Calcium Sensitive Allostery Regulates the PI(4,5)P2 Binding Site of the Dysferlin C2A Domain

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C2 domains are the second-most abundant calcium binding module in the proteome. Activity of the muscular dystrophy associated protein dysferlin is dependent on the C2A domain at the N-terminus of the protein, which couples calcium and PI(4,5)P2 binding through an unknown mechanism. Using solution state nuclear magnetic resonance spectroscopy we confirm the phosphoinositide binding site for the domain and find that calcium binding attenuates millisecond to microsecond motions at both in the calcium binding loops and the concave face of the C2A, including a portion of the phosphoinositide binding site. Our results support a model whereby increasing calcium concentrations shift the phosphoinositide binding pocket of C2A into a binding-competent state, allowing for calcium dependent membrane targeting. This model contrasts with the canonical mechanism for C2 domain-phosphoinositide interaction and provides a basis for how pathogenic mutations in the C2A domain result in loss of function and disease.

Introduction

C2 domains are lipid-binding modules that can integrate calcium and lipid signals (1, 2). Although sequence similarity can be quite low between C2 domains, the tertiary structure of the 8-stranded β-sandwich core of the domain is typically conserved and unaffected by calcium (3, 4). By contrast, the structure of the calcium binding loops at the end of C2 domains are diverse, tune the domain’s sensitivity to calcium, and facilitate binding to anionic or zwitterionic lipid headgroups (5, 6). Separate from this calcium-lipid binding pocket, some C2 domains also contain a conserved swath of basic and accessory residues along the concave side of the domain, the polybasic cluster, binds phosphatidylinositol 4,5-bisphosphate (PI(4,5)P2) and other phosphoinositide species (7–10). A causal link between calcium and phosphoinositide binding has been noted for several of these C2 domains (11–14) but a universal structural one has yet to be defined.

The muscle protein dysferlin contains seven predicted C2 domains (Figure 1), but only the N-terminal C2A is reported to bind both PI(4,5)P2 (13) and calcium (6, 13). Cell-based assays have confirmed the importance of this domain for dysferlin function (15, 16), with either mutation or domain truncation resulting in functional deficiency and loss of membrane localization. For example, the muscular dystrophy-associated missense mutation V67D within the C2A domain results in loss of colocalization between dysferlin and PI(4,5)P2 (17). Dysferlin and PI(4,5)P2 interact in muscle in a number of cellular contexts (Figure 1). First, imaging techniques have unveiled a pool of dysferlin within the transverse tubule system (18), a structure highly enriched in PI(4,5)P2 (19). Dysferlin interacts closely with dihydropyridine receptors (20) at the triad junction and loss of dysferlin activity results in defects in Ca2+ homeostasis within muscle (21). Dysferlin has been shown to be required for the development and maintenance of the transverse tubule system, a process which also requires the presence of PI(4,5)P2. Strikingly when overexpressed dysferlin can induce the formation of extensive transverse-tubule-like structures in non-muscle cells in a PI(4,5)P2-dependent manner (17). Second, repair molecules, including PI(4,5)P2 and dysferlin, accumulate at muscle cell membrane tears, and that dysferlin confers calcium sensitivity to membrane fusion events that mediate repair (22). Failure to repair these mechanically-induced membrane tears are responsible for several human pathologies (23).

How the C2A domain coordinates calcium signals with PI(4,5)P2 binding is unknown, and a deeper understanding of the mechanisms that govern this process is essential to develop therapeutic strategies for dysferlinopathies. In
this study we apply solution NMR spectroscopy to directly monitor the dynamics of the C2A domain under the influence of these effectors, and map the binding face of the domain that interacts with the headgroup of PI(4,5)P2, inositol 1,4,5-triphosphate (IP3). We find that binding of calcium restricts motion within the apical end of the domain, especially the calcium binding loops, as well as residues involved in PI(4,5)P2 binding. In addition, IP3 binding further restricts calcium binding loop movement, suggesting that both PI(4,5)P2 and calcium cooperate to influence domain structure and membrane association. The restriction of apical end dynamics suggests that calcium organizes the top half of the phosphoinositide binding region prior to membrane interaction in a way that may be representative of other calcium-sensitive phosphoinositide binding C2 domains.

