism of membrane binding for synaptotagmin I was predominantly electrostatic and ionic strength sensitive, while the membrane binding properties of synaptotagmin VII were insensitive to ionic strength and mediated by both electrostatic and hydrophobic contributions[27]. To determine if electrostatics are the prevailing force driving interaction between SUV and the ferlin constructs, we measured the GP values of the C2ABC region of

otoferlin, myoferlin, and dysferlin at various NaCl concentrations (Fig. 6d). Interestingly, the measured GP values did not change appreciably at the NaCl concentrations tested, suggesting additional non-electrostatic interactions contribute to the interaction between ferlin C2 domains and membranes. To ensure that the observed salt insensitivity was not due to an artifact of the system, we also measured otoferlin C2ABC induced changes of dansyl-PE anisotropy at 150 and 500 mM NaCl (Fig. 6e). When vesicles composed of 25 % POPS, 74 % POPC, and 1 % dansyl-PE were mixed with 5 μM otoferlin, an increase in anisotropy was observed at both 150 mM and 500 mM NaCl conditions. By contrast, 10 μM of synaptotagmin I C2B increased the dansyl-PE anisotropy at 150 mM but not 500 mM NaCl. This further suggests a difference between the mechanisms otoferlin and synaptotagmin use to interact with lipid membranes.

Discussion

In this study, we provide evidence that the C2 domains of otoferlin, myoferlin, and dysferlin promote alterations in the structural state of lipid membranes. While calcium was found to enhance this activity, there was a measureable amount of activity in the absence of calcium. Calcium independent interaction between individual ferlin C2 domains and lipid membranes has been reported before, and we have found that linked C2 domains appear to retain this characteristic. Whether anchoring to the membrane surface via the transmembrane domain has an effect on this property has yet to be determined, however it is possible that tethering to the membrane may restrict the orientation of the protein and confer greater calcium sensitivity. With the exception of the otoferlin C2A domain, which does not bind calcium, all of the domains tested harbored some degree of activity and the graded loss in activity with domain truncations suggest that multiple domains act concurrently to bring about changes to the membrane[15,28]. The activity of these domains required acidic lipids but displayed little specificity beyond the need for a negatively charged headgroup. The tested ferlin domains were also found to be relatively insensitive to electrostatic screening, as high salt concentrations did not alter their activity. To more fully appreciate these conclusions, we draw comparisons to the synaptotagmin family of C2 domain proteins, the best studied of which is synaptotagmin I (syt I). Syt I is the calcium sensor for neurotransmitter release at neural synapses, and harbors two C2 domains as well as a transmembrane domain which serves to anchor the protein to presynaptic vesicles[29]. Like the ferlins examined in this study, the C2 domains of syt I binds negatively charged lipids that are enriched on the inner leaflet of the plasma membrane[13,29,30]. Also like the ferlins syt I alters the structure of lipid bilayers when bound[13]. For syt I, the mechanism is believed to involve induction of membrane curvature and clustering of acidic lipids[13,14]. However the insensitivity of the ferlin C2 domains to electrostatic screening and their ability to interact with membranes independent of calcium strongly contrasts with syt I, where calcium is required, and salt concentrations above 200 mM NaCl inhibit protein-membrane interaction[30]. This suggests differences in the underlying mechanisms utilized by syt I and the ferlins. For syt I, electrostatic repulsion between the negatively charged loops of the C2 domains and the negative charge on the membrane prevents binding in the absence of calcium[29,31]. Upon calcium binding, the electrostatic potential of the domain changes allowing for protein-membrane interaction. This electrostatic switch mechanism may not be as pronounced for the ferlin domains. Interestingly, the binding of the C2A domain of synaptotagmin VII (syt VII) with membranes is insensitive to high salt concentrations, and this may be in part due to deep membrane insertion of two phenylalanines within the calcium binding loops of the domain that would provide a hydrophobic contribution to membrane binding[27]. Likewise, the C2 domain of cytosolic phospholipase A2 also penetrates deeply into the hydrophobic region of lipids bilayers and is insensitive to high salt concentrations[12]. We note that a sequence alignment of the C2 domains of otoferlin, myoferlin and dysferlin indicate the presence of one or more hydrophobic amino acids in the loops predicted to be involved in

calcium binding, and that a recently determined solution structure of the C2A domain of myoferlin clearly shows a phenylalanine on the same loop that contains a putative calcium binding aspartate residue. Membrane insertion of hydrophobic side chains could both account in part for the insensitivity to high salt concentrations and the observed changes in lipid bilayer structure. Future studies should focus on the effects of these residues on ferlin-membrane interaction.

