Theory

Binding of $\text{Ca}^{2+}$-independent C2 domains to lipid membranes: A multi-scale molecular dynamics study

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In brief

Larsen and Sansom analyzed MD simulations of the interactions of six different $\text{Ca}^{2+}$-independent C2 domains with lipid bilayers. C2 domains interacted strongly with membranes containing PIP$_2$. For PTEN and SHIP2, simulations of their phosphatase plus C2 domains allowed the roles of the two domains in membrane localization to be compared.

Highlights

- Binding of $\text{Ca}^{2+}$-independent C2 domains to membranes was explored by MD simulation

- C2 domains from KIBRA, PI3KC2α, RIM2, PTEN, SHIP2, and Smurf2 were compared

- C2 domains formed longer-lived interactions with lipid bilayers containing PIP$_2$

- For PTEN and SHIP2, simulations of their phosphatase plus C2 domains were performed
Theory

Binding of Ca\textsuperscript{2+}-independent C2 domains to lipid membranes: A multi-scale molecular dynamics study

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SUMMARY

C2 domains facilitate protein interactions with lipid bilayers in either a Ca\textsuperscript{2+}-dependent or -independent manner. We used molecular dynamics (MD) simulations to explore six Ca\textsuperscript{2+}-independent C2 domains, from KIBRA, PI3KC2\textsubscript{a}, RIM2, PTEN, SHIP2, and Smurf2. In coarse-grained MD simulations these C2 domains formed transient interactions with zwitterionic bilayers, compared with longer-lived interactions with anionic bilayers containing phosphatidylinositol bisphosphate (PIP\textsubscript{2}). Type I C2 domains bound non-canonically via the front, back, or side of the β sandwich, whereas type II C2 domains bound canonically, via the top loops. C2 domains interacted strongly with membranes containing PIP\textsubscript{2}, causing bound anionic lipids to cluster around the protein. Binding modes were refined via atomistic simulations. For PTEN and SHIP2, CG simulations of their phosphatase plus C2 domains with PIP\textsubscript{2}-containing bilayers were also performed, and the roles of the two domains in membrane localization compared. These studies establish a simulation protocol for membrane-recognition proteins.

INTRODUCTION

Lipid-specific membrane recognition plays a key role in the biology of eukaryotic cells. A number of families of recognition domains exist. Of especial importance are domains that recognize phosphoinositide (PI) lipids (Kutateladze, 2010; Stahelin et al., 2014), with C2 and PH domains being the most abundant families (Katan and Allen, 1999). C2 domains can enable both protein-lipid and protein-protein interactions (Corbalan-García and Gómez-Fernández, 2014; Nalefski and Falke, 1996; Zhang and Aravind, 2010) and are key players in a number of cellular signaling processes involving, e.g., ubiquitination (Wiesner et al., 2007) or (de)phosphorylation (Chen et al., 2018; Gericke et al., 2013). C2 domains bind membranes and/or other proteins and thereby bring, e.g., adjacent catalytic domains into contact with their interaction partners. One intensively studied example of a C2-domain-containing protein is the phosphatase and tensin homolog (PTEN), which consists of a C2 domain and a phosphatase domain (Lee et al., 1999; Maehama and Dixon, 1999; Zhao et al., 2004). In PTEN, C2 provides a lipid-mediated anchor to the membrane, so the phosphatase domain can dephosphorylate phosphatidylinositol trisphosphate (PIP\textsubscript{3}) to phosphatidylinositol 3,4-diphosphate (PIP\textsubscript{2}). This dephosphorylation downregulates the Akt pathway, and PTEN is thus a tumor suppressor.

All C2 domains have a similar structure (Figure 1). They are composed of around 130 residues arranged as eight β antiparallel strands (β1–β8) in two β sheets, forming a β sandwich. C2 domains have two topologies, type I or type II, which differ by a circular permutation (Corbalan-García and Gómez-Fernández, 2014; Nalefski and Falke, 1996). Calcium ions control the function of many C2-domains via Ca\textsuperscript{2+}-mediated binding and unbinding to lipid membranes (Corbalan-García and Gómez-Fernández, 2014). However, there is a group of Ca\textsuperscript{2+}-independent C2 domains with either no or little Ca\textsuperscript{2+} dependency. These are the focus of the current study.

We examine how Ca\textsuperscript{2+}-independent C2 domains interact with lipid membranes, especially those containing PI lipids. Molecular dynamics (MD) simulations have emerged as a powerful tool to examine the interactions of membrane proteins with lipids (Corradi et al., 2019; Hedger and Sansom, 2016; Manna et al., 2019) and have previously been applied to investigate the interactions of peripheral membrane proteins, especially PH domains, with PI-containing lipid bilayers (Kalli and Sansom, 2014; Lai et al., 2010, 2013; Naughton et al., 2016; Pant and Tajkhorshid, 2020; Yamamoto et al., 2016). There have been a number of MD studies of the interaction of Ca\textsuperscript{2+}-dependent C2 domains with membranes, revealing how Ca\textsuperscript{2+} ions mediate interactions between the protein and anionic lipids such as phosphatidylinerine (PS) and phosphatidylinositol phosphates (PIPs) (Alwarawrah and Wereszczynski, 2017; Banci et al., 2002; Chon et al., 2015; Jaud et al., 2007; Lai et al., 2010; Manna et al., 2008; Michaeli et al., 2017; Vermaas and Tajkhorshid, 2017). However, there have not been many simulation studies of the interactions of Ca\textsuperscript{2+}-independent C2 domains with membranes (see, e.g., Ainaas et al., 2021; Scott et al., 2020) other than for PTEN (Shenoy et al., 2012a; Treece et al., 2020), despite the growing recognition of the importance...
RESULTS

A protocol for determining and analyzing C2-domain-binding modes

To explore possible binding modes of C2 domains to lipid bilayers without bias from the initial simulation system configuration, we initiated CG simulations with the C2 domain positioned at sufficient distance that it could not "feel" the membrane, i.e., at a distance greater than the cutoff distances of the CG force field. For each simulation of an ensemble, the protein molecule was rotated through a random angle before the simulation was started (Figure 2). Each simulation was run for 2 μs, which proved long enough for the protein to encounter and interact with the bilayer, in some cases (dependent on the bilayer lipid composition) being able to dissociate and rebind. To ensure adequate sampling, based on previous experience with PH domains (Yamamoto et al., 2016) and other membrane proteins (e.g., phosphatidylinositol phosphate kinase PIP5K1A [Amos et al., 2019]), 25 repeats were run for each system. Thus, for six different C2 domains each with three different lipid membranes, a total of just under a millisecond of CG-MD simulations were performed. These were subsequently analyzed in terms of the binding orientation of the C2 domain relative to the membrane, the dependence on the membrane-lipid composition, and the energetics of the interaction.

