BIOCHEMISTRY
including biophysical chemistry & molecular biology

Article

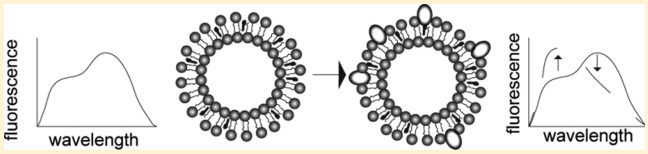
pubs.acs.org/biochemistry

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

Naomi J. Marty, Chelsea L. Holman, Nazish Abdullah, and Colin P. Johnson*

Department of Biochemistry and Biophysics, Oregon State University, Corvallis, Oregon 97331, United States

ABSTRACT: Ferlins are large multi-C2 domain membrane proteins involved in membrane fusion and fission events. In this study, we investigate the effects of binding of the C2 domains of otoferlin, dysferlin, and myoferlin on 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 with individual domains uncovered functional differences between the C2A domain of otoferlin and those of dysferlin and myoferlin, and truncation studies suggest that the effects of each subsequent C2 domain on lipid ordering appear to be additive. Finally, we demonstrate that the activities of these proteins on membranes are insensitive to high salt concentrations, suggesting a nonelectrostatic 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, membrane fission, or recruitment of other membrane-trafficking proteins.



Ferlins make up a family of eukaryotic membrane proteins that mediate membrane trafficking events.[1] The first ferlin gene characterized was expressed in *Caenorhabditis 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 because of their association with human diseases.[1] For example, mutations in otoferlin disrupt the release of a neurotransmitter 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 of myoferlin 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 five to seven C2 domains and a single-pass transmembrane region (Figure 1). Proteins with more than two tandem C2 domains are rare, and it is currently unclear 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 in helping to facilitate cellular processes.[13,14] While studies of the

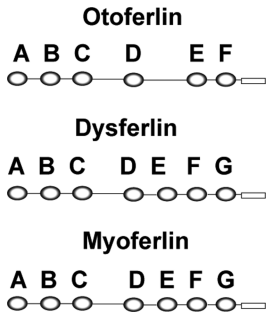


Figure 1. Schematic of ferlin proteins being studied. Otoferlin, dysferlin, and myoferlin are depicted with C2 domains as circles and the transmembrane anchor regions as rectangles.

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 of 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-termini of the protein.[18,19] We also demonstrate that this activity is conserved in myoferlin and dysferlin and is

