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Characterization of the Lipid Binding Properties of Otoferlin Reveals Specific Interactions between PI(4,5)P2 and the C2C and C2F Domains

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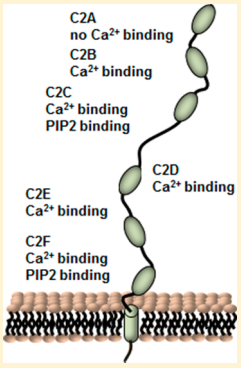
Supporting Information

ABSTRACT: Otoferlin is a transmembrane protein consisting of six C2 domains, proposed to act as a calcium sensor for exocytosis. Although otoferlin is believed to bind calcium and lipids, the lipid specificity and identity of the calcium binding domains are controversial. Further, it is currently unclear whether the calcium binding affinity of otoferlin quantitatively matches the maximal intracellular presynaptic calcium concentrations of ∼30−50 μM known to elicit exocytosis. To characterize the calcium and lipid binding properties of otoferlin, we used isothermal titration calorimetry (ITC), liposome sedimentation assays, and fluorescence spectroscopy. Analysis of ITC data indicates that with the exception of the C2A domain, the C2 domains of otoferlin bind multiple calcium ions with moderate (K_d = 25−95 μM) and low affinities (K_d = 400−700 μM) in solution. However, in the presence of liposomes, the calcium sensitivity of the domains increased by up to 10-fold. It was also determined that calcium enhanced liposome binding for domains C2B−C2E, whereas the C2F domain bound liposomes in a calcium-independent manner. Mutations that abrogate calcium binding in C2F do not disrupt liposome binding, supporting the conclusion that the interaction of the C2F domain with phosphatidylserine is calcium-independent. Further, domains C2C and C2F, not domains C2A, C2B, C2D, and C2E, bound phosphatidylinositol 4,5-bisphosphate 1,2-dioleoyl-*sn*-glycero-3-phospho(1′-myoinositol-4′,5′-bisphosphate) [PI(4,5)P2], which preferentially steered them toward liposomes harboring PI(4,5)P2. Remarkably, lysine mutations L478A and L480A in C2C selectively weaken the PI(4,5)P2 interaction while leaving phosphatidylserine binding unaffected. Finally, shifts in the emission spectra of an environmentally sensitive fluorescent unnatural amino acid indicate that the calcium binding loops of the C2F domain directly interact with the lipid bilayer of negatively charged liposomes in a calcium-independent manner. On the basis of these results, we propose that the C2F and C2C domains of otoferlin preferentially bind PI(4,5)P2 and that PI(4,5)P2 may serve to target otoferlin to the presynapse in a calcium-independent manner. This positioning would facilitate fast calcium-dependent exocytosis at the hair cell synapse.



The sense of hearing depends on reliable and temporally precise neurotransmitter release at the synapses of cochlear inner hair cells (IHCs).[1] IHCs contain sensory hair bundle structures composed of microvilli (stereocilia) arranged in rows of graded height that protrude from the apical tip of the cell. Minute displacements of the hair bundle open mechanically gated ion channels that depolarize the cell.[2] This change in membrane potential triggers an influx of calcium into the cell that in turn results in the fusion of synaptic vesicles with the plasma membrane and the release of the neurotransmitter.[2,3] Calcium-regulated exocytosis and neurotransmitter release exhibit fast kinetics in achieving exquisite temporal fidelity.[4] To aid in fidelity, IHCs contain specialized structures for tethering synaptic vesicles at release sites called synaptic ribbons, allowing for high rates of sustained synaptic transmission.[5]

The calcium-triggered fusion of vesicles with the plasma membrane is believed to be driven by the assembly of SNARE proteins.[6] However, SNARE proteins are insensitive to calcium.[7] In neurons, the protein synaptotagmin I confers calcium sensitivity to SNARE-mediated fast synchronous neurotransmission.[8,9] However, Yasunaga et al. have reported that synaptotagmin I was not detected in mature IHCs,[10] and it has been suggested that IHCs have evolved a unique calcium sensor, otoferlin, for calcium-regulated synaptic transmission.[11] However, the relationship between otoferlin and SNAREs and whether neural SNAREs are required for neurotransmitter release from hair cells have yet to be fully elucidated.[12]

Roux et al. have reported that mice lacking otoferlin were profoundly deaf and lack synaptic vesicle exocytosis in IHCs.[11] Otoferlin is also required for calcium-dependent synaptic exocytosis at immature outer hair cells (OHCs) and type I vestibular hair cells.[13,14] In addition to exocytosis, otoferlin is known to interact with the endocytotic proteins AP-2 and

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myosin-6, suggesting that otoferlin may also contribute to the replenishment of synaptic vesicles.[15−17]

Otoferlin belongs to the ferlin family of proteins and consists of six C2 domains (C2A−C2F) linked in tandem followed by a single-pass C-terminal transmembrane region.[18] C2 domains bind calcium and lipids and are found in proteins involved in membrane trafficking and signal transduction.[19−21] Johnson et al. have shown that with the exception of the C2A domain, the C2 domains of otoferlin interact with calcium and bind lipids.[22−24] In addition, otoferlin stimulates SNARE-mediated membrane fusion in a calcium-dependent manner *in vitro*, supporting the hypothesis that otoferlin acts as a synaptotagmin I-like calcium sensor for fusion.[21] However, a recent study found that synaptotagmin cannot rescue the otoferlin knockout phenotype, suggesting functional differences between the proteins.[25] In addition, Pangrsic et al. have reported that the C2F domain lacks calcium binding and did not bind to phosphatidylserine, raising questions about which domains bind calcium and the lipid binding specificity of the protein.[26] Thus, the biochemical properties of the C2 domains of otoferlin are still controversial. The lipid and calcium binding properties of synaptic proteins are critical characteristics that define and shape the release properties of a synapse, and thus, without a full quantitative characterization of these activities, an understanding of otoferlin's function in hair cells will remain elusive.

In this study, the intrinsic calcium binding properties of each C2 domain were measured using isothermal titration calorimetry (ITC). The lipid binding specificity and effects of lipids on calcium binding were also assessed using liposome sedimentation assays and laurdan fluorescence measurements. Lastly, the interaction of loop residues within the C2F domain with liposomes was tested through use of an environmentally sensitive unnatural amino acid.

EXPERIMENTAL PROCEDURES

Materials. POPS (1-palmitoyl-2-oleoyl-*sn*-glycero-3-phospho-L-serine), POPC (1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine), PI4P [1,2-dioleoyl-*sn*-glycero-3-phospho(1′-myoinositol-4′-phosphate) (ammonium salt)], and PI(4,5)P2 [phosphatidylinositol 4,5-bisphosphate 1,2-dioleoyl-*sn*-glycero-3-phospho(1′-myoinositol-4′,5′-bisphosphate) (ammonium salt)] were purchased from Avanti Polar Lipids, Inc. (Alabaster, AL). Ni^{2+}-NTA Sepharose high-performance beads were purchased from GE-Hamersham Biosciences (Pittsburgh, PA). Other common reagents were obtained from Sigma-Aldrich (St. Louis, MO).

Protein Constructs. C2 domain constructs of otoferlin were reported previously.[21,23] For the incorporation of the noncanonical amino acid acridon-2-ylalanine, mutagenesis was conducted on the pMCSG9 vector containing the otoferlin C2F domain at phenylalanine 1833 or 1746, converting the codon to a TAG site. Primers for the mutation were designed as mismatch primers to the nucleotide site of interest. Otoferlin C2C (K478A and K480A) and C2F (D1743/1831A and D1754/1837A) domains were constructed using the Stratagene QuikChange site-directed mutagenesis kit with the pMCSG9/6His-MBP-otoferlin C2C and C2F plasmid templates, respectively.