**Results**

**Resonance Assignments and Secondary Structure.** To structurally probe dysferlin C2A, we generated a recombinant form of the domain composed of residues 1-129 with a hexhistidine tag at the N-terminus. The $[^{1}H,^{15}N]$-TROSY spectra was found to be well dispersed, indicating that the domain is folded (Figure 2A). We assigned 108 of the 120 non-proline residues, and of the unassigned residues, 75% (9 out of 12) were in the loop regions between β-strands. Of the remaining three residues two were located next to the C-terminus and one is adjacent to calcium binding loop 3 (CBL3) (Figure 2B). As shown in Figure S1, secondary structure prediction based on Talos+ showed good agreement with the existing crystal structure of the dysferlin C2A domain (PDB: 4IHB) (24) and the recently reported solution structure (PDB: 7KRB) (25). This indicates that the structure of the protein in solution is similar to that observed in the crystal structure, under calcium saturating conditions.

**Calcium Reduces the Flexibility of the C2 Domain.** To first gain insight into the influence of calcium on the structure of C2A, we collected $[^{1}H,^{15}N]$-TROSY spectra of $^{15}N$ labeled dysferlin C2A samples at calcium concentrations ranging from 0 to 5 mM Ca$^{2+}$ (Figure 3A). Instead of fast exchange (µsec) as has been reported for many C2 domains (26–28), lowering the calcium concentration pushed residues in the calcium binding loops into the immediate exchange regime. We found that lowering the calcium concentration resulted in a significant decrease in peak intensity for all three CBLs, which disappeared between 4.5 and 3 mM Ca$^{2+}$. However, several peaks never dropped below 50% of their original value (Figure 3B). These observations suggest that these residues of dysferlin C2A may comprise a stable core region that accounts for approximately half of the protein opposite of the calcium binding loops. Interestingly, the stable core does not include all the β-strands. The ends of the strands located near the calcium binding loops show reduced intensity at lower calcium levels, indicating that these residues are also undergoing increased dynamics. Thus, the protein may be sampling an alternative state in the absence of calcium. To further investigate the dynamic behavior of dysferlin C2A we turned to nuclear spin-relaxation measurements as a function of calcium concentration. $^{15}N$ T$_1$, T$_2$ and heteronuclear NOE (HetNOE) were collected in the presence of 5, 3, and 1 mM CaCl$_2$. We analyzed the spin-relaxation data using modelfree formalism (29) and the results are summarized in Figure 4 and Tables S1-3. At 5 mM CaCl$_2$, the order parameters (S$^2$s) are generally between 0.8 and 1.0 in regions of secondary structure, with some deviations below 0.8 in loop regions and the termini. The S$^2$s decrease with decreasing calcium concentration across the protein, consistent with an overall reduction of rigidity in the structure. Samples with 1 mM calcium were found to have the lowest average S$^2$ values of 0.869 ± 0.127, while 3 and 5mM calcium concentrations resulted in higher average S$^2$ values of 0.897 ± 0.071 and 0.942 ± 0.061 respectively. We note that the number of residues that we could observe was reduced in the 3 and 1 mM samples as peaks began to disappear, especially in the CBL regions, indicating that these regions undergo increasing intermediate exchange as the calcium concentration decreases.

In addition to the order parameter, modelfree analysis also provides a measure of chemical exchange, R$_{ex}$, due to mo-
tion on the millisecond to microsecond timescale. We found that the number of residues displaying an $R_{\text{ex}}$ term and the magnitudes of the $R_{\text{ex}}$ terms increased with decreasing calcium concentration. $R_{\text{ex}}$ terms in the CBL regions, where measurable, increased in number and magnitude as calcium concentration decreased. We also found that several other residues showed an increase in the number and magnitude of $R_{\text{ex}}$ terms with decreasing calcium concentration. These residues mapped to the concave face of the $\beta$-sandwich fold. Overall, these results indicate that dysferlin C2A is flexible at “low” calcium concentrations and the flexibility is progressively reduced with increasing calcium (Figure 4).