Received: April 5, 2013
Revised: July 9, 2013
Published: July 16, 2013

dx.doi.org/10.1021/bi400432f | *Biochemistry* 2013, 52, 5585−5592

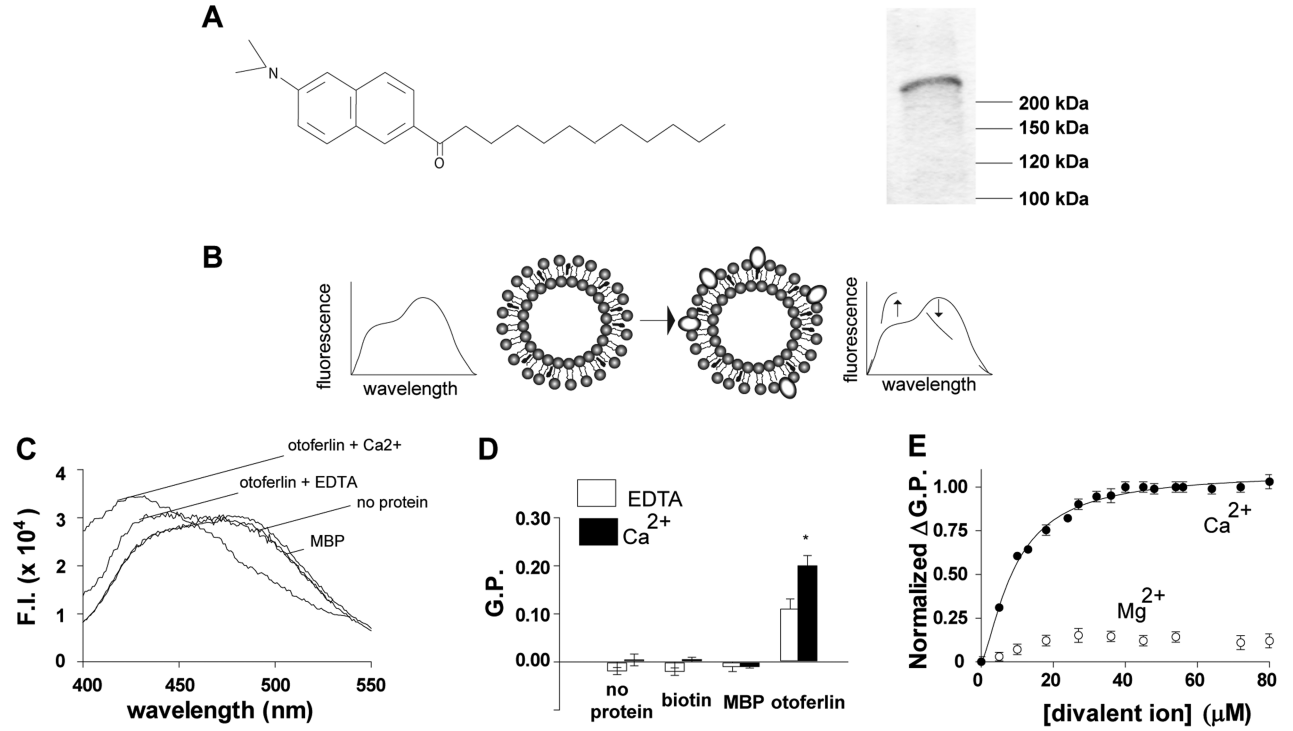


Figure 2. Otoferlin alters the lipid order of SUV. (A) Structure of the laurdan fluorescent probe and SDS−PAGE image of otoferlin ABCDEF construct (~250 kDa). (B) Schematic illustrating the qualitative changes in laurdan emission spectra. Arrows in the spectra indicate the decrease in long wavelength emission and increase in shorter wavelength intensity that corresponds to the increased level of lipid order and decreased level of bilayer hydration. (C) Laurdan emission spectra of SUV in the presence and absence of 10 μM MBP or 2 μM otoferlin ABCDEF with or without 300 μM Ca^{2+} . (D) Mean values (±SD) of the GP 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 the normalized GP shift in the presence of 2 μM otoferlin ABCDEF as a function of free Ca^{2+} or Mg^{2+} concentration (±SD). The Ca^{2+} data were fit with a one-site equation, and the fitted parameters were as follows: $K_d = 9.3 \pm 0.3$ μM, $R^2 = 0.997$, and $N = 3$.

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 domain of otoferlin differs from the equivalent domains in dysferlin and myoferlin. Studies of truncated constructs suggest that the domains have an additive effect on lipid membranes, with shorter constructs lacking the potency of larger multidomain 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 Ni^{2+}-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, New Haven, CT), and pcDNA3.1 otoferlin plasmid (gift from C. Petit, Institut Pasteur et Université Pierre et Marie Curie, Paris, France) were used as templates for amplification of *Homo sapiens* dysferlin (GenBank entry AF075575.1), *Homo sapiens* myoferlin (GenBank entry AF075575.1), and *Mus musculus* otoferlin (GenBank entry AY586513.1), respectively. Forward and reverse primers were designed to amplify the coding sequences with 5′ and 3′ sites for the insertion of LIC 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 multidomain constructs, the primers were designed for the following amino acids: dysferlin C2ABC (amino acids 1−574), otoferlin C2ABC (amino acids 1−553), otoferlin C2DEF (amino acids 960−1885), otoferlin C2ABC-DEF (amino acids 1−1885), and myoferlin C2ABC (amino acids 1−530). Dysferlin C2A D16A was constructed using the Stratagene QuikChange site-directed mutagenesis kit with the pMCSG9/6His-MBP-dysferlin C2A plasmid template. Resulting constructs were expressed as a fusion with six-His MBP (maltose binding protein). Multidomain constructs were transformed into Rosetta BL2, while single-C2 domain constructs were transformed into BL21. Expressed six-His-tagged constructs were affinity-purified using nickel IMAC resin followed by buffer exchange into 20 mM Tris-HCl (pH 7.5) and 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 of 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