Expression and Purification of the His-MBP-C2 Domains. The pMCSG9 vectors containing the otoferlin C2 domains were transformed into BL21 *Escherichia coli* cells. The bacterial cultures (OD_{600} = 0.6) were induced for 3−4 h at 37 °C with 1 mM IPTG. The C2F F1746 and F1833 acridon-2-ylalanine noncanonical amino acid constructs were expressed in autoinduction medium with 1 mM acridon-2-ylalanine using a previously reported method.[27,28] The cells were lysed by sonication in lysis buffer containing protease inhibitors (0.5 mM PMSF, 1−2 μg/mL aprotinin, leupeptin, and pepstatin A). The lysis buffer contained 20 mM Tris-HCl (pH 7.5) and 150 mM NaCl. The soluble fraction of the lysate was incubated with Ni-NTA resin for 3 h at 4 °C, and the Ni-NTA resin was washed with lysis buffer containing Tris-HCl, 150 mM NaCl, and 20 mM imidazole before the bound protein was eluted with Tris-HCl buffer containing 500 mM imidazole. Purified proteins were extensively dialyzed in ITC buffer [20 mM Tris-HCl (pH 7.5) and 150 mM NaCl] and concentrated using an Ultrafree-10 centrifugal filter unit (Millipore Inc., Bedford, MA). The protein concentrations were determined by UV absorbance using extinction coefficients of each protein based on sequence. Figure 1 of the Supporting Information shows a representative sodium dodecyl sulfate−polyacrylamide gel electrophoresis (SDS−PAGE) gel illustrating the purity of the C2 domains of otoferlin.

Isothermal Titration Calorimetry. Isothermal titration calorimetry was conducted using a Nano ITC instrument (TA Instruments). The calcium binding experiments were conducted at 37 °C, and lipid binding was conducted at 30 °C. The proteins were dialyzed extensively in buffer containing 20 mM Tris-HCl (pH 7.5) and 150 mM NaCl. Stock calcium chloride solutions were prepared in the corresponding buffers of each protein and were loaded into a 50 μL syringe. This titrant was injected with a stirring speed of 250 rpm at discrete intervals of 180 s. Calcium was added in 1 μL injections 45 times for each experiment, and the heat evolved per injection was measured. Small unilamellar vesicles (SUVs) were used to determine the binding of lipids to the C2F domain of otoferlin in the absence or presence of 1 mM calcium chloride. The lipid suspension contained the same calcium concentration as the buffer. The concentration of the C2F domain of otoferlin ranged from 40 to 400 μM, and that of the lipid suspension varied from 5 to 10 mM. The lipid suspensions were added as 1 μL injections 45−47 times with a stirring speed of 250 rpm at discrete intervals of 180 s. The heat of dilution was determined by adding the titrant to the corresponding buffer in the absence of protein and was subtracted to obtain the effective heat of binding. All ITC data were analyzed using Nano ITC analysis software.

Phospholipid Vesicles. The preparation of SUVs was performed according to reported methods.[29] Briefly, chloroform solutions composed of 25% POPS and 75% POPC, 50% POPS and 50% POPC, 95% POPC and 5% PI(4,5)P2, 95% POPC and 5% PI(4)P, or 100% POPC were mixed and dried under a stream of liquid nitrogen gas and then dried under vacuum for 3 h. The dried lipids were resuspended in buffer and extruded 20 times through a 50 nm filter (Avanti Polar Lipids, Inc.) to produce small unilamellar vesicles (SUVs).

Sedimentation Assay. For the binding assay, the C2 domains of otoferlin (5 μg) were mixed with SUVs (100 μg) in buffer [20 mM Tris (pH 7.5) and 100 mM NaCl] with calcium (10, 100, and 1 mM) or EGTA (1 mM). The mixture was incubated for 1 h at 37 °C and centrifuged at 85000*g* for 45 min in a TA-100 ultracentrifuge (Beckmann Instruments). SDS−PAGE gel data presented for calcium titration experiments consist of total protein control (total input), supernatant (soluble fraction), and pellet (lipid-bound fraction).

Fluorescence Spectroscopy. Fluorescence spectra were recorded on a PTI QuantaMaster fluorometer with 5 nm

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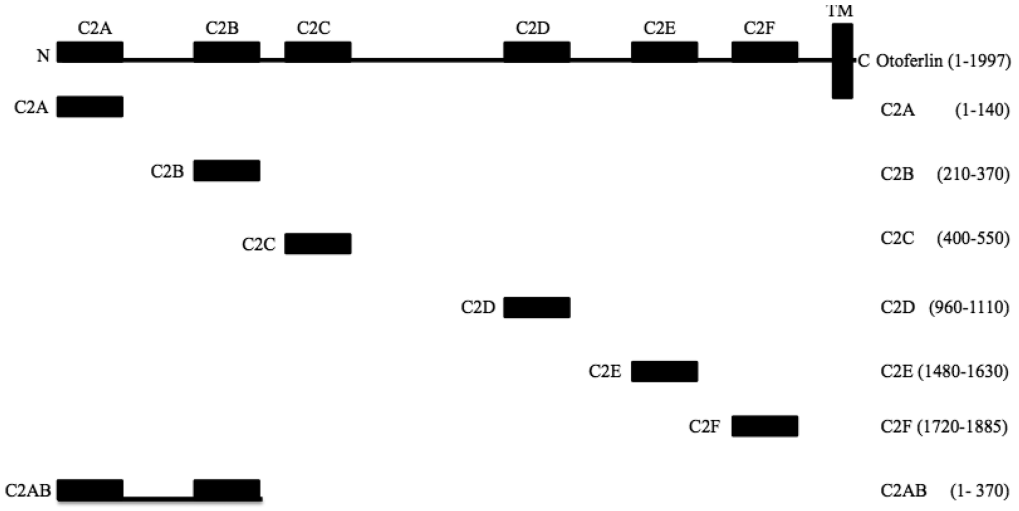

Figure 1. Schematic diagram of full-length otoferlin and the otoferlin constructs used in this study. Otoferlin is composed of six C2 domains and a transmembrane segment (TM). Recombinant proteins composed of C2 domains were generated according to the amino acid designations listed at the right.

excitation and emission slit widths. Assays were conducted at 37 °C in a quartz micro cuvette. The fluorescence intensity of oto-C2F F1833Acd and F1746Acd (2 μM) was observed in the presence of liposomes composed of 100% POPC and 45% POPS, 50% POPC, and 5% PI(4,5)P2 in the presence of calcium or EDTA. Data were collected using FelixGX set at 1.0 nm intervals with an integration time of 0.1 s. Laurdan experiments were conducted as described previously.[23] Briefly, excitation at 350 nm was used, and the generalized polarization (GP) value was calculated using emission values at 430 and 480 nm. The reported values represent means ± the standard deviation (SD) for three samples.

RESULTS

Characterization of the Calcium Binding Properties of Otoferlin. We used ITC to determine the intrinsic calcium binding properties of the C2 domains of otoferlin in solution, adapting an approach previously described for the C2 domains of PKC and synaptotagmin I.[30,31] ITC measures the heat exchange associated with binding by titrating the ligand to the macromolecule. It also provides a complete set of thermodynamic parameters of ligand−macromolecule interaction, including the binding affinity and changes in enthalpy and entropy. We performed the titration of isolated domains of otoferlin with calcium chloride (an overview of the constructs tested is given in Figure 1).