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ABREVIATIONS

biotin-PE	1,2-dioleoyl-sn-glycero-3-phosphoethanolamine-N-biotinyl
BSA	bovine serum albumin
GMPV	giant membrane plasma vesicles
GP	general polarization
MBP	maltose binding protein
POPC	1-palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine
POPG	1-palmitoyl-2-oleoyl-sn-glycero-3-phospho-(1′-rac-glycerol)
POPS	1-palmitoyl-2-oleoyl-sn-glycero-3-phospho-L-serine
SUV	small unilamellar vesicle

References

1. Lek A, Evesson FJ, Sutton RB, North KN, Cooper ST. Ferlins: regulators of vesicle fusion for auditory neurotransmission, receptor trafficking and membrane repair. Traffic. 2012; 13:185–194. [PubMed: 21838746]
2. Washington NL, Ward S. FER-1 regulates Ca2+-mediated membrane fusion during C. elegans spermatogenesis. J Cell Sci. 2006; 119:2552–2562. [PubMed: 16735442]
3. Ohsako T, Hirai K, Yamamoto MT. The Drosophila misfire gene has an essential role in sperm activation during fertilization. Genes Genet Syst. 2003; 78:253–266. [PubMed: 12893967]
4. Dulon D, Safieddine S, Jones SM, Petit C. Otoferlin is critical for a highly sensitive and linear calcium-dependent exocytosis at vestibular hair cell ribbon synapses. J Neurosci. 2009; 29:10474–10487. [PubMed: 19710301]
5. Roux I, Safieddine S, Nouvian R, Grati M, Simmler MC, Bahloul A, Perfettini I, Le Gall M, Rostaing P, Hamard G, Triller A, Avan P, Moser T, Petit C. Otoferlin, defective in a human deafness form, is essential for exocytosis at the auditory ribbon synapse. Cell. 2006; 127:277–289. [PubMed: 17055430]
6. Pangrsic T, Lasarow L, Reuter K, Takago H, Schwander M, Riedel D, Frank T, Tarantino LM, Bailey JS, Strenzke N, Brose N, Müller U, Reisinger E, Moser T. Hearing requires otoferlin-dependent efficient replenishment of synaptic vesicles in hair cells. Nat Neurosci. 2010; 13:869–876. [PubMed: 20562868]
7. Bansal D, Miyake K, Vogel SS, Groh S, Chen CC, Williamson R, McNeil PL, Campbell KP. Defective membrane repair in dysferlin-deficient muscular dystrophy. Nature. 2003; 423:168–172. [PubMed: 12736685]
8. Kobayashi K, Izawa T, Kuwamura M, Yamate J. Dysferlin and animal models for dysferlinopathy. J Toxicol Pathol. 2012; 25:135–147. [PubMed: 22907980]

