Structural basis for Ca\(^{2+}\)-mediated interaction of the perforin C2 domain with lipid membranes

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Running title: Ca\(^{2+}\)-dependent membrane binding of perforin C2

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Background: Perforin is a critical component of immune homeostasis, responsible for clearing virally infected cells.

Results: The molecular details of calcium binding by the perforin C2 domain are revealed.

Conclusion: Calcium-mediated structural rearrangement activates perforin for membrane binding.

Significance: The C2 domain regulates membrane binding by calcium-dependent events, which have now been defined for a mammalian perforin C2.

Natural killer (NK) cells and cytotoxic T-lymphocytes (CTL) deploy perforin (Prf) and granzymes to kill infected host cells. Perforin, secreted by immune cells, binds target membranes to form pores that deliver pro-apoptotic granzymes into the target cell. A crucial first step in this process is interaction of its C2 domain with target cell membranes, which is a calcium-dependent event. Some aspects of this process are understood, but many molecular details remain unclear. To address this, we investigated the mechanism of Ca\(^{2+}\) and lipid binding to the C2 domain by NMR spectroscopy and X-ray crystallography. Calcium titrations, together with dodecylphosphocholine (DPC) micelle experiments, confirmed that multiple Ca\(^{2+}\) ions bind within the calcium binding regions (CBR), activating perforin with respect to membrane binding. We have also determined the affinities of several of these binding sites and shown that this interaction causes a significant structural rearrangement in CBR1. Thus, it is proposed that Ca\(^{2+}\)-binding at the weakest affinity site triggers changes in the C2 domain that facilitate its interaction with lipid membranes.

Cell-mediated immunity is an essential host cell defence system against invasion by viruses, bacteria, and other agents of disease. Cytotoxic lymphocytes (CLs), cytotoxic T-lymphocytes (CTLs) and natural killer cells eliminate virally infected or oncogenic target cells. In concert with an immune response, the pore-forming protein perforin and the protease granzyme are released by CTLs into the synaptic cleft with target cells. Perforin binds target cell membranes and oligomerizes to form pores or lesions through which cytotoxic granzymes enter the cytosol of the target cell, with the subsequent initiation of apoptosis (1-5). Perforin-deficient CLs are unable to kill target cells or to clear many viral infections in the host cells, resulting in immune dysregulation and the development of the serious disease Familial Hemophagocytic Lymphohistiocytosis Type II (FHL-2) (2,6).

Perforin consists of three domains, an N-terminal membrane attack complex perforin-like / cholesterol-dependent cytolysin (MACPF/CDC) pore-forming domain, a central epidermal growth factor (EGF) domain, and a C-terminal membrane- and Ca\(^{2+}\)-binding C2 domain (Fig. 1A). The MACPF domain includes...
the central machinery of pore formation. Briefly, this domain mediates perforin oligomerisation and deploys two membrane-spanning regions (transmembrane hairpin-1 [TMH]-1 and TMH-2) that enter the membrane as amiphathic β-hairpins. The central EGF domain forms a 'shelf-like' assembly connecting the MACPF and C2 domains (7) (Fig. 1A). The role of this region remains to be fully understood. Finally, the C2 domain is critical for initial interaction with lipid membranes of the target cell.

Most C2 domains are found in intracellular proteins and are accordingly functional with respect to membrane binding in the presence of low concentrations of Ca\(^{2+}\). The perforin C2 domain is unusual in that it requires high (>150 μM) concentrations of Ca\(^{2+}\). Perforin is thus activated to bind membranes only in the extracellular environment, where the Ca\(^{2+}\) concentration is estimated to be between 1 and 3 mM (8-10). It is suggested that the requirement for extracellular concentrations of Ca\(^{2+}\) provides a critical control point for perforin function and helps regulate unwanted perforin activity within the cell (11).

The perforin C2 domain comprises eight β-strands in a β-sandwich fold (7). Three Ca\(^{2+}\)-binding regions (CBRs) contain conserved Asp residues and mediate the interaction with both Ca\(^{2+}\) and membranes. Different types of C2 domain bind different numbers of Ca\(^{2+}\) ions. For example, the synaptotagmin I (SytI) C\(_{2}\)B domain binds two Ca\(^{2+}\) ions (12,13). In contrast, the SytI C\(_{2}\)A domain coordinates three Ca\(^{2+}\) ions (14,15). However, some C2 domains, for example the PKC Apl II (16), can interact with membranes without first binding Ca\(^{2+}\).

In the original crystal structure of perforin (7), two Ca\(^{2+}\) ions were observed in the CBRs, which were scavenged from the environment and therefore presumably interact with the strongest binding sites. One Ca\(^{2+}\) ion (site II) was coordinated within CBR1 and CBR2 by conserved Asp residues (D435 and D483). The second Ca\(^{2+}\) ion was coordinated by a non-conserved Asp residue (D490) and found outside the CBR3 in an unusual binding position that appears to be unique to the perforin C2 domain (Fig. 1). Currently it is unknown how many Ca\(^{2+}\) ions are coordinated by perforin in the fully liganded state. However, the crystal structure of a related (39% identity) C2 domain only protein (SmC2P1 from *Scophthalmus maximus*) (Fig. 1B) revealed three Ca\(^{2+}\) ions coordinated within the CBRs (17).

In the CBRs of the perforin C2 domain, two pairs of conserved and solvent-exposed aromatic residues in CBR1 (W427 and Y430) and CBR3 (Y486 and W488) have been shown to be critical for membrane binding, as substitution of all four residues with alanine resulted in complete loss of function in effector-target cell- and red blood cell lysis-based assays (17). It is likely that these four aromatic residues are thus, essential for tight interaction of perforin C2 domain with lipid membranes. By analogy with SmC2P1, it is
suggested that Ca\(^{2+}\)-binding in the CBRs induced a conformational change in CBR1, and subsequently that the relative positions of the four aromatic residues changed to orientations facilitating interaction with lipid membranes (17). However, there is no direct structural evidence of Ca\(^{2+}\)-dependent rearrangements of the CBR1 in the perforin C2 domain and the numbers of bound Ca\(^{2+}\) ions and their binding sites remain unclear.

To address these questions, we have investigated the interactions of perforin C2 domain with Ca\(^{2+}\) ions by both NMR spectroscopy and X-ray crystallography, using highly soluble and stable variants of mouse perforin C2 domain in which W427, Y430, Y486 and W488 were mutated to alanine. We propose a detailed Ca\(^{2+}\)-binding mechanism of the perforin C2 domain and a role for bound Ca\(^{2+}\) in its interaction with lipid membranes.