dx.doi.org/10.1021/bi400432f | *Biochemistry* 2013, 52, 5585−5592

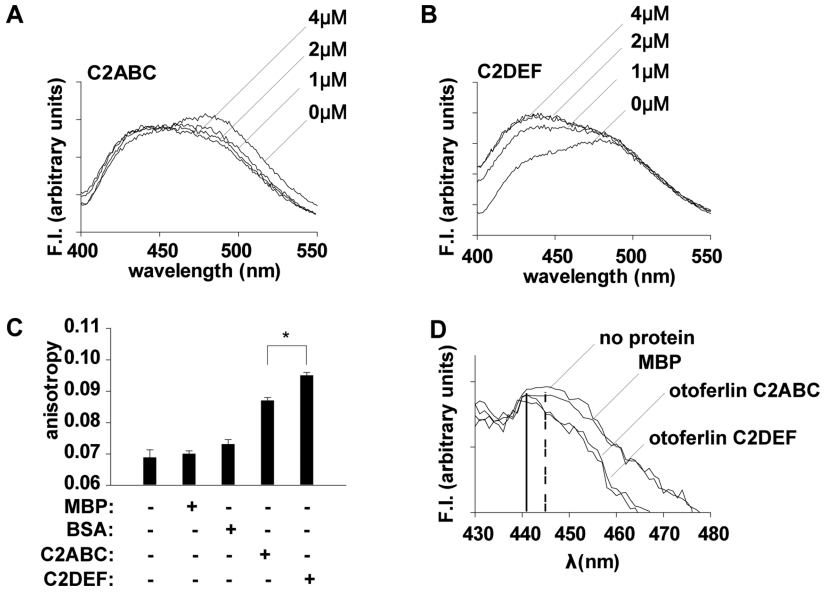


Figure 3. Otoferlin C2ABC and C2DEF constructs alter the lipid order of SUV and GPMV. Titration of otoferlin (A) C2ABC and (B) C2DEF from 0 to 4 μM shifts the emission spectra of laurdan to shorter wavelengths. (C) Mean laurdan anisotropy values (±SD) of SUV in the absence of protein, or mixed with 10 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.

purification, necessitating the longer construct of amino acids 1−574.

Small Unilamellar Vesicle Preparation. Vesicles were prepared as described previously.[13,15] Briefly, lipids dissolved in chloroform 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 1 mM and extruded using a membrane with a 50 nm cutoff. The 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 were washed with PBS and subsequently incubated with 2 mM DTT, 25 mM formaldehyde, and small (micromolar) amounts of laurdan at 37 °C for 60 min. The resulting vesicles were imaged under bright field using an epi-fluorescent microscope to verify the formation of vesicles.

Protein Fluorescence Measurements. Steady state fluorescence measurements were conducted using a QM-40 instrument with Glan Thompson polarizers (Photon Technology International, Birmingham, NJ) at 37 °C. All fluorescence measurements were conducted in samples containing 20 mM 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

$$GP = (I_{430} - I_{480})/(I_{430} + I_{480})$$

where I_{430} and I_{480} are the emission intensities at 430 and 480 nm, respectively. Anisotropy measurements were calculated using the following equation

$$r = (I_{VV} - I_{VH})/(I_{VV} + 2I_{VH})$$

where I_{VV} and I_{VH} correspond to the parallel and perpendicular emission intensities, respectively. Reported values represent the mean ± the standard deviation (SD) 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 cofloatation 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 maximum that is sensitive to the ordering of lipids in bilayers[18,19] (Figure 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 laurdan is immersed in a highly ordered lipid bilayer, its emission spectrum possesses a maximum at 430 nm, while in more disordered (greater degree of hydration) bilayers, the maximum occurs at longer wavelengths, typically in the range of 480 nm. The normalized intensity ratio at these two wavelengths is often termed the general polarization (GP) value and ranges from −1 (most disordered state) to 1 (most