9. Doherty KR, Cave A, Davis DB, Delmonte AJ, Posey A, Earley JU, Hadhazy M, McNally EM. Normal myoblast fusion requires myoferlin. Development. 2005; 132:5565–5575. [PubMed: 16280346]
10. Eisenberg MC, Kim Y, Li R, Ackerman WE, Kniss DA, Friedman A. Mechanistic modeling of the effects of myoferlin on tumor cell invasion. Proc Natl Acad Sci U S A. 2011; 108:20078–20083. [PubMed: 22135466]
11. Nalefski EA, Falke JJ. The C2 domain calcium-binding motif: structural and functional diversity. Protein Sci. 1996; 5:2375–2390. [PubMed: 8976547]
12. Nalefski EA, Wisner MA, Chen JZ, Sprang SR, Fukuda M, Mikoshiba K, Falke JJ. C2 domains from different Ca2+ signaling pathways display functional and mechanistic diversity. Biochemistry. 2001; 40:3089–3100. [PubMed: 11258923]
13. Hui E, Johnson CP, Yao J, Dunning FM, Chapman ER. Synaptotagmin-mediated bending of the target membrane is a critical step in Ca(2+)-regulated fusion. Cell. 2009; 138:709–721. [PubMed: 19703397]
14. Lai AL, Tamm LK, Ellena JF, Cafiso DS. Synaptotagmin 1 modulates lipid acyl chain order in lipid bilayers by demixing phosphatidylserine. J Biol Chem. 2011; 286:25291–25300. [PubMed: 21610074]
15. Johnson CP, Chapman ER. Otoferlin is a calcium sensor that directly regulates SNARE-mediated membrane fusion. J Cell Biol. 2010; 191:187–197. [PubMed: 20921140]
16. Therrien C, Di Fulvio S, Pickles S, Sinnreich M. Characterization of lipid binding specificities of dysferlin C2 domains reveals novel interactions with phosphoinositides. Biochemistry. 2009; 48:2377–2384. [PubMed: 19253956]
17. Davis DB, Doherty KR, Delmonte AJ, McNally EM. Calcium-sensitive phospholipid binding properties of normal and mutant ferlin C2 domains. J Biol Chem. 2002; 277:22883–22888. [PubMed: 11959863]
18. Bagatolli LA, Gratton E, Fidelio GD. Water dynamics in glycosphingolipid aggregates studied by LAURDAN fluorescence. Biophys J. 1998; 75:331–341. [PubMed: 9649390]
19. Bagatolli LA, Sanchez SA, Hazlett T, Gratton E. Giant vesicles, Laurdan, and two-photon fluorescence microscopy: evidence of lipid lateral separation in bilayers. Methods Enzymol. 2003; 360:481–500. [PubMed: 12622164]
20. Jiménez JL, Bashir R. In silico functional and structural characterisation of ferlin proteins by mapping disease-causing mutations and evolutionary information onto three-dimensional models of their C2 domains. J Neurol Sci. 2007; 260:114–123. [PubMed: 17512949]
21. Baumgart T, Hammond AT, Sengupta P, Hess ST, Holowka DA, Baird BA, Webb WW. Large-scale fluid/fluid phase separation of proteins and lipids in giant plasma membrane vesicles. Proc Natl Acad Sci U S A. 2007; 104:3165–3170. [PubMed: 17360623]
22. Ramakrishnan NA, Drescher MJ, Drescher DG. Direct interaction of otoferlin with syntaxin 1A, SNAP-25, and the L-type voltage-gated calcium channel Cav1.3. J Biol Chem. 2009; 284:1364–1372. [PubMed: 19004828]
23. Pangršič T, Reisinger E, Moser T. Otoferlin: a multi-C2 domain protein essential for hearing. Trends Neurosci. 2012; 35:671–680. [PubMed: 22959777]
24. Dinic J, Biverståhl H, Mäler L, Parmryd I. Laurdan and di-4-ANEPPDHQ do not respond to membrane-inserted peptides and are good probes for lipid packing. Biochim Biophys Acta. 2011; 1808:298–306. [PubMed: 20937246]
25. Harris FM, Best KB, Bell JD. Use of laurdan fluorescence intensity and polarization to distinguish between changes in membrane fluidity and phospholipid order. Biochim Biophys Acta. 2002; 1565:123–128. [PubMed: 12225860]
26. Keller H, Lorizate M, Schwille P. PI(4,5)P2 degradation promotes the formation of cytoskeleton-free model membrane systems. Chemphyschem. 2009; 10:2805–2812. [PubMed: 19784973]
27. Brandt DS, Coffman MD, Falke JJ, Knight JD. Hydrophobic contributions to the membrane docking of synaptotagmin 7 C2A domain: mechanistic contrast between isoforms 1 and 7. Biochemistry. 2012; 51:7654–7664. [PubMed: 22966849]

28. Helfmann S, Neumann P, Tittmann K, Moser T, Ficner R, Reisinger E. The crystal structure of the C_2A domain of otoferlin reveals an unconventional top loop region. J Mol Biol. 2011; 406:479–490. [PubMed: 21216247]

29. Chapman ER. How does synaptotagmin trigger neurotransmitter release? Annu Rev Biochem. 2008; 77:615–641. [PubMed: 18275379]

30. Hui E, Gaffaney JD, Wang Z, Johnson CP, Evans CS, Chapman ER. Mechanism and function of synaptotagmin-mediated membrane apposition. Nat Struct Mol Biol. 2011; 18:813–821. [PubMed: 21642967]

31. Murray D, Honig B. Electrostatic control of the membrane targeting of C2 domains. Mol Cell. 2002; 9:145–154. [PubMed: 11804593]