**EXPERIMENTAL PROCEDURES**

*Protein expression and purification* - The C2 quad mutant (W427A/Y430A/Y486A/W488A) was cloned into the phagemid vector pComb3X for expression of the protein in the periplasmic space of *E. coli* Top10F\(^{\text{+}}\) (Life Technologies) with C-terminal HA and His\(_6\) tags (Fig. 1B). For crystallography, residues 410-535 of murine perforin (C2 quad\(_{410-535}\)) were cloned into pComb3X by introduction of 5\(^{\prime}\) and 3\(^{\prime}\) asymmetric SfiI sites using standard molecular biology techniques. For NMR, residues 410-526 (C2 quad\(_{410-526}\)) were cloned into pComb3X and, in order to avoid an unpaired cysteine in the construct, C524 was mutated to serine by quick-change mutagenesis according to the manufacturer’s protocol (Stratagene). The clones for expression of the D429N, D435N, D483N, D490 and D491N variants of C2 quad\(_{410-526}\) were constructed using pComb3X as the template to introduce the mutation by overlap PCR with flanking 5\(^{\prime}\) EcoRI and 3\(^{\prime}\) NcoI sites. The amino acid sequences of the constructs used in this study are shown in Fig. 1B. For expression of all constructs, cells were grown overnight in 3 mL of SB (Super Broth) media contained 0.5% glucose (starting culture) at 37 \(^{\circ}\)C and subsequently inoculated into 100 mL of SB media (sub-culture). The sub-culture was grown at 37 \(^{\circ}\)C until an OD\(_{600}\) of 0.5-0.6, and then transferred into 4 L of SB media (main culture). The main culture was continuously grown at 37 \(^{\circ}\)C to an OD\(_{600}\) of 0.6. The temperature was reduced to 23 \(^{\circ}\)C and protein expression was induced by addition of 10 \(\mu\)M isopropyl \(\beta\)-D-1-thiogalactopyranoside (IPTG) followed by incubation for \(~20\) h. Uniformly \(^{15}\)N- or \(^{13}\)C/\(^{15}\)N-labelled proteins were produced by using a high-cell density method (18) with modifications. Cells were grown using a similar procedure to that for expression of unlabelled protein, except that all SB media contained 0.5% glucose to prevent leaky protein expression and the main culture was grown overnight at 37 \(^{\circ}\)C without IPTG induction. After harvesting cells
from 4 L of main culture, cells were resuspended in 2L of minimal media containing $^{15}$NH$_4$Cl as the sole nitrogen source or $^{15}$NH$_4$Cl and $^{13}$C-glucose as the sole nitrogen and carbon sources for the expression of $^{15}$N- or $^{13}C^{15}$N-labelled proteins, respectively. Cells were incubated for 1 h at 23 ºC to adapt to minimal media and protein expression was induced by adding 10 µM IPTG. Cells were harvested ~20 h after IPTG induction.

Purification of both the C2 quad$_{410-535}$ and C2 quad$_{410-526}$ mutants was performed by periplasmic protein extraction with osmotic shock (19). The cell pellets were resuspended in binding/resuspension buffer: 50 mM Tris/Cl (pH 8.0), 300 mM NaCl, 20 mM imidazole and 0.02% NaN$_3$ (typical volume is 5 mL per 1g wet cells). An osmotic shock buffer (50 mM Tris/Cl (pH 8.0), 150 mM NaCl, 1 M sucrose and 2 mM EDTA) was added into the cell mixture with a 1:1 volume ratio and mixed well. After incubation at room temperature for 30 min, the osmotically shocked cells were collected by centrifugation at 10,000 rpm for 20 min. The pellets were resuspended in release buffer (5 mM MgSO$_4$ and protease inhibitor (Roche), typical volume 1 mL per 1g osmotic shocked cells) and incubated on ice for 30 min. The supernatant containing periplasmic proteins were collected by centrifugation at 15,000 rpm for 20 min and added to an equal volume of binding/resuspension buffer, then ~2% (v/v) Ni$^{2+}$-charged chelating Sepharose (GE Healthcare) was added. After 1.5 h incubation at 4 ºC, the mixture was transferred into a column and the supernatant was isolated from the resin by gravity flow. The resin was washed with 10 column volumes of binding/resuspension buffer followed by the same volume of wash buffer; 50 mM Tris/Cl (pH 8.0), 300 mM NaCl, 40 mM imidazole and 0.02% NaN$_3$. The purified protein was eluted in 20 mL elution buffer (50 mM Tris/Cl (pH 8.0), 300 mM NaCl, 300 mM imidazole and 0.02% NaN$_3$). The eluted fraction was applied onto a Superdex 75 10/300 GL or 16/60 size exclusion column (GE Healthcare) equilibrated in either crystallography buffer (25 mM HEPES pH 7.4, 150 mM NaCl with 0.02% NaN$_3$) or NMR buffer (20 mM Tris/Cl (pH 8.0), 300 mM NaCl and 2 mM EDTA). The peak fractions were analysed by SDS-PAGE and significant protein containing fractions pooled.

Crystallographic analysis - Crystallisation was carried out by the hanging drop method with a 1:1 mixture of protein and mother liquor at 20 ºC with the protein concentrated to 9.75 mg/mL. Apo-C2 quad$_{410-535}$ crystals were obtained in 0.1M MES pH 6.0, 0.2 M NaCl and 20% (w/v) polyethylene glycol 2000 monomethyl ether. Crystals of holo-C2 quad$_{410-535}$ were obtained in 0.1 M HEPES sodium pH 7.5, 0.2 M calcium chloride dihydrate and 14% v/v polyethylene glycol 400. The crystals were flash-cooled in liquid nitrogen using 25% (v/v) glycerol as the cryoprotectant. Data sets were collected at the Australian Synchrotron MX2 beamline at 100K
(20). The data were merged and processed using XDS (21), POINTLESS and SCALA (22). Five per cent of the data set was flagged as a validation set for calculation of the R_free.

Molecular replacement (MR) was carried out using the C2 domain of 3NSJ as a search probe (7). One molecule was found per asymmetric unit cell and an initial model was generated using PHENIX. Model building was performed using COOT (23), and refinement was performed using PHENIX (24). Crystallographic and structural analysis was performed using the CCP4 suite (25) unless otherwise specified. The figures were generated using MacPYMOL (26) and the structural validation was performed using MolProbity (27). All atomic coordinates and structural factors were deposited in the PDB under codes 4Y1S (apo-C2 quad410-535) and 4Y1T (holo-C2 quad410-535).

NMR spectroscopy - All NMR measurements were conducted in 20 mM HEPES buffer (pH 7.0) and 150 mM NaCl with 10% 2H2O at 25 ºC on a Bruker Avance 600 MHz NMR spectrometer equipped with a cryoprobe. Backbone resonance assignments of C2 quad410-526 were obtained from analysis of 3D HNCA, HN(CO)CA, HNCA CB and CBCA(CO)NH spectra of a 0.5 mM uniformly 13C- and 15N-labelled sample. The side chain amide resonances of Asn and Gln were assigned by 15N NOESY-HSQC spectra (70 ms mixing time) of the same sample. Ca2+ titrations were monitored by 1H-15N SOFAST-HMQC spectra (28) of 0.15 mM uniformly 15N-labelled samples with 200 ms relaxation delay. Spectra were acquired at 13 different [Ca2+] up to 30 mM. The backbone resonance assignments in 30 mM [Ca2+] were verified by 3D HNCA and CBCA(CO)NH spectra of a uniformly 13C- and 15N-labeled sample. The Ca2+ titration experiments induced non-linear chemical shift perturbations (CSPs), so the Ca2+ concentration-dependent CSPs were calculated by

$$\Delta\delta_{obs} = \sum_{i=1}^{n} \Delta\delta_i$$

where

$$\Delta\delta_i = \left[\left(\delta_i - \delta_{i-1}\right)^2 + 0.14(\delta_i - \delta_{i-1})\right]^{1/2}$$

where n is the total number of titration points and i is i-th titration point at each [Ca2+] from 0 to 30 mM. \(\delta_i\) is the chemical shift of either 1H or 15N at i-th [Ca2+]. \(\Delta\delta_i\) is the CSP between the [Ca2+] of interest and the next lower [Ca2+]. If the chemical shifts at the next lower [Ca2+] were not detected because of exchange broadening, the observed chemical shifts at the nearest lower [Ca2+] were used for subtraction.