CG simulations of C2/membrane encounter

Six C2 domains were investigated, three type I and three type II (Table 1). The type I C2 domains were from KIBRA (PDB: 6FJD), PI3KC2α (PDB: 6BU0), and RIM2 (PDB: 2BWQ), and the type II C2 domains from PTEN (PDB: 1D5R), Smurf2 (PDB: 2JQZ), and SHIP2 (PDB: 5OKM). Here we focus on a comparative overview of their simulated interactions with lipid bilayers of varying composition. Three types of membranes were included in the study: zwitterionic (phosphatidylcholine [PC]), anionic (80% PC and 20% PS), and anionic membrane including a PI lipid (80% PC, 15% PS, and 5% phosphatidylinositol biphosphate [PIP2]). We use the shorthand notations PC, PC:PS, and PC:PS:PIP2 to denote these membranes. PC:PS:PIP2 is a model membrane for the negatively charged inner leaflet of mammalian plasma human membrane, which is relevant as all the C2 domains under consideration bind to this membrane in vitro. The PIP2 content (5%) is somewhat higher than typical overall values of a plasma membrane (1%–2%) but is comparable with the concentration of PI lipids used for in vitro studies of C2 binding to a plasma membrane mimic (e.g., Stahelin et al., 2003). As our simulation box is rather small, we increased the PIP2 content to allow for the possibility of multiple PIP2 molecules clustering around a bound C2 domain.

As can be seen from the example of the Smurf2-C2 domain (Figures 2B and 2C), a single simulation in the presence of either a PC or a PC:PS:PIP2 bilayer can be used to demonstrate the influence of the lipid bilayer composition on the protein-membrane interaction by monitoring the minimum distance between the protein and bilayer as a function of time. In this example, the C2 domain makes multiple encounters with the PC bilayer before finally binding to the surface after ~1 μs (Figure 2B). In contrast, in the presence of the PI-containing bilayer, the initial encounter occurs within the first 0.1 μs (Figure 2C), leading to binding of the...
C2 domain to the membrane, with no subsequent dissociation over the course of the 2-μs simulation.

We repeated this analysis for the 25 repeats for all six C2 domains and three lipid compositions (Figure 3). The variation among repeats is relatively large, especially for simulations with PC bilayer. Such stochastic behavior is, however, expected for single-molecule events, and the overall pattern seen for the Smurf2-C2 is conserved for all C2 domains: the simulations with a PC bilayer lead to multiple reversible contacts between the C2 and the membrane. For the anionic PC:PS bilayer, in most cases multiple encounters are seen, on average leading to a longer-lasting interaction of the protein and the membrane. In contrast, in the presence of a PC:PS:PIP2 bilayer, in all cases the initial encounter of C2 domain and bilayer leads to a protein/membrane interaction that persists for the remainder of the simulation. These simulations, especially those with PC:PS, enable us to come up with an initial, approximate ranking of the strength of interactions of C2 with a bilayer. Thus, comparing two C2 domains from PIP phosphatases (i.e., PTEN and SHIP2), we may contrast PTEN-C2, which on average forms long-lasting interactions with both PC and PC:PS bilayers, with SHIP2-C2, which forms multiple reversible interactions with both PC and PC:PS bilayers and only forms long-lasting interactions with a PC:PS:PIP2 bilayer. Fitting exponential decays to the averaged minimum protein-lipid distance versus time data for these simulations (Figure S1 and Table S1) supports this interpretation.

The mode(s) of binding of the C2 domains were characterized by calculation of protein-lipid distance versus orientation density maps, which is an approach previously adopted for comparing PH domains (Yamamoto et al., 2016). The orientation of the C2 domain relative to the bilayer was quantified as the $R_{zz}$ component of the rotation matrix, $R_{zz}$, with respect to a reference frame (Figure 4). Note that $R_{zz} = 1$ means that the orientation along z is the same as the reference, whereas a value of $-1$ means that the protein is rotated 180°. (We only consider this one component of the orientation, as the lipid bilayer is isotropic in the xy plane.) The last frame of the first repeat of the ensemble was used as a reference frame in the initial calculation of $R_{zz}$, and subsequently all $R_{zz}$ values were recalculated with respect to a selected primary binding mode in the system with PC:PS:PIP2 bilayer once this mode
was determined. The same reference was used for the systems with PC and PC:PS bilayers. We found multiple binding modes for the isolated C2 domains, showing up as dense areas in the distance/orientation maps (Figure 4). In most cases, the modes were the same for zwitterionic membrane (PC), anionic membrane (PC:PS), and anionic membrane with PI (PC:PS:PIP2), and the most visited mode was in many cases the same for all membrane compositions (Figure 4). However, the C2 domains spend more time unbound and away from the membrane for the PC membrane, while the presence of PS and PI enhanced membrane-protein contacts.

One primary binding mode was selected for each C2 domain (two for PI3KC2α). The selection criteria were: (1) the selected mode was probable, i.e., that the mode was frequently visited; (2) the mode was physically reasonable, i.e., that domains adjacent to the C2 domain in the intact protein would not overlap with the membrane; and (3) the mode was productive, i.e., adjacent catalytic domains were in contact with the bilayer. Interestingly, some modes are intermediates, as can be seen by monitoring each trajectory and following the path toward the final binding mode. Comparable dynamic behavior has previously been observed for other membrane binding proteins (e.g., K-Ras4A [Li and Buck, 2017]). In the current study we have, however, chosen to focus on the primary binding mode(s) rather than the details of the interaction pathway.

We evaluated the possible sensitivity of our results to changes in the lipid bilayer composition by systematic changes in the latter, using the PTEN C2 domain as a test case. We selected the PTEN domain because it had been seen to bind in two modes and had already been shown to exhibit some degree of sensitivity to changing the bilayer from zwitterionic (PC) to anionic (PC:PS) to anionic with PI (PC:PS:PIP2) (Figures 3 and 4). We therefore further explored the effects of: (1) increasing the fraction of PS from 20% to 40%; (2) replacing PIP2 by PIP or PIP3; and (3) inclusion of cholesterol alongside PC, PS, and PIP2. The results of these simulations are shown in Figures S2 and S3. These data do not suggest any major effects of varying the lipid head groups present, which is encouraging in that it shows our basic result of PIP2 promoting C2 binding is robust to changes. Notably, the simulations suggest that binding of the C2 domains is relatively insensitive to a change from, e.g., PIP2 to PIP3, at least in the CG simulations, despite PIP3 being the substrate for PTEN. This is broadly consistent with experimental studies, as reviewed by, e.g., Harishchandra et al. (2015), which indicate comparable binding affinities for PIP2 and PIP3.