Overall, five of the six domains of otoferlin bound calcium (Figure 2). The C2A domain of otoferlin did not bind calcium, in agreement with previous studies.[22,23] The measured heats of binding for domains C2B−C2F were fit using a two-site binding model that assumes that more than one ligand can bind independently. The best-fit K_d values ranged from 25 to 95 μM for the first site and from 400 to 750 μM for the second (Figure 3). Binding of calcium to the moderate-affinity site is exothermic, whereas binding of calcium to the low-affinity site is endothermic. The fitted values are listed in Table 1. Typically, C2 domains interact with calcium using aspartate residues located in the loop regions of the domains, and to explicitly test whether aspartate residues are required for otoferlin−calcium interaction, we tested two double aspartate mutants in C2F (D1743/1831A and D1754/1837A). Both mutants failed to bind calcium (Figure 2). We also conducted titrations with a tandem C2AB domain of otoferlin to probe for interdomain effects on calcium binding (Figures 2 and 3). The C2AB construct bound calcium with apparent K_d values of 50 and 475 μM (Table 1). This negligible deviation from the calcium affinity of the isolated C2B domain suggests that the C2A domain and linker between the domains have no effect on the calcium binding affinity of the C2B domain.

The C2 Domains of Otoferlin Bind Liposomes. We next sought to determine if the calcium binding affinities of the domains are modified in the presence of lipids. We therefore performed sedimentation assays on 75% PC/25% PS and 50% PC/50% PS liposomes mixed with C2 domains of otoferlin in the presence of calcium or EDTA.[32] The C2A domain of otoferlin bound liposomes poorly regardless of the presence of calcium, while the C2B domain bound liposomes in the absence of calcium, with ≥100 μM calcium enhancing C2B−liposome interaction (Figure 4A,B). By contrast, binding between liposomes and the C2C−C2E domains was sensitive to low concentrations of calcium (~5−10 μM) (Figure 4A,B and Figure of the Supporting Information). Although the C2F domain binds calcium, binding to PS/PC liposomes was calcium-independent (Figure 4A,B and Figure 2 of the Supporting Information). We found that although the C2F mutants D1743/1831A and D1754/1837A failed to bind calcium, the mutants did bind to PS/PC liposomes (Figure 4C). These results indicate that the calcium and lipid membrane binding activities of C2F are independent. We also conducted sedimentation assays with 50% PS and found that higher levels of PS did not enhance binding, suggesting that 25% PS is saturating (Figure 3 of the Supporting Information). None of the domains bound 100% PC liposomes under any conditions, indicating the requirement for anionic lipids (Figure 4D). As a negative control, PS/PC liposome sedimentation assays were also conducted with the maltose binding protein, which does not bind liposomes (Figure 4E).

We next tested each C2 domain for PI(4,5)P2 binding by conducting sedimentation assays on liposomes composed of 95% PC and 5% PI(4,5)P2. Only the C2C and C2F domains of otoferlin were found to bind PI(4,5)P2, and binding was calcium-independent (Figure 5A,B). Many C2 domains,

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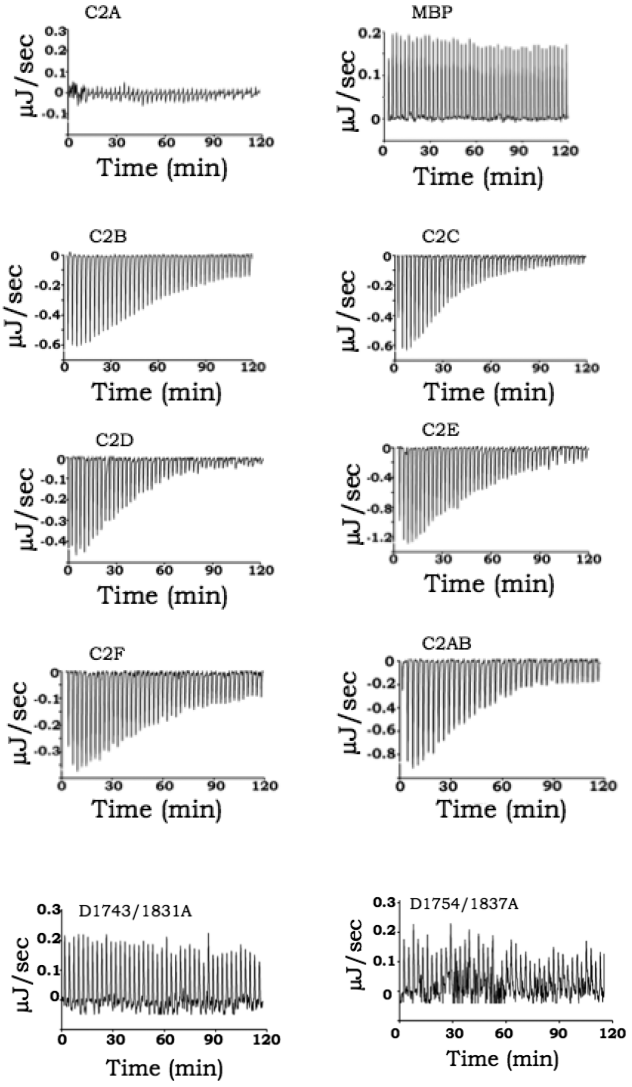

Figure 2. Representative thermograms for the interaction of $CaCl_2$ with the C2 domains of otoferlin. Titrations were performed at 37 °C in 20 mM Tris buffer (pH 7.5) and 150 mM NaCl. The following concentrations of C2 domains were used: 350 μM C2A, 420 μM C2B, 400 μM C2C, 390 μM C2D, 450 μM C2E, 390 μM C2F, 380 μM C2AB, 320 μM C2F D1743/1831A, and 340 μM C2F D1754/1837A. The syringe contained 20−30 mM $CaCl_2$.

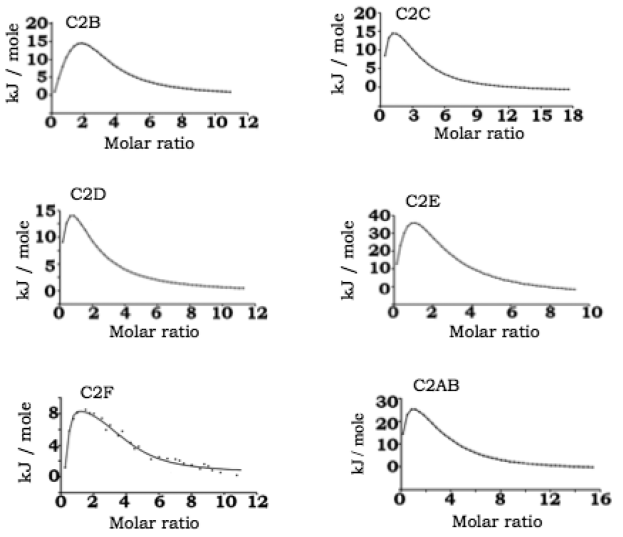

Figure 3. ITC data for the interaction of $CaCl_2$ with the C2 domains of otoferlin. The panels show the integrated heat as a function of the Ca^{2+}/protein ratio after subtraction of the heat of dilution. The solid line in the panel corresponds to the best fit to a two-site model. The values for the fitted parameters are listed in Table 1.

including the C2B domain of synaptotagmin, interact with PI(4,5)P2 in a calcium-independent manner through a set of conserved basic lysine residues. These lysine residues (K478 and K480) appear to be conserved in the C2C domain of otoferlin. To investigate whether the lysine residues contribute to PI(4,5)P2 binding, we generated K478A, K480A, and double (K478/480A) mutants. When tested, PI(4,5)P2 binding for all mutants was attenuated relative to that of the wild type (Figure 5C,D). However, lysine mutants retained the ability to bind PS/PC liposomes in a calcium-dependent manner (Figure 5E). Thus, the calcium-independent PI(4,5)P2 binding activity of C2C is distinct from the calcium-dependent PS binding activity.