The DPC titration experiments were carried out by adding aliquots of a 1M stock solution of 2H38-DPC (Sigma-Aldrich) in the same buffer. The chemical shift perturbations, \(\Delta\delta\), upon addition of DPC was calculated using \((\Delta\delta^H)^2 + (0.14\Delta\delta^{15}N)^2)^{1/2}\) (29), where \(\Delta\delta^H\) and \(\Delta\delta^{15}N\) are the chemical shift differences of the backbone amide proton and nitrogen resonances at 50 mM.
[DPC] compared with the absence of DPC. A mean dissociation constant, $K_d$, for the DPC micelles was obtained from the dependence of $^1$H chemical shifts of selected residues (D429, D485, D489 and D490) upon [DPC] for C2 quad$_{410-526}$, using equation 2 (30)

$$\Delta \delta_1^H = \frac{[M_0] - \text{cmc}}{K_d \times n + [M_0] - \text{cmc}}$$  \hspace{1cm} (2)

where $\Delta \delta_1^H$ is the difference between the backbone amide proton chemical shift at each [DPC] and the shift in the absence of DPC, $\Delta \delta_{max}^1$H is the chemical shift difference at saturating [DPC], $[M_0]$ is the monomeric [DPC], cmc is the critical micelle concentration (1.5 mM (30)) and $n$ is the aggregation number of DPC (54 (30)).

The chemical shifts of backbone amide $^{15}$N and $^1$H, $^{13}$C$\alpha$ and $^{13}$C$\beta$ were deposited in the BMRB under accession number 25481.

RESULTS

Expression and purification of perforin C2 domain mutants - Expression of the perforin C2 domain in isolation at levels required for crystallography and NMR spectroscopy has not been successful to date, despite extensive attempts over many years (17). We reasoned that the four aromatic residues in the CBRs (W427, Y430, Y486, and W488) (7) represent the major barrier to expression, possibly through driving association of the C2 domain with E. coli membranes during protein expression (Fig. 1A). By mutating these four hydrophobic aromatic residues to alanine (Fig. 1B), using the crystal structure of perforin to accurately define the domain boundaries, and exploiting periplasmic expression, two different C2 mutants W427A/Y430A/Y486A/W488A were expressed successfully (C2 quad$_{410-535}$ and C2 quad$_{410-526}$).

The crystal structure of C2 quad$_{410-535}$ - The structure of apo-perforin remains unknown as the perforin structure contains the aforementioned two Ca$^{2+}$ ions (7). Thus, we determined the crystal structure of C2 quad$_{410-535}$ in both the partially and fully calcium-liganded states (Fig. 2 and Table 1). In the 1.6 Å apo-C2 quad$_{410-535}$ structure (Fig. 2A and B) a single Ca$^{2+}$ ion was coordinated in the non-canonical position at residue D490, which mirrors one of the two atoms bound in the full-length perforin structure. This Ca$^{2+}$ ion was modelled as a 0.5 fractional occupancy, which indicates that, despite the presence of EDTA some Ca$^{2+}$ was scavenged during the purification process. The 2.6 Å holo-C2 quad$_{410-535}$ structure revealed five Ca$^{2+}$ ions, four of which occupy the groove in the jaws of the C2 domain in addition to the non-canonical Ca$^{2+}$ ion coordinated by D490 (Fig. 2A and C). In comparing the apo- and holo-C2 quad$_{410-535}$
structures, the most striking feature is the absence of the 427-431 portion of CBR1 loop (Fig. 2A and B), consistent with predictions that this region in perforin is mobile in the absence of calcium (17).

The most significant structural rearrangement driven by Ca\(^{2+}\) binding involves CBR1. This region contains the critical lipid binding residues W427 and Y430 (both mutated to alanine in this protein) that make key contacts with Ca\(^{2+}\) ions in site I, II and III. Here Ca\(^{2+}\) binding is achieved through T432 and D429. T432, which is the only Ca\(^{2+}\) binding residue visible in electron density in the apo structure, moves 6.8 Å (Fig. 2B). D429 moves 11.4 Å, from its position in the full-length perforin structure to engage Ca\(^{2+}\) (Fig. 2C), thus driving this significant structural rearrangement in CBR1. Elsewhere, conformational changes in the side chains of D485 and D491 in CBR3 position these residues such that they interact with site IV, I and II. Finally, a modest rearrangement of N454 on CBR2 completes the coordination of site III (Fig. 2D and E).

CBR3 undergoes only modest conformational changes upon calcium binding; indeed, the backbone atoms of the previously identified lipid binding residues 486 and 488 (both alanine in this protein) remain essentially unchanged between the apo and holo forms. Thus, the key event driven by Ca\(^{2+}\) binding must be the conformational change in CBR1 and the repositioning of W427 and Y430 into an orientation suitable for interacting with membranes. These data are consistent with previous studies showing that the W427A and Y430A mutations caused the most substantial functional defects with respect to perforin membrane binding (17). In comparison to the SmC2P1 structure (PDB codes: 3W56, 3W57), similar positioning of Ca\(^{2+}\) ions at sites I, II and III was observed. This bonding pattern differs from that of MUNC13-C2B, which is the most structurally similar C2 domain characterised previously (7,17,31). Unlike, full-length perforin and the structures of apo- and holo-C2 described here, SmC2P1 does not coordinate Ca\(^{2+}\) at the non-canonical position.

In the murine perforin structure, the CBR1 loop is stabilized via crystal contacts; our new data confirm the suggestions that CBR1 is largely mobile in the absence of Ca\(^{2+}\). Given that the site II Ca\(^{2+}\) is occupied in murine perforin (7) we reason this site is probably the strongest affinity Ca\(^{2+}\) binding site inside the jaws of the CBRs. However, Ca\(^{2+}\) binding to site II alone is clearly insufficient to drive conformational change in CBR1. Further, since site IV makes no contact with CBR1 we reason that binding of Ca\(^{2+}\) to site I or III must represent the key event that brings W427 and Y430 into a position suitable for interacting with membranes. To further study these events, and in order to understand the order of Ca\(^{2+}\) binding events, we conducted NMR studies.

Analysis of Ca\(^{2+}\)-binding mode of the C2 quad
we undertook \( \text{Ca}^{2+} \) titration experiments in solution, monitored by NMR. The \(^{1}\text{H}^{15}\text{N} \text{SOFAST-HMQC} \) spectrum of the \( \text{C2 quad}_{410-535} \) used for crystallography showed a number of random coil chemical shifts in the central region of the spectrum, mostly arising from additional non-native residues at the C-terminus introduced during cloning (Fig. 1B). Therefore, we created a new construct in which the C-terminal residues were removed after residue 526 (C2 quad\(_{410-526}\)) and only a hexa-His-tag was attached at the C-terminus (Fig. 1B). The \(^{1}\text{H}^{15}\text{N} \text{SOFAST-HMQC} \) spectrum of this new construct was less cluttered in the central region (Supplementary Fig. S1A).