**Calcium Shifts the Dynamic Equilibrium of C2A Between Two Structural States.** Given our observation of calcium dependent $R_{\text{ex}}$ terms that indicate chemical exchange processes on the microsecond to millisecond timescale, we performed chemical exchange saturation transfer (CEST) experiments to quantify these motions. $^{15}$N CEST experiments were performed at 1, 3, and 5 mM calcium (Figure 5 and Table 1). Chemical exchange was observed primarily in or near the CBLs at 5 mM calcium, consistent with the high order parameters and relatively few $R_{\text{ex}}$ terms identified in our modelfree analysis. Global modeling for these residues showed that the 99.8% of the protein is in the calcium bound state, with only 0.02% in the alternate state. At 3 mM calcium we found 13 residues that showed CEST profiles with chemical exchange. Each of these profiles was individually fit to a two-state model of exchange and to a global two-state exchange model as described in the methods and summarized in Table 1. All residues undergoing chemical exchange, as directly observed by CEST, were either in the CBL regions or the concave face of the $\beta$-sandwich fold. The global modeling shows that at 3 mM calcium, dysferlin C2A is 97.6% in the calcium bound state and 2.4% in the alternate state. At 1 mM calcium, many of the resonances exhibit reduced intensity, presumably due to chemical exchange. The reduced intensity of the resonances resulted in a reduction in the number of residues that we could characterize using CEST. In this case we could identify 6 residues that showed chemical exchange by CEST. Performing a similar modeling as used for the 3 mM calcium data, we determined the individual and global exchange parameters. Again, residues undergoing exchange were found to either be in the CBL regions or the concave face of the $\beta$-sandwich fold. As expected, the population of the calcium bound (87.5%) and alternate state (12.5%) shifted towards the alternate state. Our results show that there is a dynamic equilibrium between the calcium bound and alternate states even at relatively high calcium concentrations.

**The C2A Domain of Dysferlin Binds IP3 via its Polybasic Cluster.** The soluble PI(4,5)P2 headgroup analog inositol 1,4,5-trisphosphate (IP3), was titrated into a $^{15}$N labeled sample of dysferlin C2A at 5 mM Ca$^{2+}$. Chemical shift perturbations (CSPs) were measured over the course of the titration and final values were plotted against the residue number to map the IP3 binding site. The titration of the C2A domain with IP3 resulted in significant chemical shift perturbations, ranging from 0.255 to the $3\sigma$ cut-off of 0.0275 ppm, indicative of direct interaction between IP3 and C2A. Chemical shift perturbations (CSPs) were measured over the course of the titration and final values were plotted against the residue number to map the IP3 binding site. The titration of the C2A domain with IP3 resulted in significant chemical shift perturbations, ranging from 0.255 to the $3\sigma$ cut-off of 0.0275 ppm, indicative of direct interaction between IP3 and C2A. Chemical shift perturbations (CSPs) were measured over the course of the titration and final values were plotted against the residue number to map the IP3 binding site. The titration of the C2A domain with IP3 resulted in significant chemical shift perturbations, ranging from 0.255 to the $3\sigma$ cut-off of 0.0275 ppm, indicative of direct interaction between IP3 and C2A. Chemical shift perturbations (CSPs) were measured over the course of the titration and final values were plotted against the residue number to map the IP3 binding site. The titration of the C2A domain with IP3 resulted in significant chemical shift perturbations, ranging from 0.255 to the $3\sigma$ cut-off of 0.0275 ppm, indicative of direct interaction between IP3 and C2A. Chemical shift perturbations (CSPs) were measured over the course of the titration and final values were plotted against the residue number to map the IP3 binding site. The titration of the C2A domain with IP3 resulted in significant chemical shift perturbations, ranging from 0.255 to the $3\sigma$ cut-off of 0.0275 ppm, indicative of direct interaction between IP3 and C2A. Chemical shift perturbations (CSPs) were measured over the course of the titration and final values were plotted against the residue number to map the IP3 binding site. The titration of the C2A domain with IP3 resulted in significant chemical shift perturbations, ranging from 0.255 to the $3\sigma$ cut-off of 0.0275 ppm, indicative of direct interaction between IP3 and C2A. Chemical shift perturbations (CSPs) were measured over the course of the titration and final values were plotted against the residue number to map the IP3 binding site. The titration of the C2A domain with IP3 resulted in significant chemical shift perturbations, ranging from 0.255 to the $3\sigma$ cut-off of 0.0275 ppm, indicative of direct interaction between IP3 and C2A. Chemical shift perturbations (CSPs) were measured over the course of the titration and final values were plotted against the residue number to map the IP3 binding site. The titration of the C2A domain with IP3 resulted in significant chemical shift perturbations, ranging from 0.255 to the $3\sigma$ cut-off of 0.0275 ppm, indicative of direct interaction between IP3 and C2A. Chemical shift perturbations (CSPs) were measured over the course of the titration and final values were plotted against the residue number to map the IP3 binding site.
interaction surface between IP3 and C2A and is consistent with previous observations that PI(4,5)P2 binding to C2A is attenuated in the absence of calcium (13).