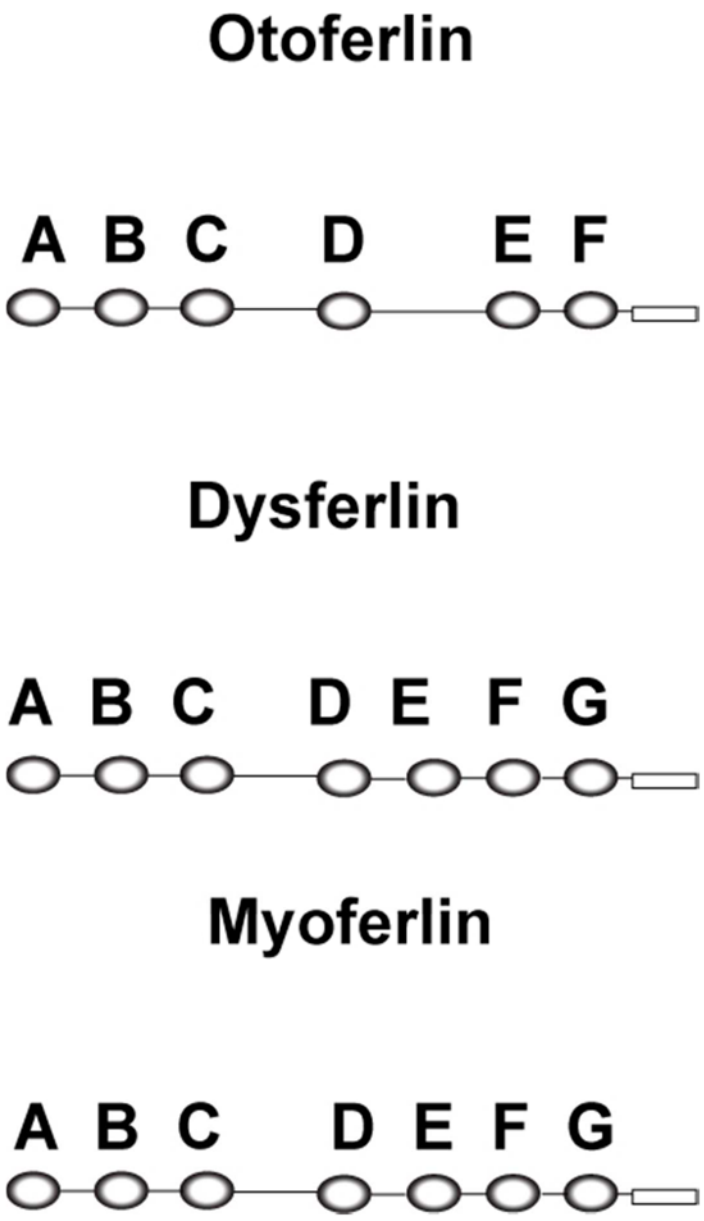

Figure 1.
Schematic of ferlin proteins under study. Otoferlin, dysferlin, and myoferlin are depicted with C2 domains as circles, and the transmembrane anchor regions as rectangles.