Two views of the energetics of C2/membrane-lipid interactions

To quantitatively compare the binding modes of the different C2 domains, we calculated binding free energies for each system. First, a potential of mean force (PMF) was calculated using umbrella sampling with the center-of-mass distance between protein and membrane as reaction coordinate (Figure 2D). This provides an estimate of a free energy curve for the interaction of the protein with the membrane. The C2 domains bound most strongly to PC:PS:PIP2 membrane, less tightly to anionic membrane without PIP2 (PC:PS), and even more loosely to PC membranes (Table 1 and Figure S4). The binding strengths had approximate ratios of 1:2:4 to 1:2:5 across the three membranes (PC versus PC:PS versus PC:PS:PIP2, Table 1).

Next, free energy perturbation (FEP) calculations provided additional information on the binding strength of specific PIP2 lipid head groups with the C2 domain (Figure 2E and Table 2). The results of these simulations are shown in Figures S2 and S3. These data do not suggest any major effects of varying the anionic lipids present, which is encouraging in that it shows our basic result of PIP2 promoting C2 binding is robust to changes. Notably, the simulations suggest that binding of the C2 domains is relatively insensitive to a change from, e.g., PIP2 to PIP3, at least in the CG simulations, despite PIP3 being the substrate for PTEN. This is broadly consistent with experimental studies, as reviewed by, e.g., Harishchandra et al. (2015), which indicate comparable binding affinities for PIP2 and PIP3. Perhaps surprisingly, given in vitro experimental data suggesting PTEN binding is not altered by the presence/absence of cholesterol (Das et al., 2003), the inclusion of 25% cholesterol speeds up binding of the C2 domain. This effect is partly due to a reduction of the area per lipid, resulting in increased surface charge density (Doktorova et al., 2017) (Figure S1). Distance/Rzz analysis (Figure S3) suggests that the modes of binding in the presence of cholesterol are very similar to those in the corresponding PC/PS/PIP2 simulations. The acceleration of C2 binding by cholesterol is intriguing and will form the subject of a subsequent more detailed study of lipid effects on C2 domain interactions.

Table 1. Summary of CG association simulations and PMFs

| Domain       | PDB ID | Topology | Binding orientation | PMF well depth\(^a\) (kJ/mol) |
|--------------|--------|----------|---------------------|-------------------------------|
|              |        |          |                     | PC       | PC-PS   | PC-PS-PIP2 |
| KIBRA-C2     | 6FJD   | type I   | side B              | 9.8 ± 1.0 | 26.0 ± 1.5 | 48.0 ± 1.4 |
| PI3KC2α-C2b | 6BUO   | type I   | front               | 15.0 ± 0.9 | 28.6 ± 1.3 | 64.1 ± 1.2 |
|              |        |          | back                | 22.7 ± 0.6 | 42.8 ± 0.7 | 115.2 ± 2.6 |
| RIM2-C2a     | 2BWQ   | type I   | side A              | 32.5 ± 1.1 | 49.8 ± 0.9 | 120.4 ± 2.4 |
| PTEN-C2      | 1D5R   | type II  | top                 | 15.4 ± 2.7 | 27.0 ± 4.2 | 77.7 ± 4.3 |
| SHIP2-C2     | 5OKM   | type II  | top                 | 8.5 ± 1.1  | 10.4 ± 0.9 | 23.1 ± 1.0 |
| Smurf2-C2    | 2JQZ   | type II  | top                 | 19.9 ± 2.3 | 38.2 ± 1.0 | 81.5 ± 2.0 |

\(^a\)As defined in Figure S4.
Figure 3. Minimum protein-lipid distances as a function of time for all simulations
For each simulation ensemble the minimum protein-lipid distance is shown as a function of time, with the different colors corresponding to the 25 repeats within the ensemble. A distance of <0.5 nm corresponds to a contact between the protein and the lipid bilayer. For each C2 domain, simulations are shown for interactions with a PC, a PC:PS, and a PC:PS:PIP2 bilayer. See also Table S1; Figures S1 and S2.
Figure 4. Density maps of the orientation and distance of the C2 domains relative to the lipid bilayer

For each simulation ensemble, a density map of the orientation and distance of the C2 domain relative to a lipid bilayer is given. Each density map represents the relative frequency (on a logarithmic color scale from purple [low] to yellow [high]), averaged across time and all 25 simulations in an ensemble, of the orientation and distance of the C2 domain relative to the bilayer. The orientation is given by $R_{zz}$, which is the $zz$ component of the rotation matrix of the C2 domain with respect to a reference structure at $R_{zz} = 1$ (see text for details of the reference structure). Note that $R_{zz} = -1$ means that the protein is rotated 180° with respect to the reference structure. The distance shown is the $z$ component of the vector between the centers of mass of the bilayer and the C2 domain. See also Figure S3.
Theory

Simulation was performed (Figure 2F) to refine the binding modes at coarse-grain resolution to atomistic resolution, and a 200-ns simulation was applied to the proteins in the CG simulations, which preserves secondary structure and tertiary fold.

In the last step of the protocol, each system was converted from experimental data (compare Tables 1 and 2). For systems with small absolute binding energy, on the other hand, there is no such discrepancy. In the following, we compare the binding modes observed in the simulations with structural and other experimental data where available.

**Smurf2**

The N-terminal C2 domain localizes Smurf (SMAD-Specific E3 Ubiquitin Protein Ligase 2) to the membrane (Feng and Derijck, 2005; Kavsak et al., 2000; Wiesner et al., 2007). Smurf2-C2 showed one dominant binding mode in our CG simulations, which is the same for all three membranes (Figure 4). This is a canonical top binding mode with loops L12, L56, and L78 facilitating lipid binding (Figure 5). These loops were previously determined as phospholipid binding sites by nuclear magnetic resonance (NMR) (Wiesner et al., 2007). Weisner et al. further determined that Smurf-C2 binds PIP1, PIP2, and PIP3 by lipid overlay assays. The Smurf-C2 stays in the same binding mode during 200-ns atomistic simulations for all three membranes (Figure 4). Notably, Smurf-C2 is the only protein out of the six investigated that stays bound to the PC membrane throughout the atomistic simulations. Smurf2-C2 shares 87% sequence identity with the C2 domain of Smurf1 (Figure S5), and they have similar functions and structure (Koganti et al., 2018), which make it relevant to compare them. Scott et al. (2020) used MD simulations to explore interactions of InsPs with Smurf1-C2 and observed a canonical top binding mode, in line with our results. Moreover, Smurf1-C2 has been crystallized with negatively charged sulfates bound, also on the top of the C2 domain (PDB: 3PYC; Figure S5).