Complete backbone resonance assignments of C2 quad\(_{410-526}\) (Supplementary Fig. S1B) were obtained by conventional triple-resonance experiments, except for proline residues, two residues at the N-terminus (410, 411), and residues 461, 471, and 472. Residues 461, 471, and 472 are located in loop regions in the crystal structure and are subject to exchange broadening among more than two conformers and/or exchange with water molecules, resulting in undetectably broad resonances under our solution conditions.

Prior to \( \text{Ca}^{2+} \) titrations, protein samples were treated with EDTA to ensure that residual \( \text{Ca}^{2+} \) ions were completely removed, and then the EDTA was removed. The 13 spectra with different \( \text{Ca}^{2+} \) concentrations are superimposed in Fig. 3. The chemical shifts of many resonances in the CBRs changed smoothly with increasing \( \text{Ca}^{2+} \), indicating that the exchange between free and bound \( \text{Ca}^{2+} \) is fast on the chemical shift time scale (Fig. 3A). However, several of these chemical shift changes were non-linear, as exemplified by the resonances of G428 or D483. This reflects the fact that different \( \text{Ca}^{2+} \)-bound states have different backbone chemical shifts and several states contribute simultaneously to the observed chemical shifts at any given [\( \text{Ca}^{2+} \)]. Complex titration profiles were also observed for the resonances of A484 and D490 (Fig. 3B and 3C).

The calcium titrations were conducted at a protein concentration of 0.15 mM and a [\( \text{Ca}^{2+} \)] range of 0-30 mM. The initial chemical shift perturbations (Fig. 3B and 3C) were largely complete by 0.3 mM \( \text{Ca}^{2+} \), implying \( K_d \) values < 0.05 mM. The next transition is evident at around 3 mM, reflecting \( K_d \) values < 0.4 mM for those binding events, whereas the chemical shift perturbations at the highest [\( \text{Ca}^{2+} \)] were still incomplete at 30 mM, implying a \( K_d \) value of 5-10 mM for the final \( \text{Ca}^{2+} \) binding event. At the typical physiological \( \text{Ca}^{2+} \) concentrations of the extracellular space, approximately 1-3 mM (8-10), the weakest \( \text{Ca}^{2+} \) binding site would therefore not be fully occupied.

As expected, the number of \( \text{Ca}^{2+} \)-binding sites is five based on the crystal structure of holo-C2 quad\(_{410-535}\) and the \( K_d \) values at several of those sites are expected to be similar to one another. Therefore, the changes in backbone
amide chemical shifts are separately summarised up to 3 mM and from 3 to 30 mM [Ca^{2+}] in Fig. 4A. The residues perturbed by ≥0.2 ppm at 3 and 30 mM [Ca^{2+}] are mapped on the crystal structure of holo-C2 quad_{410-535} in red and orange, respectively, in Fig. 4B. The significantly perturbed residues up to 3 mM [Ca^{2+}] are found in the vicinity of the five Ca^{2+} ions, indicating that the Ca^{2+} binding mode of the C2 quad mutant in solution (C2 quad_{410-536}) is the same as in the crystal (C2 quad_{410-535}). In addition, significant additional CSPs were subsequently observed in CBR1 and CBR3 up to 30 mM [Ca^{2+}].

As noted above, most of the perturbed resonances were detected at all [Ca^{2+}], but some disappeared with increasing [Ca^{2+}] (e.g. T431 in Fig. 3A). This is a consequence of intermediate exchange among two or more Ca^{2+}-free and multiple Ca^{2+}-bound states for these resonances. Several of those resonances reappeared at high [Ca^{2+}] (e.g. N455 and D485 in Fig. 3A), indicating that they are in a single major conformation at the highest concentrations examined. By contrast, the resonances of A430, T431, T432 and A486, shown in yellow in Fig. 4B, disappeared with increasing [Ca^{2+}]. These residues presumably sample more than one conformation even at the highest [Ca^{2+}], which is close to saturation of all Ca^{2+}-binding, and these conformers are exchanging with one another on an intermediate time scale that gives rise to peak broadening. These residues are involved in the quad mutation sites, which contain the hydrophobic aromatic residues Y430 and W486 in the wild-type perforin C2 domain.

**Detailed Ca^{2+}-binding mechanism of the C2 quad mutant** - To analyse details of the Ca^{2+}-binding mechanism of C2 quad_{410-526}, we mutated the conserved Ca^{2+}-binding Asp residues and non-conserved D490 to Asn (D429N, D435N, D483N, D491N and D490N) and repeated the Ca^{2+} titration experiments. Two distinct areas of Ca^{2+} titration spectra for each mutant are shown in Fig. 5 (extended spectra are shown in Supplementary Fig. S2-S5). The Ca^{2+} titration spectra of the mutants showed significant differences from those of C2 quad_{410-526}, confirming that these Asp residues bind Ca^{2+} ions, in agreement with the crystal structure of the holo-C2 quad_{410-535}.

In the D491N mutant (Fig. 5), significant differences in the perturbation curves were observed for G428, N455 and A484 in comparison with the Ca^{2+} titration spectra of C2 quad_{410-526} (Fig. 3). Since the crystal structure of holo-C2 quad_{410-535} shows that the carboxyl group of D491 coordinates two Ca^{2+} ions at sites I and IV (Fig. 2), mutation of D491 to Asn substantially reduced the Ca^{2+} binding affinity at these sites. A turning point in the titration curves was still observed at 3 mM [Ca^{2+}], as seen for G428 and D483, indicating that the weakest affinity site still exists in the D491N mutant. Therefore, the weakest affinity site is either site II or III. Since site II was occupied by Ca^{2+} scavenged from the buffer in the crystal structure.
of full-length perforin (7) we argue that site III must be the weakest affinity site.

Site III was confirmed as the weakest affinity site by examination of the D483N mutant. Ca\(^{2+}\) titration spectra of the D483N mutant showed that the resonances of G428 and N483 continued to shift beyond 3 mM [Ca\(^{2+}\)], suggesting that the weakest affinity site is maintained in this mutant (Fig. 5). The crystal structure of the holo-C2 quad\(_{410-535}\) showed that the carboxyl group of D483 interacts with two Ca\(^{2+}\) ions at sites I and II but not site III (Fig. 2), so the observation of chemical shift perturbations at [Ca\(^{2+}\)] >3 mM in this mutant supports the conclusion that site III is the weakest affinity site. This was also supported by Ca\(^{2+}\) titration experiments of the D429N and D435N mutants. The carboxyl groups of both D429 and D435 coordinate a Ca\(^{2+}\) ion at site III (Fig. 2). In the Ca\(^{2+}\) titration spectra of the D429N mutant, similar chemical shift perturbation profiles to those for the D491N mutant were observed for resonances of G428, N455 and D483 at low [Ca\(^{2+}\)], but beyond 3 mM [Ca\(^{2+}\)], in contrast, the chemical shift perturbations were very small, indicating loss of the weakest affinity site (Fig. 5). Similarly, the chemical shift changes of the D435N mutant at high [Ca\(^{2+}\)] (> 3 mM) were as small as those of the D429N mutant (Fig. 5), confirming the absence of the weakest affinity site.