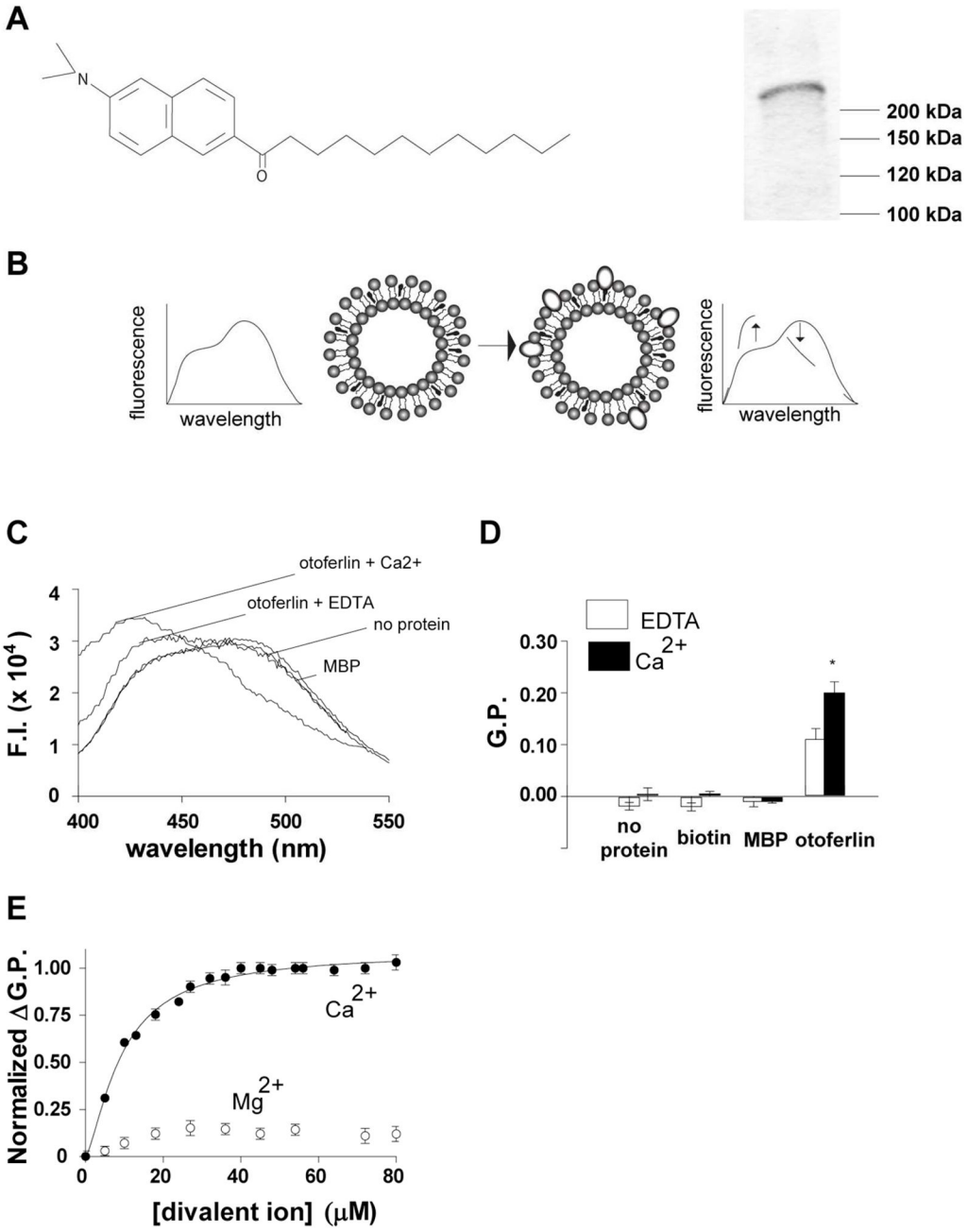

Figure 2.

Otoferlin alters the lipid order of SUV. (A) Structure of laurdan fluorescent probe and SDS-PAGE image of otoferlin ABCDEF construct (~250 kD). (B) Schematic illustrating the qualitative changes in laurdan emission spectra. Arrows in the spectra indicate the decrease in long wavelength emission and increase in shorter wavelength intensity that corresponds to the increased lipid order and decreased bilayer hydration. (C) Laurdan emission spectra of SUV in the presence and absence of 10 μM MBP or 2 μM otoferlin ABCDEF +/− 300 μM Ca^{2+}. (D) Mean values (± sd) of the G.P. shift of the laurdan emission in 500 μM calcium (black bars) or 100 μM EDTA (white bars) in the absence of protein, with 2 μM otoferlin ABCDEF, 10 μM MBP, and biotin aggregated vesicle samples. N = 4 *P < 0.05 between

EDTA and Ca^{2+} conditions. (E) Plot of normalized G.P. shift in the presence of 2 μM otoferlin ABCDEF as a function of free Ca^{2+} or Mg^{2+} (± sd). The Ca^{2+} data was fitted with a one site equation and the fitted parameters were K_d = 9.3 ±0.3 μM, R^2 =0.997, N = 3.