dx.doi.org/10.1021/bi400432f | *Biochemistry* 2013, 52, 5585−5592

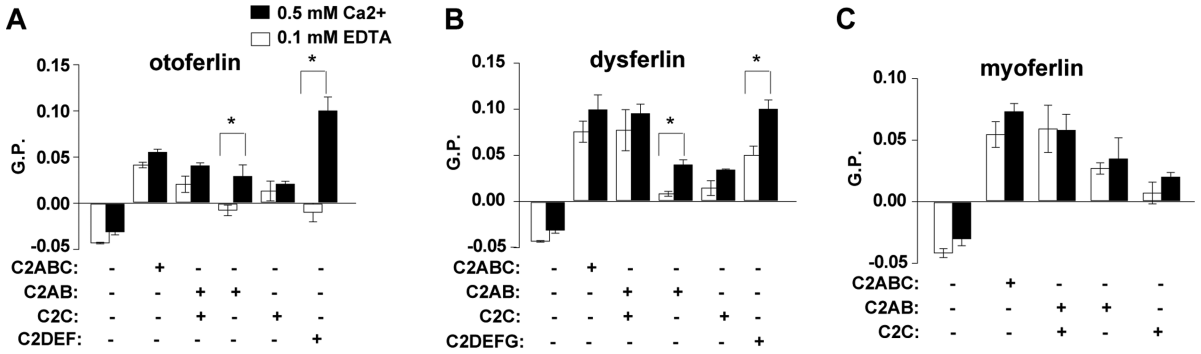


Figure 4. GP values of SUV in the presence of (A) otoferlin, (B) dysferlin, or (C) myoferlin C2ABC, C2AB, C2C, or a combination of C2AB and C2C as indicated. All proteins were added to a final concentration of 5 μM. Measurements were conducted in either EDTA (white bars) or calcium (black bars). $N = 3$. *$P < 0.05$.

ordered state). GP values are not biased by phospholipid headgroup charge, nor are they affected by the penetration of protein 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 Figure 2B and summarized in Figure 2C,D, addition of 10 μM maltose binding protein (MBP) did not elicit any shift in the laurdan spectra while addition of a recombinant otoferlin construct (2 μM) 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 (Figure 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 because of biotin−avidin-induced aggregation of vesicles (Figure 2D). To further characterize the role of calcium in enhancing the effects of otoferlin on SUV, we conducted calcium and magnesium titrations (Figure 2E). Calcium enhanced the effect of otoferlin on SUV in a dose-dependent manner with a K_d of 9.3 ± 0.3 μM. Over 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 are divalent cation specific. On the basis of 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) (Figure 3A,B). When tested, both the C2ABC and C2DEF protein fragments blueshifted the spectra in a dose-dependent manner, with the C2DEF construct appearing to be more potent within the concentration range tested (0−4 μM). Thus, domains within both the N- and C-termini of the protein alter the lipid packing of synthetic SUV.

It is generally believed that laurdan is not sensitive to binding of the peripheral membrane 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 5 μM protein was observed, indicating that the rotation of the laurdan probe was slowed (Figure 3C). In accordance with the observed GP values for C2ABC and C2DEF, the change in anisotropy was greater for the C2DEF region than for the C2ABC construct. By contrast, no change in anisotropy was observed when either 5 μM bovine serum albumin or 5 μM MBP was tested. The decreased degree of rotation of the laurdan probe upon binding of protein to SUV supports the conclusion that the proteins directly alter the physical properties of the lipid bilayer, including the fluidity.

The experiments in Figures 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 multidomain 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 shows a peak in the 440−450 nm range, suggesting that unlike the SUV, the GPMV membranes possess a greater degree of lipid packing, most likely because of the ordering effect of cholesterol (Figure 3D). However, addition of 5 μM otoferlin C2ABC or C2DEF further shifted the emission maxima to shorter wavelengths (Figure 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