**KIBRA-C2**

Kidney and brain expressed protein (KIBRA) is a multi-functional protein with around 20 known binding partners and is central in many cellular processes, e.g., membrane trafficking (Mlinik et al., 2012). KIBRA contains a C2 domain, which facilitates membrane binding in a Ca2+-independent manner (Posner et al., 2018). KIBRA-C2 displays two dominant binding modes in our simulations, but the most frequent binding mode is with β7 and β8 facing the bilayer (Figure 5), i.e., with side B (Figure 1) toward the lipid surface. This binding mode is consistent with previous NMR, X-ray crystallography, and MD data for the lipid-binding site of KIBRA-C2 (Posner et al., 2018), as it involves β1 and β8 and binds in a non-conventional way (i.e., not via the not loops). The second binding mode (Figure 4) is unphysical, as the N and C termini are buried in the bilayer and KIBRA-C2 is a central domain. During the 200-ns atomistic simulation, the

### Table 2. Interaction free energies of PIP2 head groups

| Domain     | Binding mode | FEP for each of the four bound PIP2 molecules \( ^a \) (kJ/mol) | Total FEP (kJ/mol) | \( \Delta \)PMF \( ^b \) (kJ/mol) |
|------------|--------------|---------------------------------------------------------------|--------------------|-----------------------------|
| KIBRA-C2   | side B       | 6, 7, 7, 6                                                    | 26.0 ± 3.9         | 22.0 ± 2.1                  |
| PI3KC2α-C2b | front        | 9, 7, 10, 7                                                  | 33.3 ± 4.0         | 35.5 ± 1.8                  |
|           | back         | 10, 7, 4, 10                                                | 30.9 ± 4.1         | 72.4 ± 2.7                  |
| RIM2-C2a   | side A       | 11, 21, 15, 12                                              | 58.0 ± 3.6         | 70.6 ± 2.6                  |
| PTEN-C2    | top          | 10, 7, 9, 7                                               | 32.8 ± 4.0         | 50.7 ± 5.0                  |
| SHIP2-C2   | top          | 4, 4, 4, 4                                                | 15.7 ± 2.7         | 12.7 ± 1.3                  |
| Smurf2-C2  | top          | 6, 6, 9, 8                                                | 27.9 ± 4.4         | 43.3 ± 2.5                  |

\( ^a \)Energy calculated by changing the head group of each PIP2 in the upper leaflet to PC while keeping the other PIP2 molecules unaltered. Mean of five repeats. Error on the total FEP is calculated by the standard deviation from the five repeats.

\( ^b \)PMF well depth difference between binding to PC:PS (80:20) and binding to PC:PS:PIP2 (80:15:5). See also Figures S5–S7.

for binding to PC membranes, larger for PC:PS binding, and largest for PC:PS:PIP2 membranes. A notable exception is that \( \Delta \)PMF is significantly larger than FEP for PI3KC2α. This is a general trend for C2 domains with large absolute binding PMF (compare Tables 1 and 2). For systems with small absolute binding energy, on the other hand, there is no such discrepancy.

### Binding modes, AT-MD, and comparison with experimental data

In the last step of the protocol, each system was converted from coarse-grain resolution to atomistic resolution, and a 200-ns simulation was performed (Figure 2F) to refine the binding modes (Figure 5). All C2 domains with type II topology bound with the top facing the membrane (Figure 5 and Table 1). The C2 domains with a type I topology, on the other hand, bound with either the side, front or back, toward the membrane (Figure 1), i.e., with its longest axis parallel to the membrane. This is reflected in the PMF calculations through the distance with the minimum binding energy; the type II C2 domains have lowest energy at distances between 3.1 and 3.5 nm, whereas the type I C2 domains have lowest energy at distances between 3.6 and 4.0 nm, for the productive mode (Table 1). In all simulations with PIP2 present, the PIP2 molecules clustered around the C2 domain (Figure 5) with all four PIP2s in the upper leaflet bound for most of the time.

The contacts between basic (Arg and Lys) side chains of C2 and the phosphates of PIP2 were analyzed for the binding modes observed in the atomistic simulations (Figure 5). Interestingly, there is a correlation between the number of contacts and the depth of the free energy well in the corresponding PMF (Figures S4 and S8; Table 1), indicating that electrostatic interactions between basic side chains of C2 and the PIP2 head groups are dominant but not the sole determinants of the interactions.

The C2 domains generally stayed in their initial bound configuration with PIP2 throughout the atomistic simulation, whereas C2 domains bound to PC:PS rotated more and unbound in one case, and C2 domains bound to pure PC membranes unbound in all but one case (Figure 6). The conformation of the proteins did not change, with Cα-root-mean-square deviations not exceeding 0.3 nm as compared with the crystal structure for any of the C2 domains. This justifies the use of an elastic network applied to the proteins in the CG simulations, which preserves secondary structure and tertiary fold.
C2 domain changed orientation slightly so the front faced the membrane (Figure 6) when bound to either PC:PS or PS:PS:PIP2 (the location of the front is shown in Figure 1). This mode is, however, still consistent with experimental data, as it involves β1, β2, and β8 in its lipid association. When initially bound to a PC membrane, the C2 domain diffused away from the bilayer during the AT simulation.

**RIM2-C2a**

RIM2 (Rab3 Interacting Molecule 2) is involved in synaptic vesicle priming (Dai et al., 2005; Södhof, 2004). It contains two C2 domains, one in the middle of the protein (C2a) and one in the N terminus (C2b). C2a was studied here. RIM2-C2a has a dominant binding mode in our simulations, with the front of the C2 domain binding the membrane (Figure 5) for all three membranes (Figure 4). A large binding energy to membranes for RIM2-C2 compared with the other C2 domains (Table 1) is likely due to a highly polar surface, with a positive patch on side A (β7 and β8) and a negative patch on side B (β4 and β5) (Dai et al., 2005), resulting in strong binding between side B and negatively charged membranes. RIM2 C2 has one particularly strongly bound PIP2 (with an FEP energy of 21 kJ/mol; Table 2). This lipid is bound to β4 of RIM2-C2, suggesting a specific PIP2-binding pocket at this location. In the atomistic simulations, the protein retained its orientation with a PIP2-containing membrane, underwent a rotation with PC:PS, and dissociated from the PC bilayer (Figure 6). RIM2-C2a has been crystallized with sulfates, which were suggested to bind at the same location as negatively charged PIP head groups (PDB: 2BWQ) (Dai et al., 2005). These sulfates are bound at a positively charged patch at the bottom of RIM2-C2a (Figure S6). In our simulations, a PIP2 head group was also bound at this site (Figure S6) (with binding energy of 11 kJ/mol; Table 2), suggesting that this is a specific PIP2-binding site.