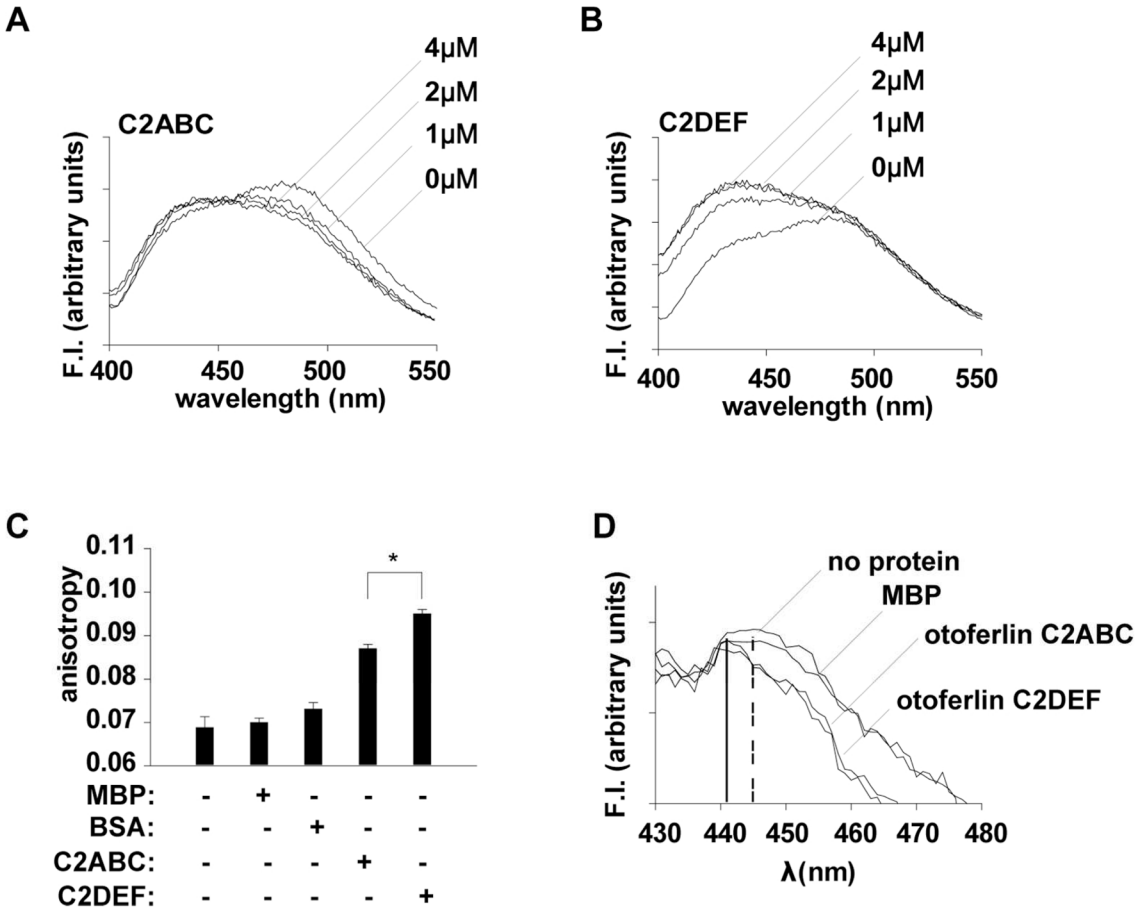

Figure 3.

Otoferlin C2ABC and C2DEF constructs alter the lipid order of SUVs and GPMVs. (A) Titration of otoferlin C2ABC and (B) C2DEF from 0 to 4 μM shift the emission spectra of laurdan to shorter wavelengths. (C) Mean laurdan anisotropy values (± sd) of SUV in the absence of protein, or mixed with 10 μM MBP, 5 μM BSA, 4 μM C2ABC, or 4 μM C2DEF. Increases in anisotropy were observed for otoferlin but not MBP or BSA. Anisotropy samples contained 500 μM free calcium. N = 3, *P < 0.05. (D) Representative spectra of laurdan labeled GPMV in the absence or presence of otoferlin. Addition of 5 μM C2ABC or C2DEF to samples containing laurdan labeled GPMV results in a shift of the emission maxima to shorter wavelengths (solid lines), while the addition of 10 μM MBP does not change the wavelength maxima (dashed line). Samples contained 500 μM free calcium.