The Structure of CBL3 is Stabilized by Interaction of IP3. To look into the dynamics in the IP3-bound state we collected nuclear spin-relaxation data on an IP3-saturated sample and further analyzed the data using modelfree formalism. The results are summarized in Figure 4 and Table S4. Overall, we found that the $S^2$s of the IP3-saturated sample were modestly reduced. It is important to note that IP3 is known to directly interact with calcium, which could lower the free calcium concentration in the sample. Lowering the free calcium concentration would be expected to lower the $S^2$ across the protein based on our dynamics data at lower calcium concentrations. In contrast to the rest of the protein, the $S^2$ appears little changed or higher in CBL3 and surrounding regions in the IP3-saturated state when compared to the overall average $S^2$ in the IP3-saturated state. The higher $S^2$ indicates that the CBL3 is at least as rigid as the whole protein, when IP3 is bound. In contrast, in the IP3-unbound state, the $S^2$ for CBL3 appears lower than the average for the rest of the protein indicating that CBL3 is less rigid than the whole protein in the absence of IP3. In addition, $R_{ex}$ terms in CBL1 and 3 are attenuated in the IP3 saturated sample, indicating that chemical exchange at CBLs 1 and 3 is also reduced by IP3 binding, further supporting an increase in rigidity in the CBL’s upon IP3 binding. These data provide evidence that the structure of CBL3 is stabilized by interaction with IP3. Interestingly, the $S^2$s for CBL2 and CBL1 do not appear to indicate an increase in rigidity in the IP3 bound state, suggesting less change in the rigidity of CBL2 and CBL1 upon IP3 binding. We note that $R_{ex}$ terms in CBL1 are attenuated, indicating that slower, chemical exchange motions are attenuated in these regions upon IP3 binding. Overall, these data indicate that CBL3 is more directly involved in IP3 binding.
when compared to CBL2 and CBL1.

**Discussion**

The calcium and membrane binding properties of different C2 domains vary widely, with the properties of the domain tuned to the specific physiological role of the protein. The dysferlin C2A domain differs from the equivalent domain in other ferlins in that it binds both PI(4,5)P2 and calcium. Truncation or missense mutations of the dysferlin C2A domain result in loss of dysferlin activity and the emergence of pathology, highlighting the importance of the domain. Using solution state NMR spectroscopy, we provide a structural basis for the allosteric connection between calcium and PI(4,5)P2 binding activities of the C2A domain. First we sought to characterize the domain’s response to calcium. Overall, we found that C2A has a core that is insensitive to calcium. By contrast, increasing calcium restricted the dynamics of the calcium binding loop regions and neighboring parts of the β-sandwich fold. Motion of the loops on the mili- to microsecond timescale was found to be calcium sensitive, as evidenced by the loss of resonance intensity in and around the calcium binding loops with decreasing calcium concentration. Interestingly, the loss of intensity extends into