Previous studies have utilized the solvatochromic fluorescent membrane probe laurdan to measure the lipid binding properties of the C2 domains of otoferlin.[23] These studies determined that binding of otoferlin to laurdan harboring liposomes results in a blue shift in the emission maxima and an increase in the general polarization (GP) value of the probe, and we next sought to use the changes in GP values to determine whether PI(4,5)P2 can steer otoferlin to a preferred liposome by conducting competition assays (Figure 6). First, we measured the change in the laurdan GP value when each C2 domain of otoferlin (5 μM) was mixed with liposomes composed of 69% PC, 25% PS, 5% PI(4,5)P2, and 1% laurdan in the presence or absence of calcium (Figure 6, black bars). Next we repeated the measurements with samples containing a mixture of 69% PC, 25% PS, 5% PI(4,5)P2, and 1% laurdan and 75% PC/25% PS liposomes lacking PI(4,5)P2 and laurdan (Figure 6, white bars). For domains C2C and C2F, the change in the GP values for the mixed liposome sample was equivalent to that of samples containing only PI(4,5)P2 liposomes. This suggests that C2C and C2F domains preferentially bound to PI(4,5)P2 liposomes and did not bind PC/PS liposomes appreciably. By contrast, for domains C2B, C2D, and C2E, the change in GP in the mixed liposome sample was smaller than those of samples containing only PI(4,5)P2 liposomes, suggesting that these domains were distributed between both sets of liposomes, resulting in a smaller change in GP. Thus, the C2C and C2F domains are selectively steered to PI(4,5)P2-containing lipid bilayers.

Characterization of the Lipid Binding Properties of the Otoferlin C2F Domain. Unlike the other C2 domains of otoferlin, including C2B, the liposome binding activity of C2F appears to be completely calcium independent. However, ITC measurements indicate that the domain does bind calcium. We therefore conducted a quantitative analysis of the lipid binding activity of this domain in the presence and absence of calcium to more fully characterize any effects calcium may have on lipid binding.

ITC titrations of the C2F domain of otoferlin with PS/PC liposomes in the absence and presence of calcium are shown in Figure 7. After accounting for the fact that the C2F domain binds only anionic lipids on the outer leaflet of liposomes, we fit the data to a one-site binding model. In the absence of calcium, the C2F domain of otoferlin bound membranes with a K_d value of 92.1 ± 13.2 μM. In agreement with the sedimentation

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Table 1. Thermodynamic Parameters of Binding of Calcium to Different Otoferlin Constructs Measured by ITC

construct	K_D (μM)	ΔH (kJ/mol)	ΔS (J mol^{-1} K^{-1})	no. of bound ligand molecules (n)
C2B	K_1 = 95.4 ± 6.4	ΔH_1 = −0.5 ± 0.1	ΔS_1 = 75.3 ± 0.6	n_1 = 0.9 ± 0.1
	K_2 = 485.1 ± 12.5	ΔH_2 = 2.9 ± 0.0	ΔS_2 = 72.8 ± 0.3	n_2 = 2.0 ± 0.1
C2C	K_1 = 26.1 ± 3.0	ΔH_1 = −0.6 ± 0.1	ΔS_1 = 87.9 ± 1.0	n_1 = 1.2 ± 0.1
	K_2 = 379.4 ± 3.7	ΔH_2 = 1.9 ± 0.1	ΔS_2 = 71.6 ± 0.2	n_2 = 3.0 ± 0.0
C2D	K_1 = 51.6 ± 2.5	ΔH_1 = −0.3 ± 0.1	ΔS_1 = 78.6 ± 4.6	n_1 = 0.9 ± 0.0
	K_2 = 591.6 ± 19.4	ΔH_2 = 1.9 ± 0.1	ΔS_2 = 67.8 ± 0.9	n_2 = 1.2 ± 0.2
C2E	K_1 = 34.3 ± 1.4	ΔH_1 = −0.14 ± 0.1	ΔS_1 = 86.0 ± 0.1	n_1 = 1.1 ± 0.1
	K_2 = 771.0 ± 76.7	ΔH_2 = 1.3 ± 0.1	ΔS_2 = 63.9 ± 0.7	n_2 = 2 ± 0.0
C2F	K_2 = 25.3 ± 7.2	ΔH_2 = −0.2 ± 0.0	ΔS_1 = 88.9 ± 2.4	n_1 = 0.9 ± 0.1
	K_1 = 568.3 ± 25.5	ΔH_1 = 2.3 ± 0.0	ΔS_2 = 69.3 ± 0.3	n_2 = 1.0 ± 0.0
C2AB	K_1 = 50.4 ± 4.6	ΔH_1 = −0.3 ± 00	ΔS_1 = 92.2 ± 2.2	n_1 = 1.1 ± 0.1
	K_2 = 475.9 ± 12.6	ΔH_2 = 2.8 ± 0.0	ΔS_2 = 72.1 ± 0.3	n_2 = 2.1 ± 0.1

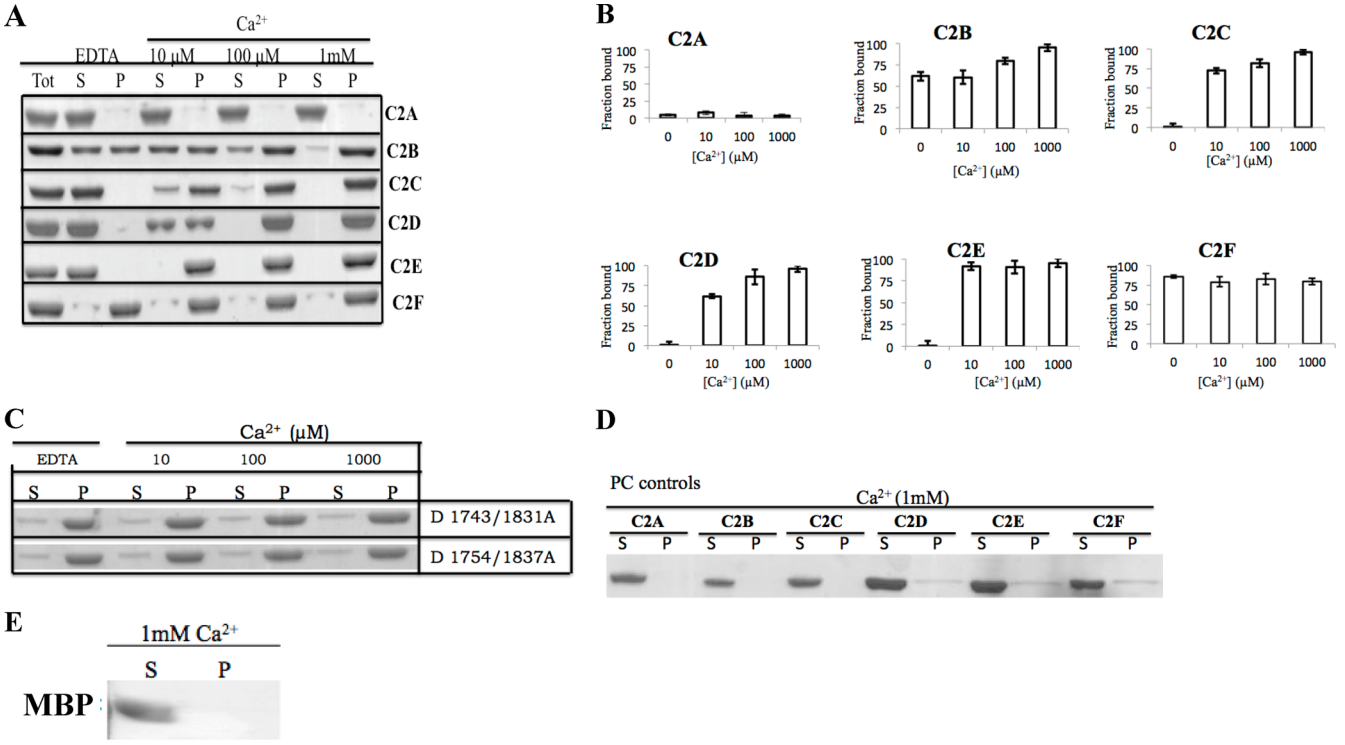

Figure 4. Association of C2 domains of otoferlin with phospholipid membranes in the presence of increasing free calcium concentrations. (A) Interaction of the C2 domains of otoferlin with liposomes composed of 25% POPS and 75% POPC (B) Quantitation of the results of the liposome binding assay from panel A. (C) Interaction of calcium binding mutants of the C2F domain (D1743/1831A and D1754/1837A) of otoferlin with liposomes composed of 25% POPS and 75% POPC (D) Sedimentation assays conducted with 100% POPC liposomes and the C2 domains of otoferlin. (E) Sedimentation assays conducted with MBP and liposomes composed of 25% POPS and 75% POPC (±standard deviation; N = 3). S denotes the supernatant and P the pellet.