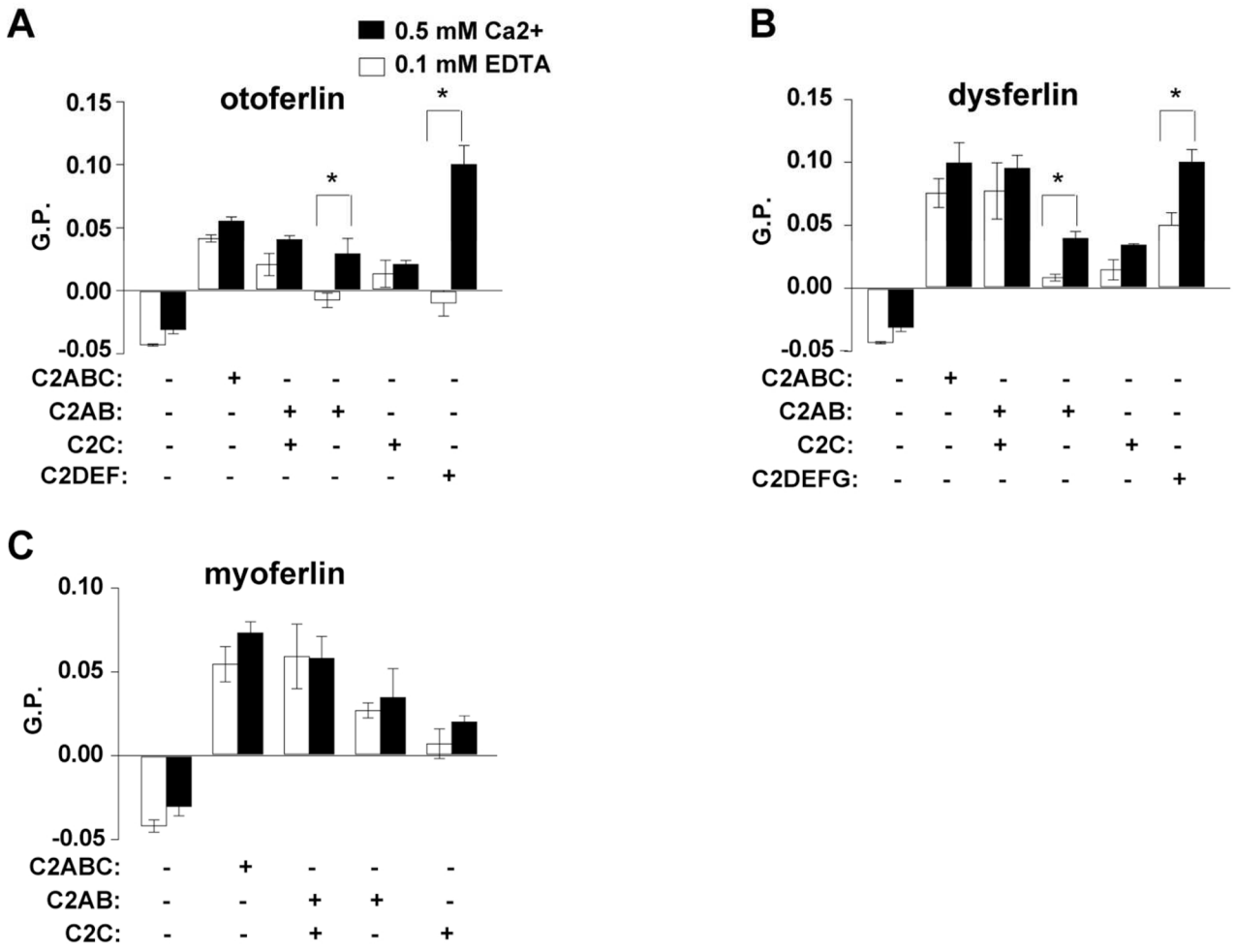

Figure 4.

GP values of SUV in the presence of (A) otoferlin, (B) dysferlin, or (C) myoferlin C2ABC, C2AB, C2C or a combination of C2AB and C2C as indicated. All proteins were added to a final concentration of 5 μM. Measurements were conducted in either EDTA (white bars), or calcium (black bars) N = 3, *P < 0.05