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**Fig. 5.** a) Residues exchanging at 3mM CaCl₂ mapped onto the structure (4IHB). b) Selected CEST traces showing increased exchange at lower calcium concentrations. Data (circles) and fits (lines) for 50 (dark), 25 (medium), and 10 (light) Hz saturation field strengths. Solid vertical lines indicate the $^{15}$N chemical shift for the major state, dotted vertical lines indicate the $^{15}$N chemical shift for the minor state, with error indicated by grey shading.
the β-strands proximal to the calcium binding loops, suggesting that the β-strands are also stabilized by calcium binding. This observation is further supported by the appearance of R_ex terms for residues in the β-strands at low calcium concentrations, indicating that residues in the β-strands are also undergoing chemical exchange. Calcium induced changes in modelfree R_ex values were also observed for CBL1, CBL2, and CBL3 supporting a role for calcium in affecting loop dynamics. Together, these changes indicate that coordination of calcium ions restricts the dynamics of both the apical loops and top regions of the β-strands. This change in the internal motion of the domain may favor specific structural conformations that are a prerequisite for the formation of a membrane-C2A complex, and thus, contribute to the calcium sensitivity of C2A-membrane binding.

PI(4,5)P2 binding by C2A is thought to be integral to the formation of the complex between C2A and the phospholipid membrane. Our results show that the headgroup IP3 interacts with the concave face of the β-sandwich fold and CBL3. This binding region agrees with the consensus site seen in type I C2 domains (8–11), though dysferlin C2A is of a type II fold. Tyr23 in β-strand 2, Lys32 and Arg34 in β-strand 3, and Asn78 on CBL3 are all conserved, the exception is a serine in place of a lysine at position 25. In addition, measurements of chemical exchange values revealed IP3 sensitivity within the CBL3, suggesting that a structural rearrangement of the loop may occur to accommodate IP3. Loop rearrangement would also distinguish the dysferlin C2A domain from rabphilin 3A C2A, which remains structurally unchanged when bound to PI(4,5)P2 (9).

Previous studies of C2A have shown that calcium binding enhances PI(4,5)P2 binding (13). Our results offer a molecular scale mechanism for this observation. Calcium binding to C2A stabilizes both the calcium binding loops and the β-strands near these binding loops, including Tyr23 and Asn78 which comprise the top region of the PI(4,5)P2 site. Our CEST results show that several key residues in this site undergo increasing chemical exchange as the calcium concentration decreases. These residues are clearly sampling a structurally different state, as indicated by the significant dif-

Fig. 6. Proposed model for dysferlin C2A action. a) Increasing calcium concentrations stabilize the calcium binding loops and adjacent regions of the domain, shifting the equilibrium to a conformation that favors PI(4,5)P2 binding b) Within the cell, calcium can trigger the C2A conformational change, which subsequently results in dysferlin anchoring to the membrane. Created with BioRender.com.
ferences in chemical shifts for the excited state. However, the observed chemical shifts for the excited state are not consistent with the expected chemical shifts for an unfolded state (Figure S1). Thus, we propose that calcium binding enhances PI(4,5)P2 binding by switching C2A from a more dynamic, PI(4,5)P2 low affinity state, to a more rigid, PI(4,5)P2 high affinity state, where the β-strands in the concave face of the β-sandwich are stabilized for PI(4,5)P2 binding. Previously it was postulated that in the case of PKCz-C2, calcium binding enhanced PI(4,5)P2 binding by facilitating access to the polybasic cluster (30). Our findings provide support for a different mechanism: the low calcium concentrations in unstimulated cells would allow the C2A domain to occupy a dynamic, low PI(4,5)P2 affinity state and thus would be predominantly unbound from membranes. Upon calcium influx, through microtears in the sarcomelema or through DHPR or ryanodine receptors in the transverse tubules, C2A is stabilized and converted from the low PI(4,5)P2 affinity state to the high PI(4,5)P2 affinity state, resulting in membrane binding and activation of dysferlin (Figure 6b).

Based on our measurements, we propose that the C2A docks with the membrane at an acute angle that includes contact between β-strands 2 and 3 on the domain and the PI(4,5)P2 headgroup in the membrane. A reorientation of CBL3 appears to help facilitate binding with the lipid headgroup, however this does not inhibit calcium ion coordination by the domain. The calcium binding region of many C2 domains stabilizes domain interaction with phosphatidyserine through a calcium bridging mechanism, and lipidosome binding assays support a similar bridging effect for dysferlin C2A (6). The inositol phosphate sensitive residues identified in our study are distinct from the region of the domain that interacts with phosphatidyserine, allowing for simultaneous membrane interactions in the presence of calcium. The observed reduction in dynamics of the CBL region of the domain upon calcium binding may serve to preorganize the loops for interaction with the phosphatidyserine lipid, and thus serve as a second method for conferring calcium sensitivity to membrane binding.