results, calcium did not alter the binding affinity, with a fitted K_d value of 81.0 ± 12.1 μM in the presence of calcium (Table 2). Positive ΔH and ΔS values were associated with binding.[33]

To quantify the effect of PI(4,5)P2 on the membrane binding activity the C2F domain, we titrated PC/PS/PI(4,5)P2 phospholipid membranes (Figure 8). The best fit of the data to a one-site binding model indicates that the C2F domain binds with a K_d value of 6.3 ± 2.7 μM. Thus, the binding affinity increased by 12-fold in the presence of PI(4,5)P2 compared to that for PS/PC membranes (Table 3). In agreement with the sedimentation assay results, we did not observe any effect of calcium on the binding of C2F to PC/PS/PI(4,5)P2 liposomes. Titrations with 100% POPC liposomes did not show appreciable binding (Figure 4 of the Supporting Information). As a negative control, titrations of 50% PS/50% PC liposomes with the maltose binding protein were also conducted (Figure 4 of the Supporting Information).

In addition to the presynaptic region, otoferlin also localizes to the Golgi and has been hypothesized to play a role in trans-Golgi trafficking.[34,35] The cytoplasmic leaflet of the Golgi membrane contains PI4P, and we therefore sought to test for the effects of calcium on C2F−PI4P binding by titrating phospholipid membranes composed of PC, PS, and PI4P in the absence and presence of calcium (Figure 8). In the absence of calcium, the C2F domain bound liposomes with a K_d value of 29.1 ± 4.39 μM. In the presence of 1 mM calcium, only modest changes in affinity were found (K_d value of 32.6 ± 0.15 μM). Thus, membrane binding is enhanced 3-fold compared to that of PS/PC membranes (Table 4).

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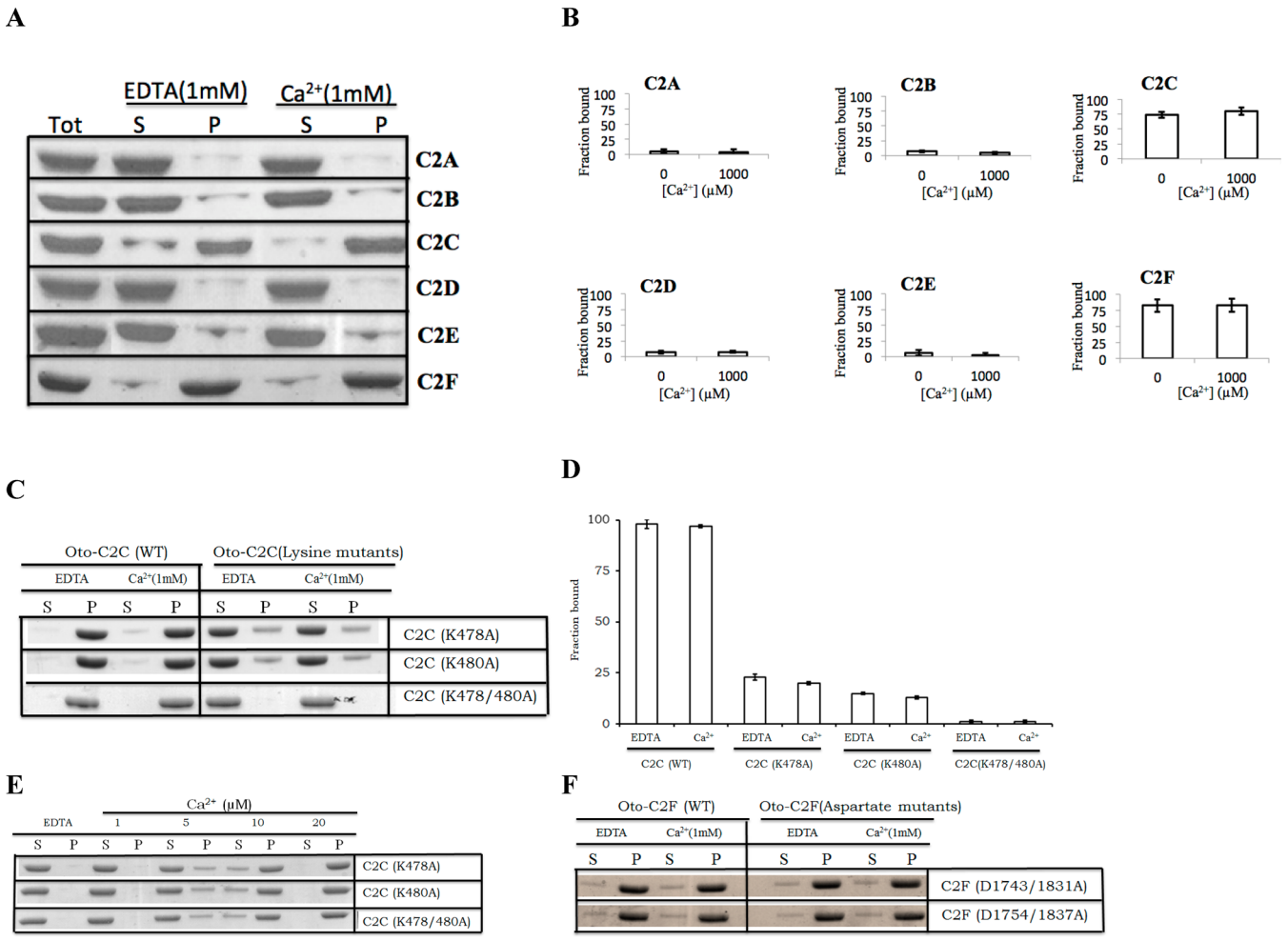

Figure 5. Association of C2 domains of otoferlin with PI(4,5)P2 in the presence or absence of calcium. (A) Interaction of the C2 domains of otoferlin with liposomes composed of 95% POPC and 5% PI(4,5)P2. (B) Quantification of results of the liposome binding assay from panel A (±standard deviation; $N = 3$). (C) Interaction of C2C WT, K478A, K480A, and K478/480A domains of otoferlin with liposomes composed of 95% POPC and 5% PI(4,5)P2 in the presence or absence of calcium. (D) Quantification of the results of the liposome binding assay from panel C (±standard deviation; $N = 3$). (E) Association of the K478A, K480A, and K478/480A mutants with liposomes composed of 25% POPS and 75% POPC. (F) Interaction of calcium binding mutants of the C2F domain (D1743/1831A and D1754/1837A) of otoferlin with liposomes composed of 95% POPC and 5% PI(4,5)P2 in the presence or absence of calcium (±standard deviation; $N = 3$). S denotes the supernatant and P the pellet.