**PI3KC2z-C2b**

PI3K (phosphoinositide 3-kinase) has a central C2 domain (C2a) and a C-terminal C2 domain (C2b). The structure of C2b has been solved by X-ray crystallography (PDB: 6BU0; note that C2b is referred to as PI3KC2zC2C in Chen et al., 2018). C2b along with the adjacent C-terminal PX domain autoinhibit kinase activity in solution, but, interestingly, PX and C2b increase enzymatic effect at PI(4,5)P2-containing membrane (Wang et al., 2018). In our simulations, PI2KC2z-C2b revealed two distinct binding modes in our simulations with PC:PS:PIP2 membranes, with, respectively, the front and the back of C2 domains facing the membrane (Figure 5). In the front binding mode, the lipids bind PI3KC2-C2 mainly via the C-terminal β strand, β8. In the back binding mode, the lipids mainly bind with β4 and the loops L34 and L67, but also via β7 and L23. Both binding modes are possible, as they do not block the neighboring PX domain in PI3KC2z, which is connected via a flexible loop at the N terminus of C2 (Chen et al., 2018). The crystallographic asymmetric unit of 6BU0 contains three copies of the C2b domain and four InsP6 molecules, which therefore define two distinct binding sites on the surface of the domain (Chen et al., 2018). The first binding site is at the front of the C2 domain and the second is at the back, with L34 and L67 contacting the bound InsP6 molecule (Figure S7). Thus, the simulations and the crystal structure both suggest these two binding modes, i.e., a front and a back binding mode. The front binding mode had four PIP2s bound in the simulations, but none of them coincided with the InsP6s from the crystal structure. Instead, the PIPs were bound close to the loops in the top and bottom of the C2 domain. The back binding mode likewise had all four PIP2s from the upper leaflet bound, one of which was bound in proximity to the binding pocket of the crystal, i.e., the PIP2 head group from the simulation was bound about 0.8 nm from the crystallographic InsP6 (Figure S7). This suggests that this may be a more specific binding pocket for PIP2s, albeit having a relatively low binding affinity (4 kJ/mol; Table 2) compared with the other bound PIPs.

**PTEN-C2 and SHIP2-C2**

Both of these C2 domains form the C-terminal half of a core enzyme structure, made up of a catalytic phosphatase (Ptase) domain followed by a membrane-targeting C2 domain (Le Coq et al., 2017; Worby and Dixon, 2014). This enables us to identify which binding modes of the isolated C2 domains are “productive.” Also, the simulated binding interactions of the isolated C2 domains may be compared with those of the core Ptase-C2 structures.

PTEN is a tumor suppressor that dephosphorylates PIP2 to PI(4,5)P2 (Worby and Dixon, 2014). The C2 domain of PTEN acts by bringing the phosphatase domain of PTEN (Ptase) close to the membrane. The isolated C2 from PTEN exhibits two
Simulations of larger fragments of PTEN and SHIP2

As noted above, for PTEN and SHIP2 the structures of the core proteins (Ptase-C2) are known, so it is possible to also simulate these and compare the modes of membrane interactions with those of the isolated C2 domains. There have been a number of previous simulation studies of the interaction of PTEN with membranes (Gericke et al., 2013; Kalli et al., 2014; Lumb and Sansom, 2013; Nanda et al., 2015; Shenoy et al., 2012a, 2012b; Treece et al., 2020), but we have simulated it here again to maintain the same simulation settings as for isolated C2, in particular the same lipid composition, namely PC:PS:PIP2 (80:15:5).

As described above, for isolated PTEN-C2 two binding modes were observed, only one of which was "physical," with the top loops pointing toward the bilayer. In the simulations of PTEN (Ptase + C2), we also obtained two binding modes (Figure 7A). One mode (~50% of the population; mode 1 in Figure 7A) was comparable with the "physical" mode for the isolated C2 domain and allowed both the Ptase and C2 domains to contact the membrane (Figure 7A). An alternative mode 2 was also observed with side A of C2 pointing toward the lipids. This mode was not seen for the isolated C2. The catalytic site of Ptase of PTEN involves the P loop, the WDP loop, and the TI loop (Lee et al., 1999), as well as an N-terminal motif covering residues 12–16 (NKRRY) (Gericke et al., 2013). In our simulated mode 1, the PIP2 molecules are in frequent contact with the N-terminal motif, the P loop, and the TI loop, and the PIPs have some contact with the WDP loop (Figure 7A). In the simulated mode 2 there is also contact between the PIPs and the N-terminal motif and the three loops, but substantially less so (Figure 7A). Therefore mode 1, which was also found for the isolated C2 domain, is likely to correspond to the productive binding mode, enabling the association of the catalytic domain with the membrane.

For the isolated SHIP2-C2 domain, as discussed above, a rather weak multi-mode interaction was observed. For the larger fragment of SHIP2 (Ptase + C2) we observed three binding modes (Figure 7B). Of these, one mode (mode 1), similar to the productive mode for the isolated C2, accounted for 36% of the population. In SHIP2, three loops are part of the catalytic site: the loop containing residues 675–684 (denoted L4 by Le Coq

binding modes in our simulations (Figure 4). In the first mode, C2 binds with the loops in the top of the domain (Figure 5), whereas the second binding mode, which has the back of C2 interacting with the bilayer, is unphysical as the Ptase domain of PTEN would clash with the membrane, as seen by comparison with the X-ray crystal structure (Lee et al., 1999) (PDB: 1DSR). Therefore, the top binding mode is the only physical binding mode observed in our simulations, and this mode is also consistent with previous MD simulations of PTEN (Kalli et al., 2014). Electrostatics is the main driver component in the lipid binding, as has also been shown in binding studies of PTEN-C2 with different membranes (Das et al., 2003), but there is no simple linear correlation between bilayer surface charge density and membrane affinity (Figure S1), suggesting that electrostatics is not the sole component.