Dysferlin is a broadly conserved membrane trafficking protein, present from sea stars (31) to humans (32, 33). Mutations in dysferlin have been directly linked to muscular dystrophies and cardiomyopathy, and dysferlin-null mouse models display muscular dystrophy-like pathologies (34). The large size of the full-length protein prohibits its utility in gene delivery therapies (35), thus we must be judicious in our design of smaller constructs more appropriate for gene delivery. A truncated dysferlin composed of the only the two most C-terminal C2 domains can rescue the membrane resealing deficiency, but it is not sufficient to completely alleviate the dystrophic phenotype in mice (36). This indicates that there is perhaps an equally important role for the N-terminal dysferlin domains in muscle tissue. Here our work argues for the importance of the N-terminal C2A domain and provides a rationale for including the domain in future gene therapy constructs.

It remains to be seen how mutations, such as V67D, that lie far from the calcium binding loops and the canonical polybasic cluster, impact the two states, however the calcium sensitive exchange beyond these regions we have identified may provide the explanation for the resulting pathology. Furthermore, while we have shown how calcium binding modulates PI(4,5)P2 affinity, interaction with other C2A binding partners may also influence the populations of the exchanging states, leading to modulation of activity. Further refinement of structural methods for probing protein-membrane interaction, like those demonstrated in this study, may shed light on these future directions.

Materials and Methods

Molecular Biology and Protein Purification. Human dysferlin cDNA (AF075575), a gift from Dr. Kate Bushby (Newcastle University), was used as a template for cloning. The C2A domain (amino acids 1-129) was cloned into the pET-28a(+) vector (Novagen) between the BamHI and HindIII restriction sites. Complementary flanking restriction sites for the dysferlin insert were generated by PCR amplification using the following primers: forward 5'–GCC CGC GGA TCC ATG CTG AGG GTC TTC ATC CTC TAT GCC-3' reverse 5'–GCC CGC AAG CTT TTA AGC TCC AGG CAG CGG-3'. BL21 DE3 cells harboring the expression plasmid were cultured overnight at 37°C in Luria-Bertani broth containing 50 µg/mL kanamycin and 1% w/v glycerol and were used to seed 1 liter cultures of Luria-Bertani broth containing 50 µg/mL kanamycin at a ratio of 1:1000. 15N and 15N/13C isotopically labeled protein was produced by bacteria grown in MJ9 media containing 1 g/L 15NH4Cl and 2 g/L of 12C or 13C glucose. These were then grown to an optical density of 0.6 at 37°C and induced with 0.5 mM isopropyl β-D-1-thiogalactopyranoside (IPTG). Protein was expressed for 16 hours at 18°C. Cultures were centrifuged at 4000 rpm at 4°C for 20 minutes and resuspended in lysis buffer: 50 mM HEPES pH 8, 250 mM NaCl, 10% (v/v) glycerol, 5 mM CaCl2, 1 mM phenylmethanesulfonyl fluoride (PMSF), and 1 µM leupeptin, pepstatin A, and aprotonin. Cells were lysed using a Microfluidics M-11P microfluidizer at 18,000 psi. 0.5% CHAPS (w/v) was added to the total lysate and left to rock for 1 hour on ice. Soluble fractions were then obtained by centrifugation in a Beckman J2-21 centrifuge at 20,000 x g at 4°C for 20 minutes. Clarified lysate was bound to HisPur Cobalt resin (Thermo Scientific) for 2 hours with rocking at 4°C. Beads were then washed with 20 column volumes of lysis buffer and protein was eluted with 50 mM HEPES, 150 mM NaCl, 5 mM CaCl2, and 200 mM imidazole. Purity of the elution fractions was confirmed via SDS-PAGE, pooled, and dialyzed against 50 mM HEPES, 150 mM NaCl, 5 mM CaCl2 and 1 mM 1,4-dithiothreitol (DTT). The hexahistidine purification tag was cleaved from isotonically labeled samples by thrombin during dialysis into a low salt buffer (50 mM HEPES, 20 mM NaCl, 5 mM CaCl2, 1 mM DTT, pH 7). The cleaved protein was further purified by cation exchange chromatography (HiTrap™ SP HP, GE). Samples were then dialyzed into NMR buffer (50 mM HEPES, 100 mM KCl, 5 mM CaCl2, 5 mM DTT, pH 7), concentrated, and
supplemented with 1 mM sodium azide, 10% D2O, and 2-2 dimethylsilapentane-5-sulfonic acid prior to data collection.