The C2B domain of synaptotagmin I docks to PI(4,5)P2 in a calcium-independent manner, and elevations in intracellular calcium levels induce a reorientation of the C2 domains such that side chains found in the calcium binding loops of both C2 domains insert into the lipid bilayer. Specifically, IAEDANS fluorescence-based studies have identified loops 1 and 3 of both C2A and C2B as directly interacting with lipid bilayers.[36,37] If the C2F domain of otoferlin acts similarly, it would provide an explanation for the calcium binding activity of the domain. In support of this, a sequence alignment indicates that phenylalanine 1833 in otoferlin C2F is found at the equivalent position as the membrane penetrating phenylalanine 234 in synaptotagmin C2A. To determine if F1833 resides at the C2 domain–lipid binding interface, F1833 was replaced with acridon-2-ylalanine (Acd), a noncanonical amino acid with an environmentally sensitive fluorescence emission spectrum (Figure 9A).[28] When it was tested, the fluorescence emission profile for Acd-labeled C2F changed significantly when it was mixed with PS/PC/PI(4,5)P2 liposomes (Figure 9B). The observed fluorescence change was unaltered by calcium. No change in fluorescence was observed when the domain was mixed with 100% PC liposomes (Figure 9B). In addition, when F1746, which is located on the opposite side of the domain from F1833, was replaced with Acd, no change in the emission spectra was observed for liposomes regardless of lipid composition or the presence of calcium. These results suggest the lipid binding region of C2F is restricted to the putative calcium binding loops of the domain and that the interaction with anionic lipids in the bilayer is calcium-independent. Results of sedimentation assays conducted with Acd C2F domain were indistinguishable from those of the wild-type C2F domain, suggesting that the Acd did not appreciably alter the properties of the domain (Figure 9C).

DISCUSSION

Otoferlin is believed to be a calcium sensor required for exocytosis in inner hair cells, as well as neurotransmitter release in immature outer hair cells. Given the importance of otoferlin's calcium and lipid binding properties for neurotransmitter release, the goal of our work was to measure the intrinsic calcium binding affinity and calcium-mediated interaction of the

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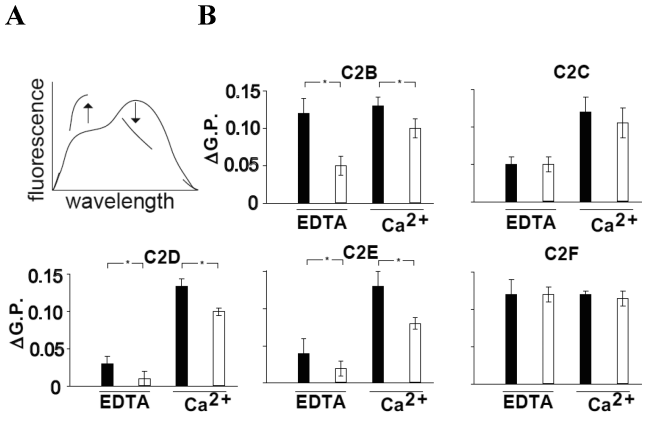

Figure 6. PI(4,5)P2 steers C2C and C2F domains. (A) Schematic illustrating the changes in laurdan emission spectra upon C2 domain binding. Arrows indicate the decrease in long wavelength emission and the rise in shorter wavelength intensity accompanying C2 domain binding. (B) Mean change in GP values (±standard deviation) in samples containing 5 μM C2 domain and either POPC/POPS/PI(4,5)P2/laurdan liposomes (black bars) or a mixture of POPC/POPS and POPC/POPS/PI(4,5)P2/laurdan liposomes (white bars) in either 100 μM EDTA or 500 μM calcium (N = 3; *P < 0.05).

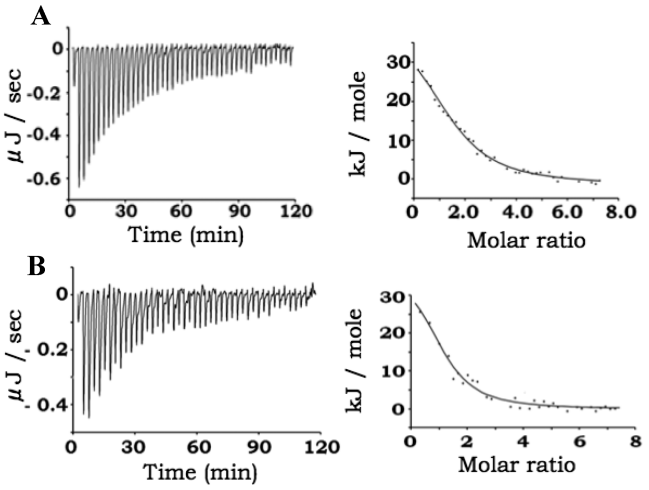

Figure 7. Representative thermograms for the interaction of C2F with 50% PC/50% PS liposomes in the presence or absence of calcium. (A) Representative thermograms and integrated heat changes after subtracting the heat of dilution for the titration of 360 μM C2F with 10 mM lipid vesicles in the presence of 1 mM $CaCl_2$. (B) Thermograms and integrated heats of binding after subtracting the heat of dilution for the titration of 360 μM C2F with 10 mM lipid vesicles in the absence of calcium. The binding isotherms were fit using a one-site model. The values of the fitted parameters are summarized in Table 2.

Table 2. Thermodynamic Parameters of POPC/POPS (50:50) SUVs Binding to the Otoferlin C2F Domain in the Presence and Absence of Ca^{2+}

Ca^{2+}	K_D (μM)	ΔH (kJ/mol)	ΔS (J mol^{-1} K^{-1})
without	K_1 = 92.1 ± 13.2	ΔH_1 = 1.8 ± 0.1	ΔS_1 = 75.9 ± 0.4
with	K_1 = 81.0 ± 12.1	ΔH_1 = 1.8 ± 0.1	ΔS_1 = 76.9 ± 0.4

C2 domains of otoferlin with anionic lipid membranes. Figure 10A summaries the results of our studies.

Otoferlin Binds Calcium with Moderate to Low Affinity in the Absence of Membranes. With the exception

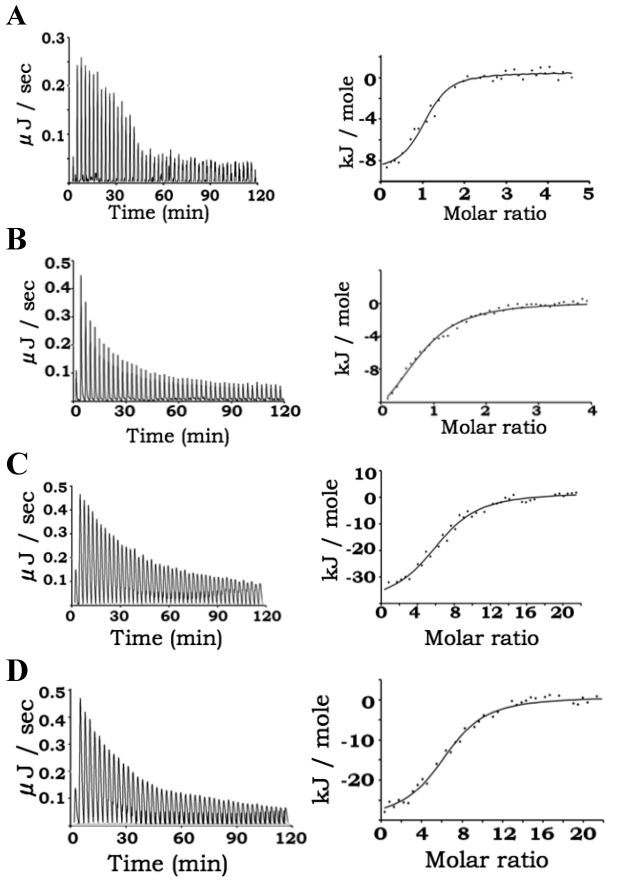

Figure 8. Representative thermogram of the interaction between C2F and liposomes composed of 45% POPC, 50% POPS, and 5% PI(4,5)P2 or 45% POPC, 50% POPS, and 5% PI4P. (A) Thermogram and integrated heat changes after subtracting the heat of dilution for the titration of 360 μM C2F with 10 mM POPC/POPS/PI(4,5)P2 liposomes in the presence of 1 mM $CaCl_2$. (B) Thermogram and the integrated heats of binding after subtracting the heat of dilution for the titration of 360 μM C2F with 10 mM POPC/POPS/PI(4,5)P2 liposomes in the absence of calcium. (C) Thermogram and integrated heat changes after subtracting the heat of dilution for the titration of 80 μM C2F with 10 mM POPC/POPS/PI4P liposomes in the presence of 1 mM $CaCl_2$. (D) Thermogram and the integrated heats of binding after subtracting the heat of dilution for the titration of 80 μM C2F with 10 mM POPC/POPS/PI4P liposomes in the absence of calcium. The values of the fitted parameters are summarized in Tables 3 and 4.