SHIP2 (SH2-containing-inositol-5-phosphatase 2) dephosphorylates PIP3 to PI(3,4)P2, and its affinity toward both PIP3 and PI(4,5)P2 has been demonstrated experimentally (Chi et al., 2004; Pesesse et al., 1998). SHIP2-C2 exhibits three distinct binding modes in simulations with a PC:PS:PIP2 membrane (Figure 4). The structure of SHIP2-C2 has been determined with the adjacent Ptase domain (Le Coq et al., 2017) (PDB: 5OKM), and in two of the modes the Ptase domain is unproductively protruding out in the cytosol, with no contact with the lipids. In the productive mode Ptase is close to the membrane, facilitated by C2. This latter binding mode corresponds to the top loops binding to the membrane, in particular L12, L34, and L56 (Figure 5). Interestingly, the PMF for SHIP2-C2 reveals that it binds weakly (Figure S4) with a broad minimum encompassing all three binding modes. Furthermore, even when bound to the PIP2-containing membrane, SHIP2-C2 has some rotational freedom in the atomistic simulation (Figure 6).

Simulations of larger fragments of PTEN and SHIP2

As noted above, for PTEN and SHIP2 the structures of the core proteins (Ptase-C2) are known, so it is possible to also simulate these and compare the modes of membrane interactions with those of the isolated C2 domains.
et al., 2017), the loop consisting of residues 533–541, and the loop consisting of residues 562–568, as judged from available crystal structures (PDB: 5OKM and 4A9C) (Le Coq et al., 2017; Mills et al., 2012). Notably, in mode 1 of the simulations of SHIP2, the Ptase has frequent contacts between the bound PIP₂s and these three loops of the active site (Figure 7B). In the two other modes, on the other hand, there is little or no contact between PIP₂s and the active-site loops. Interestingly, in mode 1 the C2 seems to interact relatively weakly with the membrane, whereas the Ptase domain is more tightly bound and PIP₂ molecules cluster more around Ptase than around C2 (Figure 7B). This suggests that while the C2 domain may facilitate SHIP2-membrane interactions, in this case the Ptase domain is perhaps more important for the membrane interaction of SHIP2.

Comparing PTEN and SHIP2, these observations would suggest that whereas C2 from PTEN does, to some extent, determine the binding mode of PTEN, C2 from SHIP2 is less important in determining the binding orientation of SHIP2 and only makes a weak contribution to the avidity of binding. This is consistent with C2 from SHIP2 being the weakest binding C2 domain in the study (PMF = 23 kJ/mol), whereas C2 from PTEN has a higher membrane affinity (PMF = 78 kJ/mol).

**DISCUSSION**

We have demonstrated the utility of a simulation pipeline for exploring the interactions of Ca²⁺-independent C2 domains with anionic lipid bilayer models of cell membranes. This has allowed us to address a number of key questions by comparing the interactions of six different species of C2 domain, namely whether there is a systematic correlation between lipid binding and topology, how C2 domains bind to PIP molecules in a membrane, and whether C2 domains bind multiple PIP molecules, thus contributing to the formation of anionic lipid clusters. Here, we review the implications of these findings in more detail and discuss limitations of the method.

**Possible limitations of the Martini force field**

Using a CG force field allowed us to fully sample the encounter and binding to the lipid bilayer of the C2 domains in an unbiased manner. In these simulations, the size of the box does limit the
membrane-bending modulus, i.e., we effectively assume a flat bilayer in our simulations. Bilayer curvature effects might also be worth investigating systematically, but we consider it beyond the scope of the current study. Using a CG force field does have some known limitations. Electrostatic interactions are only approximately described in the Martini force field, so we did not investigate, e.g., the effect of salt concentration and only added sufficient Na+/Cl− ions to neutralize the system. Therefore, some electrostatic interactions may be overemphasized due to the absence of buffer-like charge screening. Some of the C2 domain/membrane binding energies are large (above 100 kJ/mol, Table 2), which has led to suggestions that the Martini force field may be too “sticky” (see, e.g., the discussions in Herzog et al., 2016; Javanainen et al., 2017). However, we have previously used the Martini force field to investigate PH domain binding to PIP-containing membranes, a system for which there is a reasonable body of experimental data (Naughton et al., 2016, 2018; Yamamoto et al., 2016). For that system, the energies were not overestimated compared with experimental values (but rather tended to be underestimated unless one allowed for multiple PIP molecules binding to each PH domain), which strengthens our justification for applying a comparable approach to the C2 system. We therefore suggest that the substantial binding energies observed are not an artifact from the force field. Moreover the C2 domains, once bound to PIP2-containing membrane, generally stay in their binding pose in the atomistic simulations (Figure 6). In the control simulations in which the protein was bound to a PC-only bilayer, the C2 domains in most cases dissociated from the bilayer during the atomistic simulations (Figure 6). Experimental data measuring membrane binding energies of the investigated C2 domains remains limited, and we hope that this study will stimulate future experimental binding studies.

Non-canonical binding of C2 domains

C2 domains were first recognized as a Ca2+-dependent protein, with a canonical binding orientation involving the calcium binding loops at the “top” of the domain (Figure 1) (Corbalan-Garcia and Gómez-Fernández, 2014; Nalefski and Falke, 1996). Several studies have, however, expanded the possible modes to include binding without calcium, and other studies have suggested that C2 domains can bind in a non-canonical orientation (Posner et al., 2018). We have systematically categorized and analyzed some of these non-canonical binding modes. We found that all of the six C2 domains bound to anionic membranes in the absence of Ca2+ ions and that three of these proteins bound in non-canonical orientations via the front, back, or side of the β sandwich (Figure 5; consult Figure 1 for definitions of front, back, etc.). Notably, our results suggest a connection between topology and binding orientation, with type I C2 domains binding non-canonically and type II C2 domains binding canonically, i.e., via the top loops.

PIP2 binding and clustering

Clustering of PIP2 was observed on inspection of the final binding modes of the C2 domain (Figure 5). Binding of multiple PIP2 to a single domain has also been reported for PH domains (Yamamoto et al., 2016), so this may be a general phenomenon for lipid-associated protein domains. Most of the binding energies for single PIP2 molecules are relatively small (<10 kJ/mol; Table 2), which may explain the lack of co-crystallized structures with, e.g., inositol phosphates in the absence of the possibility of binding to multiple PIP2s in a membrane when in a crystal.

Binding strength

We compared the binding strength of the C2 domains by different methods. From the minimum distance versus time data, we obtain the ranking (strongest binding first): (PTEN, PI3KC2α, and Smurf2) > (RIM2, KIBRA) > SHIP2. From the PMF calculations: (PI3KC2α, RIM2) > (Smurf2, PTEN) > (KIBRA, SHIP2). Finally, from the FEP calculations: RIM2 > (PI3KC2α, PTEN) > (Smurf2, KIBRA, SHIP2).