**NMR Backbone Assignments.** NMR spectra were collected at 303K on a 800-MHz Bruker Avance IIIHD spectrometer equipped with a TCI cryoprobe. Backbone resonance assignments were determined from a set of [15N, 1H] TROSY based triple-resonance experiments. (HNCA, HNCACB, HNOCACB, HNCO, HCACO). NMR spectra were processed with NMRPipe (37) and analyzed with CcpNmr Analysis (38). TALOS+ was used to calculate secondary structure propensity (39). Chemical shifts have been deposited with the BMRB under accession number 50753.

**NMR Titrations.** Peak intensities for the titration experiments were normalized to the DSS signal to correct for changes in sample concentration. For the calcium titration a sample in 5 mM CaCl2 was back titrated with 0.5 mM EDTA (ethylenediaminetetraacetic acid) dissolved in NMR buffer lacking CaCl2. D-myo-Inositol 1,4,5-tris-phosphate trisodium salt (Sigma-Aldrich) was dissolved in appropriate NMR buffer to stock concentrations of 2.057 or 20.57 mM and titrated to a final IP3:C2A ratio of 7.6:1.

**NMR Spin Relaxation.** T1 and T2 15N relaxation times and steady-state 15N-1H NOE values for all samples were obtained using standard [15N, 1H] TROSY based Bruker pulse sequences. (trt1etf3gpsite3d, trt2etf3gpsite3d, trnoetf3gpsite) Relaxation delay times for T1 experiments varied from 20 ms to 1.2 s, relaxation delay times for T2 experiments ranged from 33.9 ms to 271.4 ms. The steady-state 15N-1H NOE experiment included a total recovery delay of 8 seconds. Peak intensities were measured as peak heights, which were fit to

**NMR Chemical Exchange Saturation Transfer (CEST) Measurements.** 15N CEST data was collected using a standard Bruker pulse sequence (hsqc_cest_etf3gpsite3d) with saturation field strengths of 50, 25, and 10 Hz, with a saturation time of 0.4 seconds, and a 1.5 second recovery delay. A total of 64 planes were collected, 0.5 ppm steps from 103.0 ppm and 134.0 ppm and one reference plane. Fits to CEST intensity profiles were done using the ChemEx program (https://github.com/gbougvignes/chemex) (42). Individual fits were made for each residue to a two-state exchange model, then residues where the error in exchange rate exceeded the exchange rate itself were rejected. The remaining residues yielded similar populations and exchange rates. We subsequently globally fit these residues to the two-state exchange model, yielding global exchange parameters (Table 1). Gibbs free energies were calculated from the population differences. To identify additional residues that were in exchange, but where the chemical shift differences were small, we further fit the data for residues close to those previously fit in the global model, using the global exchange rate and populations as fixed parameters. This analysis resulted in the identification of 4 additional exchanging residues in the 3 mM Ca2+ sample.