Table 3. Thermodynamic Parameters of POPC/POPS/PIP2 (45:50:5) SUVs Binding to the Otoferlin C2F Domain in the Presence and Absence of Ca^{2+}

Ca^{2+}	K_D (μM)	ΔH (kJ/mol)	ΔS (J mol^{-1} K^{-1})
without	K_1 = 8.7 ± 1.3	ΔH_1 = −0.4 ± 0.0	ΔS_1 = 76.2 ± 5.4
with	K_1 = 6.3 ± 2.7	ΔH_1 = −0.5 ± 0.0	ΔS_1 = 86.2 ± 1.4

Table 4. Thermodynamic Parameters of POPC/POPS/PI4P (45:50:5) SUVs Binding to the Otoferlin C2F Domain in the Presence and Absence of Ca^{2+}

Ca^{2+}	K_D	ΔH (kJ/mol)	ΔS (J mol^{-1} K^{-1})
without	K_1 = 29.1 ± 4.4	ΔH_1 = −3.7 ± 0.1	ΔS_1 = 65.1 ± 0.3
with	K_1 = 32.6 ± 0.2	ΔH_1 = −2.8 ± 0.0	ΔS_1 = 71.2 ± 0.0

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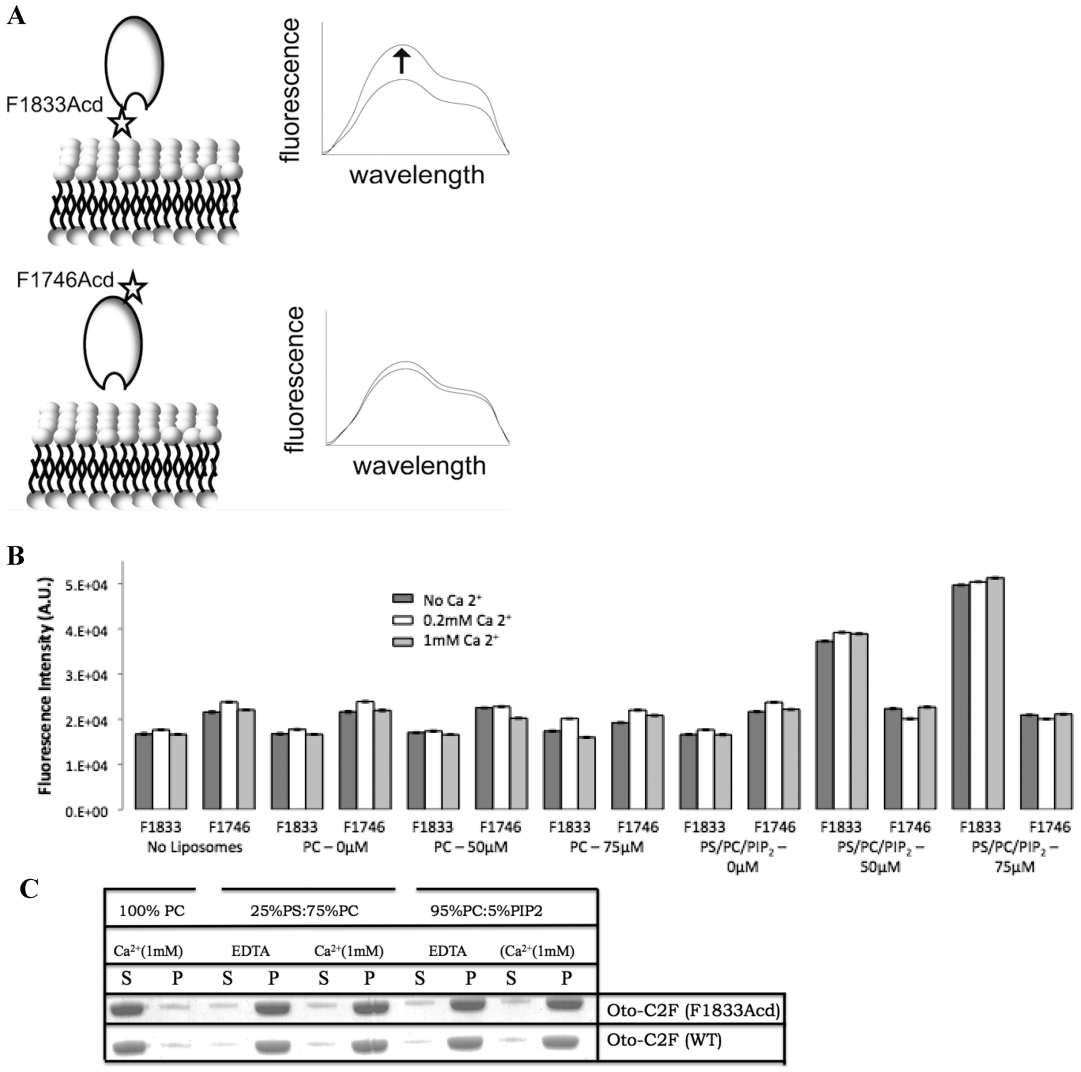

Figure 9. Flurescence intensity of C2F-acridon-2-ylalanine (Acd) in the presence of varying liposome and calcium concentrations. (A) Schematic illustrating the two acridone-2-ylalanine-labeled C2F domains and their interaction with liposomes. (B) C2F-Acd fluorescence intensity measured in the presence or absence of calcium and liposomes. The fluorescence intensity of C2F F1833Acd and F1746Acd did not increase in the presence of 100% POPC liposomes. A marked increase in fluorescence was observed for F1833Acd but not F1746Acd in the presence of 45% POPS/50% POPC/5% PI(4,5)P2 lipsomes. The addition of calcium did not affect the fluorescence for any of the samples. Error bars represent the standard deviation (N = 3). (C) Interaction of the F1833Acd and wild-type C2F with liposomes composed of 100% POPC, 25% POPS and 75% POPC, or 95% POPC and 5% PI(4,5)P2. S denotes the supernatant and P the pellet.

of C2A, the C2 domains of otoferlin bound calcium ions with moderate (K_d = 25−95 μM) and low affinity constants (K_d = 400−700 μM) in solution. The moderate-affinity site is in agreement with the work of Johnson et al., who reported that the apparent dissociation constant for calcium binding ranged from 13 to 25 μM.[22] Their results may represent calcium binding to the first site but not the second site. Mutations to the conserved aspartate residues of the C2F domain (D1743/1831A and D1754/1837A) of otoferlin abolished calcium binding. Measurements of tandem domains did not reveal any cooperative binding properties, suggesting that the domains bind calcium independently. Remarkably, despite variations in the composition of the putative calcium binding loops across the domains, all of otoferlin's C2 domains were determined to possess similar binding affinities. This contrasts with the otoferlin homologue dysferlin, which displays greater variation in calcium binding affinities, ranging from approximately 1 μM to 1 mM.[38] In comparison to those of synaptotagmin I, otoferlin's calcium binding affinities are equivalent or slightly higher, as the C2B domain of synaptotagmin binds calcium with solution K_d values in the range of 300−600 μM.[39,40]