So the consensus is that PI3KC2α, PTEN, RIM2, and Smurf2 form strong interactions whereas KIBRA and SHIP2 form weaker interactions. However, we note that (in part due to the limitations of the CG approach) we have not explored either PIP2 versus PIP1 or P(4,5)P2 versus P(3,4)P2 in terms of the strength of binding interactions. A more detailed examination of the strength and specificity of binding of PIP species would require development of robust atomistic simulation estimates of the energetics of protein binding to lipids (see Pant and Tajkhorshid, 2020 for an example of this) and also a consideration of possible effects of ionization states of PIP head groups and the influence of bound cations (see, e.g., Bilkova et al., 2017; Slochower et al., 2013).

Driving force for lipid binding

The main driving force for the binding of C2 to the membranes appears to be electrostatics (see, e.g., Figure S8). However, protein-lipid interactions can also reflect the geometry around the binding sites as seen, e.g., for K-Ras4A (Li and Buck, 2017). We have not made a systematic investigation of this but do not observe a simple correlation between surface charge density and binding of the PTEN C2 domain (Figure S2), which suggests that electrostatics is not the sole factor controlling C2 binding. The impact of cholesterol suggests that lipid ordering may also be of importance.

Methodology

MD simulations allow for a systematic investigation of several related membrane-recognition proteins, providing direct comparison between them. This would in most cases be challenging to do experimentally for six or more proteins. CG-MD allows us to both monitor binding of domains to bilayers of differing lipid composition and undertake free energy calculations. The consistency observed for PMF and FEP calculations (Table 2) suggests that the energy calculations are converged. Convergence is an issue for energy calculation of protein-membrane binding, as shown for PMF calculations of PH domains binding to a PIP-containing membrane (Naughton et al., 2016, 2018) and PIP2 bound to Kir2.2 integral membrane protein (Corey et al., 2019). In both cases, 500- to 1,000-ns sampling was needed per umbrella window to obtain convergence (we used about 80 windows per protein/membrane combination in the present study). FEP needed 200–300 ns per window to obtain convergence for PIP2 bound to Kir2.2 (Corey et al., 2019) (we used 20 windows). These accumulated simulation times are not feasible with AT-MD, so we did the energy calculations before converting to atomistic resolution.
The CG force field may not, on the other hand, describe the energies accurately, so experimental benchmarking will be needed in the future to verify the absolute values of the energetics calculations.

We believe that the established method is applicable to other membrane-recognition domains. Similar computational approaches have already been exploited for comparative studies of PH domains (Naughton et al., 2016; Yamamoto et al., 2016), and the method can be applied to other domains as long as a sufficient number of solved structures are available.

There are hundreds of C2 domains (Nalefski and Falke, 1996), and the six included here are therefore not representative for all. We decided to limit the scope to include only C2 domains that (1) are Ca\textsuperscript{2+}-independent, (2) are interacting with the inner membrane leaflet, and (3) are solved structurally. This limits the number of possible C2 domains to include. However, as new structures are solved the methods can be applied to these and results compared with the present results, to obtain more certain knowledge about the binding orientation, affinity, and specificity of Ca\textsuperscript{2+}-independent C2 domains.

There are only relatively limited experimental data on lipid-protein interactions of the Ca\textsuperscript{2+}-independent C2 domains investigated. We hope that this study will stimulate further biophysical experiments. Of particular interest would be a systematic examination of C2 domains from different proteins under the same protocol, to provide a benchmark dataset for comparison with computational analysis.

Conclusions

We have investigated six different Ca\textsuperscript{2+}-independent C2 domains. These are all constituents of multi-domain proteins that interact with the cell membrane and play a key role in signaling. Using multi-scale MD, we investigated their binding modes, including binding orientation, binding affinity, and lipid specificity. We found that binding orientation and structural topology were related: type I C2 domains bound to the anionic membranes via the sides, front, or back of the β sandwich, whereas type II C2 domains bound via the loops at the top of the structure. Calculated binding free energies revealed significant binding differences between the six C2 domains, but these binding energies were not systematically correlated with the domain topology. Moreover, the domains, in general, only bound and stayed bound to negatively charged membranes, i.e., those containing PS and/or PIP\textsubscript{2}, and bound significantly more strongly to PIP\textsubscript{2}-containing membranes. PIP\textsubscript{2} clustered around the C2 domains upon binding, which suggests that C2 lipid interaction generally involves binding of several clustered PIPs, as also observed for the related PH domain (Yamamoto et al., 2016). PS, on the other hand, did not cluster around the C2 domains, nor did PC. We have thus provided an overview of expected binding modes, binding affinity, and binding specificity for non-canonical C2 domains, which improves our overall understanding of the cellular roles of C2 domains.

Equally important, we have presented a transferable methodology that can be utilized for novel structures of C2 domains and can readily be expanded for investigations of other protein domains interacting with lipid bilayer models of cell membranes. Advances in both spatially resolved lipidomics (Tsuji et al., 2019) and simulations of complex membrane models (Marrink et al., 2019) combined with systematic simulation methodologies will in the future allow us to reliably predict patterns of protein/membrane recognition (Irvine et al., 2019).

STAR METHODS

Detailed methods are provided in the online version of this paper and include the following:

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SUPPLEMENTAL INFORMATION

Supplemental information can be found online at https://doi.org/10.1016/j.str.2021.05.011.

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AUTHOR CONTRIBUTIONS

M.S.P.S. and A.H.L. conceptualized the project. A.H.L. carried out the simulations, analyzed the results, and wrote the original draft. M.S.P.S. and A.H.L. co-wrote and edited the final manuscript.

DECLARATION OF INTERESTS

The authors declare no competing interests.

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STAR METHODS

KEY RESOURCES TABLE

| REAGENT or RESOURCE | SOURCE | IDENTIFIER |
|---------------------|--------|------------|
| Deposited data      |        |            |
| KIBRA C2 structure  | Posner et al., 2018 | PDB: 6FJD |
| PI3KC2α C2 structure| Chen et al., 2018   | PDB: 6BU0 |
| RIM2                | Dai et al., 2005    | PDB: 2BWQ |
| PTEN                | Lee et al., 1999    | PDB: 1O5R |
| Smurf2              | Wiesner et al., 2007| PDB: 2JQZ |
| SHIP2 C2 structure  | Le Coq et al., 2017 | PDB: 5OKM |

Software and algorithms

| GROMACS             | Abraham et al., 2015 | www.gromacs.org |
|---------------------|-----------------------|------------------|
| PyMOL               | maintained and distributed by www.schrodinger.com | https://pymol.org/ |
| Modeller            | Fiser et al., 2000    | https://saliab.org/modeller/ |
| Martini 2.2         | de Jong et al., 2013  | http://cgmartini.nl/ |
| alchemical-analysis script | Klimovich et al., 2015 | https://github.com/MobleyLab/alchemical-analysis |
| scripts for generating simulations | this study | https://github.com/andreashlarsen/Larsen2021-C2 |

RESOURCE AVAILABILITY

Lead contact
Further information and requests for resources should be directed to and will be fulfilled by the Lead Contact, Mark Sansom (mark.sansom@bioch.ox.ac.uk).