D429 coordinates three Ca\(^{2+}\) ions at sites I, II and III (Fig. 2). However, the D429N mutant may still be able to coordinate a single Ca\(^{2+}\) ion at site II, as its chemical shift perturbation patterns were similar to those of the D491N mutant at low [Ca\(^{2+}\)]. As mentioned previously, the D491N mutation does not affect Ca\(^{2+}\) binding at site II. In the D435N mutant, significantly different chemical shift perturbation patterns were observed for residues G428, A484, W453, N455 and D483, showing that the D435 mutation affects a different Ca\(^{2+}\)-binding site from those of D491 and D429 (Fig. 5). This is in good agreement with the crystal structure of holo-C2 quad\(_{410-535}\), in which the side chain carboxyl of D435 coordinates Ca\(^{2+}\) at sites II and III, but not sites I and IV (Fig. 2). The D435N mutant is still capable of coordinating Ca\(^{2+}\) ions at site I and IV (and the non-canonical position), and the observed titration curves reflect Ca\(^{2+}\) binding events only at these sites. In the crystal structure of holo-C2 quad\(_{410-535}\) (Fig. 2), all of the backbone amides in CBR2 are > 10 Å away from sites I and IV, implying that the resonances of backbone amides in the CBR2 should be less affected by the Ca\(^{2+}\)-binding events at these sites. Indeed, the resonances of W453, N454 and N455 in the CBR2 were almost unchanged over the entire Ca\(^{2+}\) titration in the D435N mutant.

D490 coordinates a single Ca\(^{2+}\) ion at a non-canonical position (site V) outside CBR3 (Fig. 2). Ca\(^{2+}\) titrations of the D490N mutant showed similar chemical shift perturbation patterns to those of the C2 quad\(_{410-526}\) (Supplementary Fig. S6), except that several
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resonances appeared to be affected by local conformational differences associated with the point mutation. At 30 mM [Ca²⁺], the spectrum of the D490N mutant was similar to that of C2 quad₄₁₀-₅₂₆ (Supplementary Fig S7), suggesting that Ca²⁺ bound at the non-canonical position had no significant effect on the saturated Ca²⁺-binding state of the C2 quad mutant.

Interaction of C2 quad mutant with membranes and role of Ca²⁺ ions - Because of the replacement of four hydrophobic aromatic amino acid residues with alanine, the C2 quad mutant is considered to have lost its membrane binding capacity (17). However, NMR is capable of detecting weak interactions that cannot be readily monitored by other methods. To analyse the interaction of the C2 quad mutant with membranes, dodecylphosphocholine (DPC) titration experiments were performed. DPC titration spectra of C2 quad₄₁₀-₅₂₆ at three different [Ca²⁺] (0, 2 and 30 mM) are shown in Fig. 6 (extended spectra are shown in Supplementary Fig. S8-S10). Considering the comparatively high protein concentration and the expected K_d value of Ca²⁺ at the weakest affinity site (> 5 mM), complete Ca²⁺-binding was observed at ~30 mM [Ca²⁺], even though Ca²⁺ binding at the weakest affinity site commenced at ~3 mM [Ca²⁺]. In the presence of 30 mM [Ca²⁺] (Fig. 6A), several backbone amide resonances were linearly perturbed with increasing [DPC]. Not only did chemical shifts change, but the intensities of resonances were also attenuated as a consequence of interaction with the DPC micelles, the critical micelle concentration of which is ~1.5 mM. The chemical shift perturbations upon addition of 50 mM DPC in the presence of 30 mM Ca²⁺ are plotted on the primary sequence of the C2 quad₄₁₀-₅₂₆ in Fig. 6D. Residues with chemical shift perturbations > 0.05 ppm are mapped on the surface of the crystal structure of holo-C2 quad₄₁₀-₅₃₅ (Fig. 6E). The perturbed residues were localized to a surface containing the CBRs. In addition, the side chain amide resonance of N454 (N454ε) was also strongly affected by DPC titration, disappearing beyond 3 mM DPC (Fig. 6A). The side chain of this residue is exposed and oriented downwards in the right view in Fig. 6E. These results strongly suggest a specific interaction with DPC micelles. Significantly changed ¹H chemical shifts (Δδ¹H) of D429, D485, D489 and D490 (Fig. 6D) were fitted to equation 2 (see “Experimental Procedures”) (Fig. 7A), and the dissociation constant (K_d) of C2 quad₄₁₀-₅₂₆ against DPC micelles was determined as ~1mM.

To investigate the effect of Ca²⁺ ions on the interaction with membranes, DPC titration experiments were performed at different [Ca²⁺]. In the absence of Ca²⁺ (Fig. 6B), resonances indicative of unfolded or disordered protein appeared in the central region of the spectrum at 3 mM DPC, and these resonances intensified at 10 mM DPC. These data imply that the interaction with DPC micelles partially unfolds the structure of the C2 quad₄₁₀-₅₂₆ in the absence
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To assess how many Ca²⁺ ions are required for interaction with membranes, we undertook a DPC titration in the presence of 2 mM Ca²⁺, in which the weakest affinity site should be largely unoccupied (Fig. 6C). No CSPs were observed except for the C-terminal residues. In addition, the C2 quad was partially unfolded at 10 mM [DPC], and adding increasing [DPC] resulted in loss of the original well-resolved resonances.

Importance of the weakest Ca²⁺-binding site for interaction with lipid membranes - The titration of C2 quad with DPC demonstrated that occupancy of Ca²⁺ at the weakest affinity site is critical for interactions with membranes (Fig. 6). The importance of the weakest affinity site was confirmed by DPC titrations with the D491N, D429N, D435N, D483N and D490N mutants in the presence of 30 mM [Ca²⁺], as shown in Fig. 7B-7F (extended spectra are shown in Supplementary Fig. S11-S15). DPC titration of the D491N mutant in the presence of 30 mM Ca²⁺ showed no significant perturbations of the backbone amide resonances. However, the side chain amide resonance of N454ε was clearly perturbed (Fig. 7B). The CSP of N454ε was also observed in C2 quad at 30 mM [Ca²⁺]. In addition, no unfolded resonances were observed up to 50 mM [DPC]. Therefore, the D491N mutant can still bind to DPC micelles in the proper orientation. The much smaller chemical shift change indicates that the affinity of DPC micelles became much weaker because of the loss of Ca²⁺-binding sites I and IV.

By contrast, resonances indicative of unfolding caused by non-specific interaction with DPC micelles were observed in the D429N and D435N mutants in the presence of 30 mM [Ca²⁺] with increasing [DPC] (Fig. 7C and 7D). Both mutants lose the weakest affinity site, resulting in partial Ca²⁺-bound states even at 30 mM Ca²⁺. Indeed, in the presence of 50 mM DPC, the spectra of both mutants are very similar to the C2 quad at 2 mM [Ca²⁺], in which the weakest affinity site should be unoccupied (Fig. 6C). These data support the critical role of Ca²⁺ binding at the weakest affinity site in enabling the C2 quad mutant to interact with lipid membranes. Similar destabilization was observed as the D483N mutant interacted with DPC micelles, which maintains the weakest affinity site (Fig. 7E). However, this mutant lost Ca²⁺-binding sites II and possibly I, indicating that sequential Ca²⁺ occupancy at Ca²⁺-binding sites II and III is important for proper interaction of the C2 quad mutant with DPC micelles. The DPC titration of the D490N mutant showed similar chemical shift perturbation patterns to that of the C2 quad (Fig. 7F), indicating the Ca²⁺-binding to D490 is not important for interaction with membranes.