Influence of Acidic Lipids on the Calcium Binding Properties of the C2 Domains of Otoferlin. Otoferlin bound to multiple calcium ions with apparent affinities of 20−50 and 400−700 μM in solution. These values are low relative to the calcium concentrations believed to elicit release at hair cell synapses and thus difficult to reconcile with a role for otoferlin as the calcium sensor for neurotransmitter release. However, in the presence of PS, calcium concentrations of 10 μM resulted in significant C2−liposome interaction for C2C−C2E domains, suggesting that the presence of acidic lipids enhances the calcium binding affinity for some of the domains to physiologically relevant values. This effect was not detected for C2F and was less pronounced for C2B. Thus, otoferlin possesses domains that appear to operate using an "electrostatic switch" mechanism, as well as domains that bind regardless of

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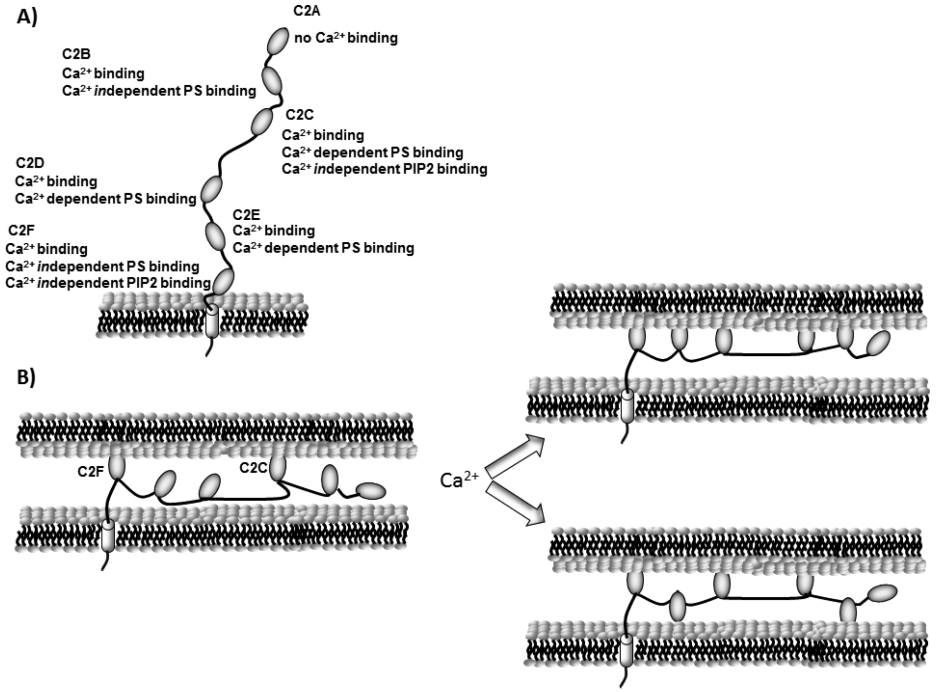

Figure 10. Summary and possible mechanisms of action. (A) Diagram of otoferlin with calcium phosphatidylserine (PS) and PI(4,5)P2 binding properties denoted for each domain. (B) Possible models of membrane binding. In the absence of calcium, synaptic vesicle-bound otofelrin contacts the presynaptic membrane through the interaction of PI(4,5)P2 with the C2C and C2F domains. Increased intracellular calcium concentrations would drive additional C2 domain–lipid interaction with either the synaptic vesicle membrane or presynaptic membrane.

calcium. Although calcium-independent membrane binding has been reported for the C2 domains of dysferlin,[38] neither C2 domain of synaptotagmin I binds appreciably to PS-containing liposomes in the absence of calcium, suggesting a difference in the mechanisms by which synaptotagmin I and otoferlin bind membranes.

PI(4,5)P2 Binding Properties of the C2 Domains of Otoferlin. PI(4,5)P2 is a major signaling molecule at the presynapse, and thus, we sought to determine if the C2 domains of otoferlin have a preference for phosphoinositide lipids. Our studies indicate that only the C2C and C2F domains of otoferlin bind PI(4,5)P2 and that binding is calcium-independent. This result differs from that of a recent study of the C2F domain that reported the calcium sensitivity of the C2F–PI(4,5)P2 interaction.[41] However, this study used free PI(4,5)P2 lipids instead of mixed composition liposomes, and thus, a direct comparison cannot be made. However, we found that aspartate mutant forms of C2F that do not bind calcium did bind PI(4,5)P2 liposomes, supporting the conclusion that PIP2 binding is calcium-independent. Many C2 domains contain a polybasic region with cationic and aromatic residues (YXK/Q Xn1 KXK) that is known to interact with the phosphate moieties of the inositol ring.[42,43] This polybasic region appears to be conserved (**YVQ** VFFAGQ **KGK**) in the C2C domain of otoferlin but does not appear to be conserved in C2F. Mutation of these lysines in C2C (K478A and K480A) weakened PI(4,5)P2 binding. However, the lysine mutants bound POPS/POPC liposomes like the wild-type C2C domain, suggesting that different residues mediate phosphatidylserine and phosphatidylinositol binding and that that these binding activities can be selectively abrogated. ITC measurements indicate that PI(4,5)P2 enhanced liposome binding for the C2F domain by 12-fold but by only 3-fold for PI4P relative to PS/PC liposomes, indicating a specificity for the bisphosphate. The C2B domain of synaptotagmin interacts with PI(4,5)P2 in a calcium-independent manner, and this interaction is believed to target the protein to the presynaptic membrane.[30,36] The C2C and C2F domains of otoferlin may act in a similar manner, targeting otoferlin to the presynaptic region of the cell and positioning it for calcium-induced neurotransmitter release (Figure 10B). Indeed, our laurdan measurements suggest C2C and C2F preferentially target PI(4,5)P2-containing liposomes.

Given that C2F–lipid membrane interaction is calcium-independent, the exact reason for the calcium binding activity of this domain is unclear. Studies of the domains of synaptotagmin have demonstrated a calcium-triggered reorientation of the C2 domains as well as penetration of hydrophobic side chains into the lipid bilayer, as demonstrated using the fluorescent probe AEDANS.[36,44] Our study using a fluorescent unnatural amino acid in one of the putative calcium binding loops indicates that the side chain interacts with liposomes in an anionic lipid-dependent manner. However, no evidence of calcium-induced changes was detected, suggesting that although the loop does interact with lipids, the mechanism may be different from that of the C2 domains of synaptotagmin. Rather than lipid interaction, calcium binding to C2F may facilitate other actions, including vesicle priming and fusion. In support of this, several studies have demonstrated calcium sensitive protein binding and membrane fusion activity *in vitro*.[22,45]

■ ASSOCIATED CONTENT

Supporting Information

Supplemental Figures 1–4. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

ABBREVIATIONS

POPS, 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phospho-L-serine; POPC, 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine; PI4P, 1,2-dioleoyl-*sn*-glycero-3-phospho(1′-myoinositol-4′-phosphate) (ammonium salt); PI(4,5)P2, phosphatidylinositol 4,5-bisphosphate 1,2-dioleoyl-*sn*-glycero-3-phospho(1′-myoinositol-4′,5′-bisphosphate) (ammonium salt); PS, phosphatidylserine; SUVs, small unilamellar vesicles; MBP, maltose binding protein; Acd, acridon-2-ylalanine.

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■ NOTE ADDED AFTER ASAP PUBLICATION

This paper was published ASAP on July 23, 2014, with a misspelling in the fourth author's name. The corrected version was reposted on July 24, 2014.