dx.doi.org/10.1021/bi400432f | *Biochemistry* 2013, 52, 5585−5592

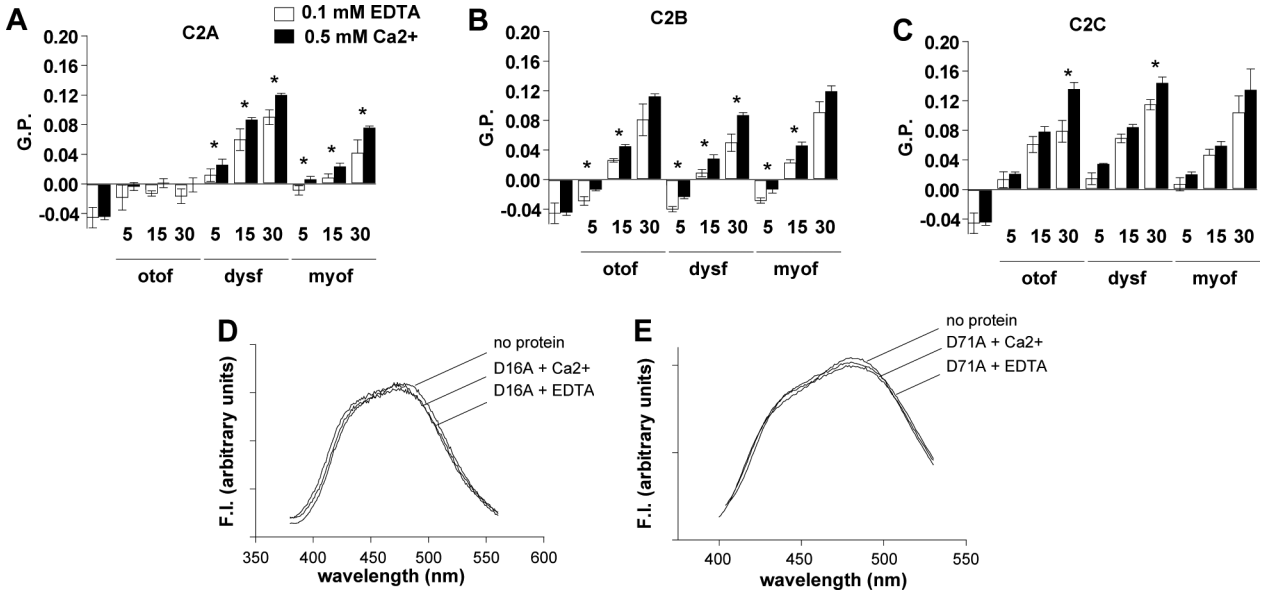


Figure 5. Lipid ordering activity of isolated C2 domains of dysferlin, otoferlin, and myoferlin. GP values for SUV in the absence or presence of 5, 15, or 30 μM otoferlin, dysferlin, and myoferlin (A) C2A constructs, (B) C2B constructs, and (C) C2C constructs. Measurements were conducted in either EDTA (white bars) or calcium (black bars). $N = 3$. *$P < 0.05$ between EDTA and Ca^{2+} conditions. Laurdan spectra for (D) dysferlin C2A D16A and (E) myoferlin C2A D71A in the absence and presence of 500 μM calcium.

ferlin family, and we next sought to determine if the results obtained with otoferlin can be generalized 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-terminal C2ABC region of these proteins, as a recent study found a greater degree of sequence divergence within this region than in the C-terminus of the proteins.[20] When tested, 5 μM 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 (Figure 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 those of SUV mixed with 5 μM C2AB and 5 μM 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 (Figure 4A). However, in similar experiments with dysferlin and myoferlin, the GP is shifted similarly in the presence of 5 μM C2ABC compared to that with 5 μM C2AB and 5 μM C2C (Figure 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 with those of C2AB.

Having established the activity of multidomain 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, and 30 μM), while the C2A domains of dysferlin and myoferlin did alter the laurdan signal in a dose-dependent manner, with dysferlin C2A appearing to be slightly more potent than myoferlin (Figure 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 to be more potent than the C2B domains (Figure 5B,C). Thus, with the exception of otoferlin C2A, all the examined C2 domains shifted the laurdan spectra in a calcium-enhanced manner (Figure 5A–C).

A previous report predicted that a mutation to residue D16 in the C2A domain of dysferlin would result in a loss of function, and it is hypothesized that this aspartate is involved in the calcium binding activity of the domain.[17] As an additional test to determine the role of calcium in the lipid ordering activity of the C2 domains, a dysferlin C2A D16A mutation was generated and tested for activity. Relative to the wild type, this mutant displayed significantly attenuated activity regardless of the presence of calcium (Figure 5D). Further, an 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 the presence of calcium (Figure 5E).

The Interaction between Ferlin C2 Domains and Membranes Is Composed of 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 with varying anionic lipid compositions. When tested, the C2ABC region of otoferlin failed to alter the measured GP when that region was mixed with SUV comprised of 0% POPS or 10% POPS (Figure 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, an increase in the GP value was observed for SUV composed of 25% POPS but not 0% POPS.