DISCUSSION

Our studies demonstrate that the C2 quad mutant binds multiple Ca²⁺ ions in the CBRs, the positions of which were identified in the crystal structure. Substitution of the four aromatic
residues W427, Y430, Y486 and W488 with alanine resulted in a loss of perforin activity (17), although these four residues are not involved in Ca\(^{2+}\) binding among known C2 structures (14-16,32-37). Indeed, the mutations of all four residues to alanine did not influence Ca\(^{2+}\) binding as confirmed by thermodynamic stability (17). The C2 quad mutant, therefore, is a suitable model for analysing Ca\(^{2+}\)-binding to the perforin C2 domain.

The crystal structures of the apo- and holo-C2 quad\(_{410-535}\) (Fig. 2C-D) demonstrate good agreement with previous results identified through mutational analyses and inferred from the C2-domain-only protein from *S. maxmius* (17,38). These structures have identified the exact positions of the co-ordinated Ca\(^{2+}\) ions and demonstrated that the C2 domain is capable of binding five Ca\(^{2+}\) ions. These structures also provided further evidence for the non-canonical Ca\(^{2+}\) ion co-ordinated at D490, which was observed previously in the full-length perforin crystal structure but is known to be non-essential for perforin function (38). CBR1 is highly mobile in the absence of Ca\(^{2+}\), placing it in a conformation that is sub-optimal for binding membranes. Upon Ca\(^{2+}\) binding, CBR1 undergoes a very large movement and the ‘jaws’ of the C2 domain co-ordinate four Ca\(^{2+}\) ions within the hydrophobic groove, predominantly mediated by Asp residues. Furthermore, Ca\(^{2+}\) binding is a critical regulatory step in the hydrophobic-dependent membrane binding of perforin, bringing the CBR1 and CBR3 into close proximity forming a hydrophobic cleft/groove.

Whilst the structures of the C2 domains do not reveal any obvious rearrangements that could possibly trigger conformational change within other domains of perforin, in particular the MACPF domain, our holo-C2 quad\(_{410-535}\) structure is the first evidence of the locations of Ca\(^{2+}\) in the C2 domain of perforin and the first indication of an additional canonical Ca\(^{2+}\) co-ordination position, site IV.

As demonstrated by our NMR titrations with Ca\(^{2+}\), the affinity of one Ca\(^{2+}\)-binding site is significantly weaker compared to other sites. From mutation analyses of the four conserved Asp residues D429, D435, D483 and D491 in the CBRs, we defined the weakest affinity site to be site III. The crystal structure of the holo-C2 quad\(_{410-535}\) showed that the side chain carboxyl group of D429 in CBR1 coordinates three Ca\(^{2+}\) ions at sites I, II and III, whereas this residue, together with the flanking two residues, is disordered in the apo form (Fig. 2). The crystal structure of perforin C2 domain revealed that the CBR1 domain swings out from the Ca\(^{2+}\)-binding pockets and the carboxyl side chain of D429 is directed toward solvent (7). Comparing the holo-C2 quad\(_{410-535}\) mutant and the perforin C2 domain, significant structural differences are seen only in the relative orientation of CBR1 (Fig. 2). A previous study (17) suggested that bound Ca\(^{2+}\) ions induced conformational rearrangement of CBR1, which then facilitated re-positioning of
the four key hydrophobic residues to interact with lipid membranes.

The crystal structure of the holo-C2 quad\textsubscript{410-535} showed that D435 binds two Ca\textsuperscript{2+} ions at sites II and III, and D491 coordinates a Ca\textsuperscript{2+} ion at site I (and site IV), which is consistent with the crystal structure of SmC2P1 (17). In contrast, D429 interacts with three Ca\textsuperscript{2+} ions at site I, II and III, which differs from SmC2P1, where this residue coordinates Ca\textsuperscript{2+} ions only at sites I and II. Based on Ca\textsuperscript{2+} titration results by NMR, D429 will be able to coordinate two Ca\textsuperscript{2+} ions at sites I and II at low [Ca\textsuperscript{2+}]. At higher than extracellular [Ca\textsuperscript{2+}], in contrast, D429 is capable of coordinating one more Ca\textsuperscript{2+} ion at site III; subsequently a conformational re-arrangement of CBR1 is induced. The coordination of Ca\textsuperscript{2+} ion at site III by the carboxyl group of D429 is also seen in other C2 domains, for example SytI (12,14) and the PKC-\textbeta (35), both of which bind three Ca\textsuperscript{2+} ions in the CBRs.

The importance of the conformational re-arrangement of CBR1 for the C2 domain interaction with membranes was demonstrated by DPC titration experiments. It is clear that bound Ca\textsuperscript{2+} stabilises the structure of the C2 quad mutant in its interaction with DPC micelles. These data are in good agreement with previous evidence indicating that perforin is stabilized in the presence of Ca\textsuperscript{2+}, as determined by a thermal stability assay (17). Importantly, stabilization of the C2 quad mutant by Ca\textsuperscript{2+} is highly dependent on the [Ca\textsuperscript{2+}], and complete stabilization requires full occupancy of Ca\textsuperscript{2+} ion at the weakest affinity site (site III). If the weakest affinity site is unoccupied, DPC micelles interact with the C2 quad mutant non-specifically, leading to partial unfolding. The Ca\textsuperscript{2+} titration spectra showed that Ca\textsuperscript{2+}-binding at the weakest affinity site starts around 3 mM [Ca\textsuperscript{2+}], which is well matched with physiological extracellular [Ca\textsuperscript{2+}]. However, the Ca\textsuperscript{2+}-free state at the weakest affinity site is still dominant around these [Ca\textsuperscript{2+}]. Because the exchange between the free and the Ca\textsuperscript{2+}-bound forms is fast in the NMR time scale, the NMR spectra are observed as a population average of the free and the Ca\textsuperscript{2+}-bound forms. Therefore, observation of the fully Ca\textsuperscript{2+} bound state requires excess Ca\textsuperscript{2+} (~30 mM), at which the Ca\textsuperscript{2+}-bound state is dominant at the weakest affinity site III. A previous study (38) demonstrated that the Ca\textsuperscript{2+} concentration required for full activation of perforin in a SRBC lysis assay is \textasciitilde250 \mu M, which is less than the physiological extracellular Ca\textsuperscript{2+} concentration. In the case of native perforin, the interaction with membrane should be tighter than the C2 quad mutant. Once Ca\textsuperscript{2+} binds to the weakest affinity site, the native C2 domain can interact with the membrane, which may occur when a small proportion of the protein is Ca\textsuperscript{2+}-bound, rather than requiring the dominant population to be in a fully Ca\textsuperscript{2+}-bound state. This allows for oligomerisation of perforin and activity at less than physiological extracellular [Ca\textsuperscript{2+}].
site III was confirmed by the results of DPC titration of the D491N mutant, in which the C2 quad_{410-526} was still stable at 30 mM Ca^{2+} even when the Ca^{2+}-binding site I was not occupied. By contrast, the site III-deficient mutants, D429N and D435N, were unstable in the presence of DPC micelles even at 30 mM Ca^{2+}. In addition, the NMR results for the D483N mutant clearly indicated that the proper conformational re-arrangement of CBR1 is required for Ca^{2+} binding at site III as well as site II. Our results are consistent with previous studies that D429, D435 and D483, but not D491, are critical residues for plasma membrane binding and cell lysis (38).