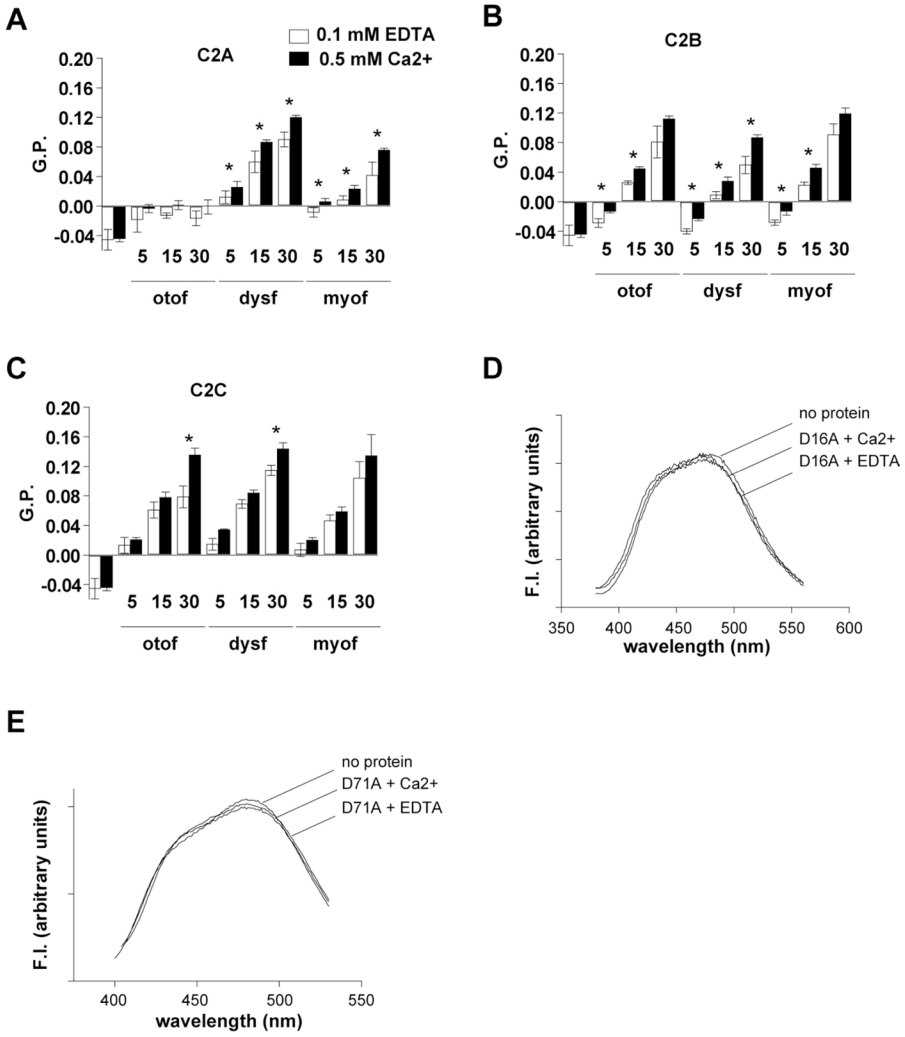

Figure 5.

The lipid ordering activity of isolated C2 domains of dysferlin, otoferlin, and myoferlin. GP values for SUV in the absence or presence of 5, 15, or 30 μM otoferlin, dysferlin, and myoferlin (A) C2A constructs, (B) C2B constructs, and (C) C2C constructs. Measurements were conducted in either EDTA (white bars), or calcium (black bars). N = 3, *P < 0.05 between EDTA and Ca2+ conditions. Laurdan spectra for (D) dysferlin C2A D16A and (E) myoferlin C2A D71A in the absence and presence of 500 μM calcium.

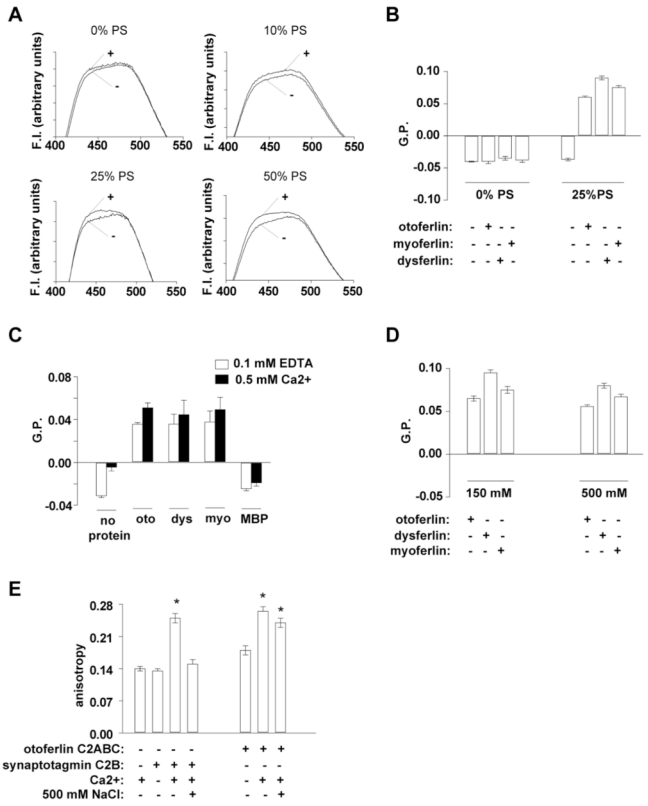

Figure 6.

Negatively charged lipids are required for ferlin activity. (A) Laurdan emission spectra of SUV composed of POPC with varying mol percentages of POPS with calcium in the presence and absence of 5 μM otoferlin C2ABC. (B) GP values of SUV composed of 0 or 25 mol % POPS mixed with 5 μM otoferlin, dysferlin, or myoferlin C2ABC in the presence of 500 μM Ca^{2+}. (C) GP values of laurdan SUV composed of POPC and 25% POPG mixed with 5μM otoferlin, dysferlin, or myoferlin C2ABC. White bars represent samples containing EDTA, and black bars correspond to measurements made in calcium. (D) GP values of SUV composed of 75% POPC and 25 % POPS mixed with 5 μM otoferlin, dysferlin, or myoferlin C2ABC in the presence of 150 or 500 mM NaCl. Samples contained 500 μM calcium. (E) Fluorescence anisotropy values for dansyl-PE SUV mixed with synaptotagmin I C2B or otoferlin C2ABC in the presence or absence of Ca^{2+} and high salt (500 mM NaCl) concentrations. N = 3, *P < 0.05 between protein and protein free samples.