dx.doi.org/10.1021/bi400432f | *Biochemistry* 2013, 52, 5585–5592

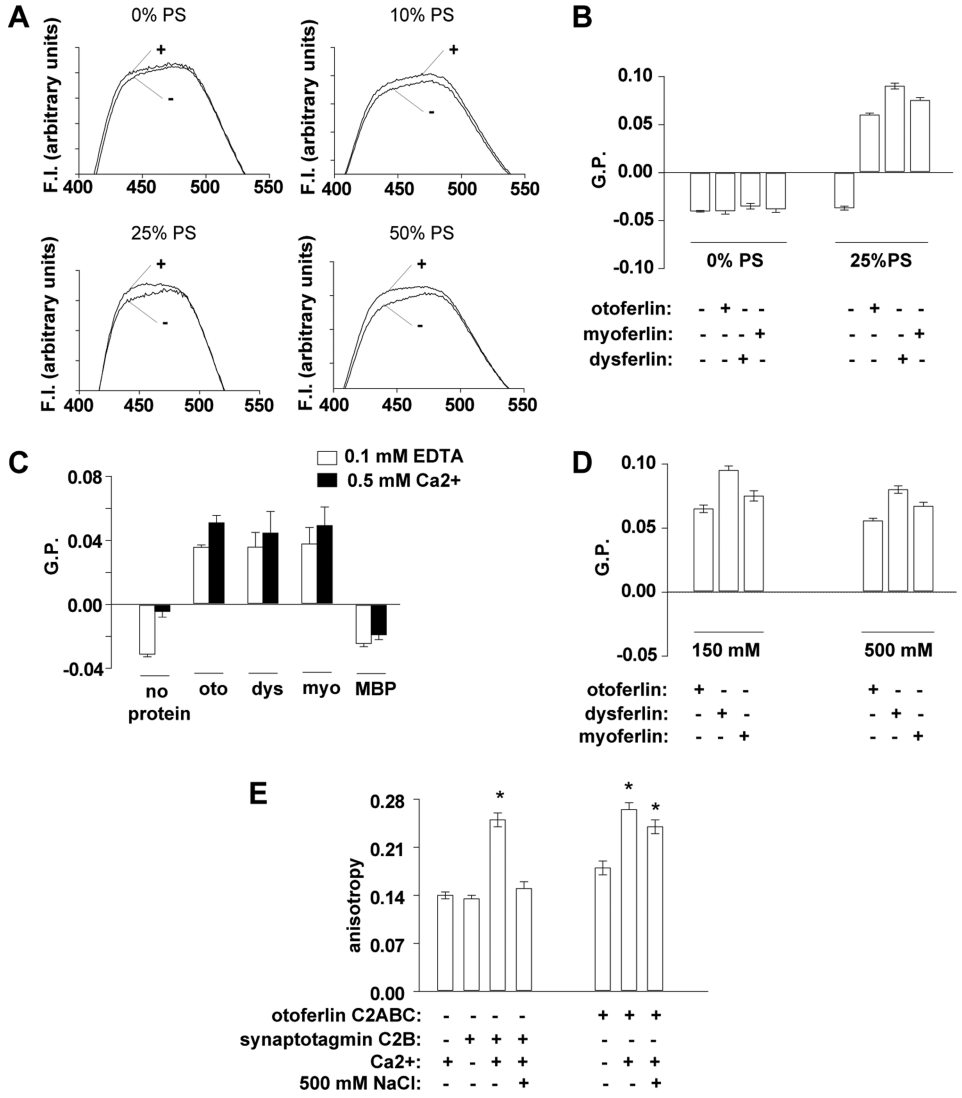


Figure 6. Negatively charged lipids are required for ferlin activity. (A) Laurdan emission spectra of SUV composed of POPC with varying mole percentages of POPS with calcium in the presence and absence of 5 μM otoferlin C2ABC. (B) GP values of SUV composed of 0 or 25 mol % POPS mixed with 5 μM otoferlin, 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 5 μM otoferlin, dysferlin, or myoferlin C2ABC. White bars represent data for 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 concentrations (500 mM NaCl). $N = 3$. *$P < 0.05$ between protein and protein free samples.

Figure 6B summarizes the results of these measurements. To explicitly test whether the interactions are electrostatic in origin, or because of highly specific interactions between the POPS headgroup and proteins, we conducted 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 Figure 6C, all three ferlin constructs increased the GP values when the constructs were mixed with POPG-containing SUV, supporting a generalized electrostatic interaction. On the basis of 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 sensitive to ionic strength, 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 (Figure 6D). Interestingly, the measured GP values did not change appreciably at the NaCl concentrations tested, suggesting additional nonelectrostatic 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 in dansyl-PE anisotropy at 150 and 500 mM NaCl (Figure 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 and 500 mM NaCl. By contrast, 10 μM synaptotagmin I C2B increased the dansyl-PE anisotropy at 150 mM NaCl but not at 500 mM

dx.doi.org/10.1021/bi400432f | *Biochemistry* 2013, 52, 5585−5592

NaCl. This further suggests a difference between the mechanisms that 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 measurable 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 of activity with domain truncations suggests 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 that serves to anchor the protein to presynaptic vesicles.[29] Like the ferlins examined in this study, the C2 domains of syt I bind 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 they are bound to syt I.[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 in a manner independent of calcium strongly contrast with the features of 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] When calcium binds, 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 lipid bilayers and is insensitive to high salt concentrations.[12] We note that a sequence alignment of the C2 domains of otoferlin, myoferlin, and dysferlin indicates 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 account in part for both 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.