The conformational re-arrangement of CBR1 enables the C2 quad_{410-526} to interact with DPC micelles thorough the CBRs. Based on our observations, we propose the following mechanism of perforin C2 domain interaction with lipid membranes. At less than extracellular [Ca^{2+}], although the C2 domain coordinates two Ca^{2+} ions in the CBRs, the CBR1 does not change conformation and is in the “inactive form”. At higher than extracellular [Ca^{2+}], three Ca^{2+} ions are bound within the CBRs. The Ca^{2+}-binding at the site III, which is the weakest affinity site, induces a conformational re-arrangement of CBR1 that leads to the “active form”, facilitating interaction with membranes (Fig. 8). The affinity of the C2 quad_{410-526} for DPC micelles is very low (~ 1 mM), which is insufficient for functional activities of perforin. Indeed, no perforin activity was observed in the full-length perforin quad mutant (17) because of the lack of four hydrophobic aromatic residues that are crucial for proper interaction with membranes. On the basis of the Ca^{2+} titration results, the regions including the quad mutations will not be fixed a single conformer even at close to saturating concentrations of Ca^{2+}, which may reduce the ability of the C2 quad_{410-526} to interact with the membrane. As indicated above, however, the mutation of four key hydrophobic aromatic residues to alanine does not affect Ca^{2+}-binding to the perforin C2 domain. Therefore, the Ca^{2+}-dependent conformational switch from the “inactive from” to the “active form” should also occur in native perforin C2 domain. On the basis of our Ca^{2+} titration results, the CBRs are not be fixed in a single conformation even at close to saturating concentrations of Ca^{2+}. This conformational plasticity may be necessary for the initial interaction with membranes. Subsequently, in the case of native perforin C2 domain, the four key hydrophobic aromatic residues would be re-arranged into the orientation required for tight binding to lipid membranes. Importantly, higher extracellular [Ca^{2+}] promotes membrane binding of the C2 domain on granule exocytosis, whereas low [Ca^{2+}] prevents premature activation of perforin (3,38-40). Our crystal and NMR data are in excellent agreement with these biological results.

In conclusion, we have clarified the mechanism of the perforin C2 domain interaction
with membranes and the role of Ca$^{2+}$ in that process. Our results represent the first observation of structural details regarding the interaction of the perforin C2 domain with lipid membranes and will facilitate further understanding of perforin function.

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**Conflict of Interests**

The authors declare that they have no conflicts of interest with the contents of this article.

**Author contributions**

HY, PJC, JCW and RSN designed experiments. HY, PJC, EWWL and RHPL performed experiments. All authors contributed to interpretation of the data. HY, PJC, JCW and RSN wrote the manuscript. All authors reviewed the results and approved the final version of the manuscript.
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**FIGURE LEGENDS**

**Figure 1. The crystal structure of murine perforin.**

(A) The crystal structure of murine perforin, PDB code 3NSJ (7), is shown with the C2 domain colored in magenta. The hydrophobic aromatic residues (W427, Y430, Y486 and W488) are shown in stick representation where the side chain density of Y486 is disordered and the extreme C-terminus is colored in blue. Two Ca\(^{2+}\) ions are indicated as magenta spheres. The MACPF and EGF domains are shown in gray. The ‘shelf-like’ region is boxed with dashed line. Disulfide bonds between C496 and C509, and C524 and C535 are represented by green and cyan sticks, respectively. The residues (410, 411, 461, 471, and 472), whose resonances were undetected in the current NMR study are shown in yellow. (B) Sequence alignment of mouse perforin (Prf) C2 and the C2 quad mutant constructs used in the analyses carried out in this study. Amino acid sequences of the mouse perforin C2 domain (C2 WT) and the C2 quad mutants used for the current crystal (C2 quad\(_{410-535}\)) and NMR studies (C2 quad\(_{410-526}\)), together with SmC2P1 are aligned. The N-terminal signal sequence and the C-terminal additional sequences including the hemagglutinin (HA) and/or His\(_6\) tag are shown in grey. The four alanine residues substituted from the WT aromatic residues are illustrated in red. Five conserved Asp residues are highlighted in yellow. The positions of Asp-Asn mutations (D429, D435, D483, D490 and D491) are colored in blue. Sequence numbers of the mouse perforin C2 domain and SmC2P1 are shown above and below the primary sequences, respectively. The positions of the CBRs are boxed. The signal sequence was cleaved during the export process at the position indicated by arrow, resulting in an alanine overhang at the N-terminus. Pairs of Cys residues that form disulfide bonds are highlighted in the same colors as in (A). In the C2 quad\(_{410-526}\), C524 was mutated to serine (cyan) to remove a free thiol in the construct.

**Figure 2. The crystal structure of apo- and holo-C2 quad\(_{410-535}\) superimposed with the C2 domain from full-length perforin.**

(A) The C2 domain of 3NSJ (magenta) superimposed with both the apo- (cyan) and holo-C2 (orange) quad\(_{410-535}\). CBR 1 and 3 are identified and the corresponding colored spheres represent the Ca\(^{2+}\) ions for each structure. The view is rotated by 90° to view the C2 domain from the membrane perspective. The CBR1 in the apo-C2 quad\(_{410-535}\) structure is disordered and the loop was not built into the density (cyan dashed line).

(B) Superimposed structures of the apo- (cyan) and holo-C2 (orange) quad\(_{410-535}\) to illustrate the re-organization that occurs in the residues involved in Ca\(^{2+}\) coordination. The residues from A427 to A431 (CBR1 – cyan dashed line) were not visible in the electron density map of the apo structure and were not
modelled into the final structure. Significant movement is observed in the CBR1, where residue T432 moves through 6.8Å, after which the CBR1 loop becomes well ordered and visible in the holo-C2 structure. Upon movement of the loop, D429 is re-positioned to engage Ca\(^{2+}\) in positions I, II and III. (C) Superimposition of the C2 domain from murine perforin and the holo-C2 quad\(_{410-535}\) further demonstrates the significant movement of residue D429 over 11Å. Throughout, key residues are represented in stick form labelled by residue number, arrows indicate movement, and disulphide bonds are represented as yellow sticks. The Ca\(^{2+}\) are numbered as described previously (17) and the additional observed Ca\(^{2+}\) numbered sequentially (IV).

(D) Key residues of the holo-C2 quad\(_{410-535}\) structure making contact with Ca\(^{2+}\).

(E) Schematic representation of Ca\(^{2+}\)-binding interactions in the CBRs. The coordinates of the side chain carboxyl groups of conserved Asp residues and D490 with Ca\(^{2+}\) ion at each site are indicated in dashed lines. The Ca\(^{2+}\) ions at each site are shown in orange spheres with corresponding site numbers. The weakest affinity site (III) is labelled in red.