Materials availability
No unique reagents or materials were generated in this study.

Data and code availability
This study did not generate new software. The simulation trajectory datasets supporting the current study have not been deposited as a public repository for MD simulation data does not yet exist. Coordinates of the models generated by this study (as representative frames from simulations revealing the interactions of C2 with PIP₂ and with cholesterol) are available from the corresponding author on request. Details of deposited coordinates employed are provided in the key resources table. Scripts for running the simulations are available at github.com/andreashlarsen/Larsen2021-C2.

EXPERIMENTAL MODEL AND SUBJECT DETAILS

No experimental models were used. The experimental data for the MD simulations consisted of the protein coordinate set (i.e. PDB files) as detailed in the key resources table.

METHOD DETAILS

Coarse-grained molecular dynamics (CG-MD) simulations
CG-MD simulations were done using the Martini 2.2 force field (de Jong et al., 2013) and performed in GROMACS 2018.6 (Abraham et al., 2015). The input structures were truncated to only contain the C2 domain, using PyMOL (The PyMOL Molecular Graphics System, version 2.0 Schrödinger, LLC) and missing residues were added using Modeller (Fiser et al., 2000). The protein was coarse-grained using the Martinize script (de Jong et al., 2013) with the default elastic network to maintain the tertiary structure. The protein was positioned above a lipid membrane using the Insane script (Wassenaar et al., 2015) with box size 7x7x18 nm³. For the simulations with the larger fragments of PTEN or SHIP2, the box size was 12x12x24 nm³. The protein placed at a minimum distance of 4.4 nm from the membrane, which is four times the VDW and electrostatic cutoff distances of 1.1 Å. 10% antifreeze water was added (Marrink et al., 2007) and the system was neutralised with Na⁺ or Cl⁻ ions. The protein was rotated through a randomly selected angle relative to the bilayer to avoid any bias in binding orientation. Three different membranes were generated for each protein: PC, PC:PS (molar ratio 80:20) and PC:PS:PIP₂.
Generating Distance vs. $R_{zz}$ Plots
Distances between the centre of mass of the protein and the centre of mass of the lipids were calculated for each frame using gmx distance (after centering the protein in the box). A reference frame was needed for calculating the rotation matrix. One frame for each protein was selected from one of the 25 simulations with membrane PC:PS:PIP$_2$. The protein from this frame was extracted, and used as reference protein, also for simulations with PC and PC:PS membranes. The rotation matrix was then calculated for each frame by comparing the protein in each frame with the reference protein using gmx rotmat, after fitting in the xy plane. $R_{zz}$ is the zz component of the rotation matrix.

Potential of mean force (PMF) calculations
PMFs were calculated using umbrella sampling with the centre-of-mass distance between protein and membrane as reaction coordinate (Figure 2E). As preparation for the PMF calculations, two steered MD simulation was performed to generate starting frames for the umbrella sampling along the reaction coordinate, using the GROMACS pull code. In the first, the protein was pushed into the bilayer, and in the second, the protein was pulled away from the bilayer. The distance between the centre of mass of the protein and centre of mass of the lipids was restrained with a harmonic potential with force constant of 1000 KJ/mol/nm$^2$ and a rate of 0.2 nm/ns in the z-direction (positive and negative direction for push and pull simulation, respectively). A position restraint with a force constant of 15,000 KJ/mol/nm$^2$ was applied on the strongly bound PIP$_2$ lipids to prevent them from being pulled out from the membrane with the protein. Frames were retrieved from the steered MD run every 0.05 nm until a centre-of-mass distance of 7.0 nm was obtained for the last frame (about 80 windows per system). Umbrella sampling was then performed by sampling each retrieved frames for 1 μs with a 2000 KJ/mol/nm$^2$ position restraint on the protein, but with the restraint on PIP$_2$ removed. The PMF was calculated using gmx wham (Hub et al., 2010), skipping the first 200 ns as equilibration, and using the bootstrap method (option nBootstrap) to get uncertainties on the PMF values.

Free energy perturbations (FEP)
Free energy perturbations were done as previously described (Corey et al., 2019, 2020), converting the Martini lipid POP2 (i.e. PIP$_2$) to Martini lipid POPC (Figure 2F). The head group beads were gradually changed, in 20 steps, as controlled by a parameter $\lambda$. Each of the four POP2 Martini lipids in the upper leaflet was converted individually to POPC, with the others kept as POP2. To keep the system neutral at all times, we gradually converted five sodium ions to neutral beads. Each frame was sampled for 1 μs, and the free energy was calculated using the alchemical-analysis script (https://github.com/MobleyLab/alchemical-analysis) and the MBAR method (Klimovich et al., 2015). The perturbation energies were also calculated for conversion of POP2 in a bilayer without bound protein. The reported energies are the difference between FEP energies for free and bound POP2 (Corey et al., 2019). Five repeats were made for calculation of uncertainties via the standard deviation.

Atomistic molecular dynamics (AT-MD) simulations
Selected frames with a membrane-bound C2 molecule were converted from CG (force field Martini 2.2) to AT (force field CHARMM36m (Best et al., 2012; Huang et al., 2017) and TIP3P water) using the CG2AT script (Vickery and Stansfeld, 2021). Atomistic simulations were run in GROMACS 2018.6 (Abraham et al., 2015). The system was first minimised, then equilibrated for 100 ps in the NVT ensemble and 100 ps in the NPT ensemble. The equilibrated system was run for 200 ns. Both equilibration steps and production run had 2 fs timesteps with 1.2 nm VDW and electrostatic cutoff distance, $\nu$-rescale temperature coupling keeping the temperature at 300 K with timeconstant of 0.1 ps, separately for protein, lipids and solvent. A Parrinello-Rahman barostat (Parrinello and Rahman, 1981) was applied to the NPT equilibration and production run with a semi-isotropic pressure coupling, keeping pressure at 1 bar with time constant of 5 ps and water compressibility of 4.5·10$^{-5}$ bar$^{-1}$. Long-range electrostatics were handled using the particle mesh Ewald method (Darden et al., 1993).

QUANTIFICATION AND STATISTICAL ANALYSIS
Statistical analysis details can be found in the relevant sections of the STAR methods and the table legends. In particular, PMF well depths (Table 1) and FEP errors were calculated as the mean plus/minus the standard deviation as detailed in the STAR methods above.

ADDITIONAL RESOURCES
No additional resources were generated by this study.