AUTHOR INFORMATION

Corresponding Author

*Department of Biochemistry and Biophysics, Oregon State University, Corvallis, OR 97330. Telephone: (541) 737-4517. E-mail: colin.johnson@oregonstate.edu.

Funding

This work was supported by National Institutes of Health Grant R00 DC-011267 and Oregon State University startup funds.

Notes

The authors declare no competing financial interest.

ABBREVIATIONS

biotin-PE, 1,2-dioleoyl-*sn*-glycero-3-phosphoethanolamine-*N*-biotinyl; BSA, bovine serum albumin; GMPV, giant membrane plasma vesicle(s); 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(s).

REFERENCES

(1) Lek, A., Evesson, F. J., Sutton, R. B., North, K. N., and Cooper, S. T. (2012) Ferlins: Regulators of vesicle fusion for auditory neurotransmission, receptor trafficking and membrane repair. *Traffic 13*, 185−194.

(2) Washington, N. L., and Ward, S. (2006) FER-1 regulates Ca^{2+}-mediated membrane fusion during *C. elegans* spermatogenesis. *J. Cell Sci. 119*, 2552−2562.

(3) Ohsako, T., Hirai, K., and Yamamoto, M. T. (2003) The *Drosophila* misfire gene has an essential role in sperm activation during fertilization. *Genes Genet. Syst. 78*, 253−266.

(4) Dulon, D., Safieddine, S., Jones, S. M., and Petit, C. (2009) Otoferlin is critical for a highly sensitive and linear calcium-dependent exocytosis at vestibular hair cell ribbon synapses. *J. Neurosci. 29*, 10474−10487.

(5) Roux, I., Safieddine, S., Nouvian, R., Grati, M., Simmler, M. C., Bahloul, A., Perfettini, I., Le Gall, M., Rostaing, P., Hamard, G., Triller, A., Avan, P., Moser, T., and Petit, C. (2006) Otoferlin, defective in a human deafness form, is essential for exocytosis at the auditory ribbon synapse. *Cell 127*, 277−289.

(6) Pangrsic, T., Lasarow, L., Reuter, K., Takago, H., Schwander, M., Riedel, D., Frank, T., Tarantino, L. M., Bailey, J. S., Strenzke, N., Brose, N., Müller, U., Reisinger, E., and Moser, T. (2010) Hearing requires otoferlin-dependent efficient replenishment of synaptic vesicles in hair cells. *Nat. Neurosci. 13*, 869−876.

(7) Bansal, D., Miyake, K., Vogel, S. S., Groh, S., Chen, C. C., Williamson, R., McNeil, P. L., and Campbell, K. P. (2003) Defective membrane repair in dysferlin-deficient muscular dystrophy. *Nature 423*, 168−172.

(8) Kobayashi, K., Izawa, T., Kuwamura, M., and Yamate, J. (2012) Dysferlin and animal models for dysferlinopathy. *J. Toxicol. Pathol. 25*, 135−147.