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**Bibliography**
1. Eric A. Naielksi and Joseph J. Falke. The C2 domain calcium binding motif: Structural and functional diversity. Protein Science. 5(12):2375–2390, December 1996. doi: 10.1002/pro.5560051201.
2. Josep Rizo and Thomas C. Südhof. C2 -domains, Structure and Function of a Universal Ca2+ -binding Domain. Journal of Biological Chemistry. 273(26):15879–15882, June 1998. doi: 10.1074/jbc.273.26.15879.
3. X. Shao, I. Fernandez, T. C. Sudhof, and J. Rizo. Solution structures of the Ca2+-free and Ca2+-bound C2A domain of synaptotagmin I: does Ca2+ induce a conformational change? Biochemistry. 37(46):16106–16115, November 1998. doi: 10.1021/bi801799b.
4. Josep Ubach, Xiangyang Zhang, Xiguang Shao, Thomas C. Sudhof, and Josep Rizo. C2 alpha binding to synaptotagmin: how many Ca2+ ions bind to the tip of a C2 domain? The EMBO Journal. 17(14):3921–3930, July 1998. doi: 10.1026/1418-0279.17.14.3921.
5. Naomi J. Marty, Chelsea L. Holman, Nazish Abdullah, and Colin P. Johnson. The C2 Domains of Otoferlin, Dystelrin, and Mdyferlin Alter the Packing of Lipid Bilayers. Biochemistry. 52(33):5585–5592, August 2013. doi: 10.1021/bi4014332.
6. Nazish Abdullah, Muruges Padmanarayana, Naomi J. Marty, and Colin P. Johnson. Quantitation of the Calcium and Membrane Binding Properties of the C2 Domains of Dystelrin. Biophysical Journal. 106(2):382–389, January 2014. doi: 10.1016/j.bpj.2013.11.4402. Publisher: Elsevier.
7. W Cho and R Satchell. Membrane binding and subcellular targeting of C2 domains. Biochimica et Biophysica Acta (BBA) - Molecular and Cell Biology of Lipids. 176(1):838–849, August 2006. doi: 10.1016/j.bbalip.2006.06.014.
8. Marta Guerrero-Valero, Cristina Ferrer-Orta, Jordi Querol-Audi, Consuelo Marin-Vicente, Ignacio Fita, Juan C. Gomez-Fernandez, Nuria Verdaguer, and Sena Corbalan-Garcia. Structural and mechanistic insights into the association of PkCaC2 domain to Phosphatidylinositol 4,5-bisphosphate. Proceedings of the National Academy of Sciences. 106(16):6603–6607, April 2009. doi: 10.1073/pnas.0810399106. Publisher: National Academy of Sciences Section: Biological Sciences.
9. Jaime Guillen, Cristina Ferrer-Orta, Monica Buxaderas, Dolores Perez-Sanchez, Marta Guerrero-Valero, Ginés Luengo-Gil, Joan Pous, Pablo Guerra, Juan C. Gomez-Fernandez, Nuria Verdaguer, and Sena Corbalan-Garcia. Structural insights into the Ca2+ and PtdIns(4,5)P2 binding modes of the C2 domains of ralphp and synaptotagmin 1. Proceedings of the National Academy of Sciences. 110(51):20503–20508, December 2013. doi: 10.1073/pnas.1316179110. Publisher: National Academy of Sciences Section: Biological Sciences.
10. Chuanjuan Wan, Bo Wu, Zhenwei Song, Jiahai Zhang, Huiping Chu, Aik Wang, Qingsong Liu, Yuyu Shi, Guishui Li, and Junfeng Wang. Insights into the molecular recognition of the granuphilin C2A domain with PI(4,5)P2. Chemistry and Physics of Lipids. 186:61–67, February 2015. doi: 10.1016/j.chemphys.2015.01.003.
11. Linn Michaeli, Irit Gottfried, Maria Bykhovskaia, and Uri Ashery. Phosphatidylinositol(4, 5)-bisphosphate targets double C2 domain protein B to the plasma membrane. Proceedings of the National Academy of Sciences. 106(16):6603–6607, April 2009. doi: 10.1073/pnas.0810399106. Publisher: National Academy of Sciences Section: Biological Sciences.
12. Christian Thirrien, Sabrina Di Fulvio, Sarah Pickles, and Michael Srinveera. Characterization of lipid binding specificities of dystelrin C2 Domains Reveals Novel Interactions with Phosphoinositides 1. Biochemistry. 48(11):2377–2384, March 2009. doi: 10.1021/bi801724s.
13. Jihong Bai, Ward C. Tucker, and Edwin R. Chapman. PIP2 increases the speed of receptorsynaptotagmin and steers its membrane-penetration activity toward the plasma membrane. Nature Structural & Molecular Biology. 11(1):36–44, January 2004. doi: 10.1038/nsmb709. Number: 1 Publisher: Nature Publishing Group.
Calcium Regulates Dysferlin C2A P(I,4,5)P Binding