(9) Doherty, K. R., Cave, A., Davis, D. B., Delmonte, A. J., Posey, A., Earley, J. U., Hadhazy, M., and McNally, E. M. (2005) Normal myoblast fusion requires myoferlin. *Development 132*, 5565−5575.
(10) Eisenberg, M. C., Kim, Y., Li, R., Ackerman, W. E., Kniss, D. A., and Friedman, A. (2011) Mechanistic modeling of the effects of myoferlin on tumor cell invasion. *Proc. Natl. Acad. Sci. U.S.A. 108*, 20078−20083.
(11) Nalefski, E. A., and Falke, J. J. (1996) The C2 domain calcium-binding motif: Structural and functional diversity. *Protein Sci. 5*, 2375−2390.
(12) Nalefski, E. A., Wisner, M. A., Chen, J. Z., Sprang, S. R., Fukuda, M., Mikoshiba, K., and Falke, J. J. (2001) C2 domains from different Ca^{2+} signaling pathways display functional and mechanistic diversity. *Biochemistry 40*, 3089−3100.
(13) Hui, E., Johnson, C. P., Yao, J., Dunning, F. M., and Chapman, E. R. (2009) Synaptotagmin-mediated bending of the target membrane is a critical step in Ca^{2+}-regulated fusion. *Cell 138*, 709−721.
(14) Lai, A. L., Tamm, L. K., Ellena, J. F., and Cafiso, D. S. (2011) Synaptotagmin 1 modulates lipid acyl chain order in lipid bilayers by demixing phosphatidylserine. *J. Biol. Chem. 286*, 25291−25300.
(15) Johnson, C. P., and Chapman, E. R. (2010) Otoferlin is a calcium sensor that directly regulates SNARE-mediated membrane fusion. *J. Cell Biol. 191*, 187−197.
(16) Therrien, C., Di Fulvio, S., Pickles, S., and Sinnreich, M. (2009) Characterization of lipid binding specificities of dysferlin C2 domains reveals novel interactions with phosphoinositides. *Biochemistry 48*, 2377−2384.
(17) Davis, D. B., Doherty, K. R., Delmonte, A. J., and McNally, E. M. (2002) Calcium-sensitive phospholipid binding properties of normal and mutant ferlin C2 domains. *J. Biol. Chem. 277*, 22883−22888.
(18) Bagatolli, L. A., Gratton, E., and Fidelio, G. D. (1998) Water dynamics in glycosphingolipid aggregates studied by LAURDAN fluorescence. *Biophys. J. 75*, 331−341.
(19) Bagatolli, L. A., Sanchez, S. A., Hazlett, T., and Gratton, E. (2003) Giant vesicles, Laurdan, and two-photon fluorescence microscopy: Evidence of lipid lateral separation in bilayers. *Methods Enzymol. 360*, 481−500.
(20) Jiménez, J. L., and Bashir, R. (2007) 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. 260*, 114−123.
(21) Baumgart, T., Hammond, A. T., Sengupta, P., Hess, S. T., Holowka, D. A., Baird, B. A., and Webb, W. W. (2007) Large-scale fluid/fluid phase separation of proteins and lipids in giant plasma membrane vesicles. *Proc. Natl. Acad. Sci. U.S.A. 104*, 3165−3170.
(22) Ramakrishnan, N. A., Drescher, M. J., and Drescher, D. G. (2009) Direct interaction of otoferlin with syntaxin 1A, SNAP-25, and the L-type voltage-gated calcium channel Cav1.3. *J. Biol. Chem. 284*, 1364−1372.
(23) Pangršič, T., Reisinger, E., and Moser, T. (2012) Otoferlin: A multi-C2 domain protein essential for hearing. *Trends Neurosci. 35*, 671−680.
(24) Dinic, J., Biverståhl, H., Mäler, L., and Parmryd, I. (2011) Laurdan and di-4-ANEPPDHQ do not respond to membrane-inserted peptides and are good probes for lipid packing. *Biochim. Biophys. Acta 1808*, 298−306.
(25) Harris, F. M., Best, K. B., and Bell, J. D. (2002) Use of laurdan fluorescence intensity and polarization to distinguish between changes in membrane fluidity and phospholipid order. *Biochim. Biophys. Acta 1565*, 123−128.
(26) Keller, H., Lorizate, M., and Schwille, P. (2009) PI(4,5)P2 degradation promotes the formation of cytoskeleton-free model membrane systems. *ChemPhysChem 10*, 2805−2812.
(27) Brandt, D. S., Coffman, M. D., Falke, J. J., and Knight, J. D. (2012) Hydrophobic contributions to the membrane docking of synaptotagmin 7 C2A domain: Mechanistic contrast between isoforms 1 and 7. *Biochemistry 51*, 7654−7664.
(28) Helfmann, S., Neumann, P., Tittmann, K., Moser, T., Ficner, R., and Reisinger, E. (2011) The crystal structure of the C_2A domain of otoferlin reveals an unconventional top loop region. *J. Mol. Biol. 406*, 479−490.
(29) Chapman, E. R. (2008) How does synaptotagmin trigger neurotransmitter release? *Annu. Rev. Biochem. 77*, 615−641.
(30) Hui, E., Gaffaney, J. D., Wang, Z., Johnson, C. P., Evans, C. S., and Chapman, E. R. (2011) Mechanism and function of synaptotagmin-mediated membrane apposition. *Nat. Struct. Mol. Biol. 18*, 813−821.
(31) Murray, D., and Honig, B. (2002) Electrostatic control of the membrane targeting of C2 domains. *Mol. Cell 9*, 145−154.