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**Figure 3. Titrations of C2 quad\(_{410-526}\) with Ca\(^{2+}\).**

(A) Overlay of \(^1\)H-\(^{15}\)N SOFAST-HMQC spectra of 150 µM \(^{15}\)N-labelled C2 quad\(_{410-526}\) recorded with increasing [Ca\(^{2+}\)]. Spectra were processed identically and plotted with the same contour levels. Resonance colours correspond to different [Ca\(^{2+}\)] as shown in the top-left corner. Resonances of interest in the main text are labelled. The chemical shift change patterns are shown in black arrows, and intensity changes are roughly represented by a gradient. The resonances of T431 decreased its intensity with increasing [Ca\(^{2+}\)] and disappeared by 0.6 mM [Ca\(^{2+}\)]. The resonances of W453 and D485 were significantly broadened at several [Ca\(^{2+}\)] in the middle of titrations. (B) and (C) Expanded spectral regions showing resonances of A484 and D490, respectively.

**Figure 4. Concentration-dependent chemical shift perturbations (CSPs) of C2 quad\(_{410-526}\) upon binding to Ca\(^{2+}\).**

(A) CSP values upon Ca\(^{2+}\)-binding were calculated up to 30 mM [Ca\(^{2+}\)] using equation 1 (see ‘Experimental Procedures’) and plotted for each residue on the primary sequence of the C2 quad\(_{410-526}\). CSPs > 0.2 ppm are coloured and labelled. Bars with CSPs up to 3 mM and with CSPs from 3 to 30 mM [Ca\(^{2+}\)] are represented in red and orange, respectively. The resonance of T434 was undetected at 3 mM [Ca\(^{2+}\)] because of exchange broadening. Asterisks mark resonances not detected because of exchange broadening beyond 0.3 mM [Ca\(^{2+}\)]. Dashed lines indicate CSP values of ≥0.2 ppm. The positions of CBRs
Ca<sup>2+</sup>-dependent membrane binding of perforin C2

are shown below the residue number. (B) Residues with significant CSPs upon Ca<sup>2+</sup>-binding are mapped on the crystal structure of holo- C2 quad<sub>410-535</sub> using the same colour representations as in (A). The positions of CBRs are labelled. Residues with undetected resonances beyond 0.3 mM [Ca<sup>2+</sup>] are shown in yellow and labelled. Right and left figures are related by a 180° rotation around vertical axis. Blue spheres indicate bound Ca<sup>2+</sup> ions.

Figure 5. Contribution of four conserved Asp residues D491, D483, D429 and D435 to Ca<sup>2+</sup>-binding.
Overlay of portion of <sup>1</sup>H-<sup>15</sup>N SOFAST-HMQC spectra of 150 µM <sup>15</sup>N-labelled C2 D491N, D483N, D429N and D435N mutants (from the top to the bottom), recorded with increasing [Ca<sup>2+</sup>]. Spectra were processed identically and plotted with the same contour levels. Resonance colours correspond to different [Ca<sup>2+</sup>] shown at the left. Resonances of interest in the main text are labelled. The chemical shift change patterns are shown in black arrows, and intensity changes are roughly represented by gradient. Different spectral regions are shown in the left and right panels.

Figure 6. Interaction of C2 quad<sub>410-526</sub> with DPC micelles.
(A) Overlay of a portion of <sup>1</sup>H-<sup>15</sup>N SOFAST-HMQC spectra of 150 µM <sup>15</sup>N-labelled C2 quad<sub>410-526</sub> recorded with increasing [DPC] in the presence of 30 mM [Ca<sup>2+</sup>]. Spectra were processed identically and plotted with the same contour levels. Resonance colours correspond to different [DPC] as shown at the right-bottom of the spectra. Significantly perturbed resonances upon addition of DPC are labelled and traced by dotted arrows. (B) and (C) Same as (A) except that spectra were recorded in the absence of Ca<sup>2+</sup> (B) and with 2 mM [Ca<sup>2+</sup>] (C), respectively. Only the boxed areas in (A) are represented. (D) CSPs values upon addition of the DPC in the presence of 30 mM [Ca<sup>2+</sup>] were calculated using \( \{(\Delta \delta^1H)^2 + (0.14\Delta \delta^{15}N)^2\}^{1/2} \) and plotted for each residue on the primary sequence of C2 quad<sub>410-526</sub>. The bars with chemical shift perturbations > 0.05 ppm (dashed line) are shown in magenta and labelled. Asterisks mark undetected resonances because of exchange broadening beyond 0.3 mM [Ca<sup>2+</sup>]. The positions of CBRs are shown below the residue number. (E) Residues with CSPs > 0.05 ppm are mapped on the surface of the crystal structure of holo-C2 quad<sub>410-535</sub> using the same colour representations as in (D). The positions of CBRs are labelled. The side chain of residue N454 is shown in a stick model. Residues with undetected resonances beyond 0.3 mM [Ca<sup>2+</sup>] are shown in yellow. Right and left figures are related by a 90° rotation around the horizontal axis. Blue spheres mark bound Ca<sup>2+</sup> ions.

Figure 7. Effects of the bound Ca<sup>2+</sup> to the C2 quad<sub>410-526</sub> upon interaction with DPC micelles
(A) Changes in chemical shifts, $\Delta\delta^1H$, of indicated amide proton resonances of the C2 quad$_{410-526}$ are plotted against [DPC]. Solid lines show the fits of the data to equation 2 (see “Experimental Procedures”). Filled triangles, diamonds, circles and squares showed $\Delta\delta^1H$ values of residues D429, D489, D490 and D485, respectively. The average dissociation constant ($K_d$) was determined as 1.1 ± 0.3 mM (B)–(F) Overlay of portion of $^1$H–$^{15}$N SOFAST-HMQC spectra of 150 µM $^{15}$N-labelled C2 D491N (B), D429N (C), D435N (D), D483N (E) and D490N (F) mutants in the presence of 30 mM [Ca$^{2+}$] recorded with increasing [DPC]. Spectra were processed identically and plotted with the same contour levels. Resonance colours correspond to different [DPC] shown in the same colour on the bottom-right side of spectra. In the D491N mutant, no significant CSPs upon adding DPC were observed in the resonances of backbone amides, but a side chain amide resonance of N454 was clearly perturbed. In the D429N, D435N and D483N mutants, resonances indicative of degradation or aggregation gradually emerged with increasing [DPC]. In the D490N mutant, the same resonances of the C2 quad$_{410-526}$ were perturbed with increasing [DPC].

**Figure 8. Model of Ca$^{2+}$-induced activation of perforin C2 domain interaction with membranes.**

Left: In the absence of Ca$^{2+}$ ions, the relative orientation of CBR1 is not properly arranged to interact with membranes results in being aggregation or degradation with interacting membranes (inactive form). Right: At higher [Ca$^{2+}$], the carboxyl group of D429 interacts with Ca$^{2+}$ at site III and CBR1 changes the relative orientation to readily interact with membranes properly (active form). This re-arrangement of CBR1 allows the re-positioning of key aromatic residues to enable membrane binding. The CBR1 is drawn in magenta and D429 and D435 are shown in stick models. The crystal structures of the apo- and holo-C2 quad$_{410-535}$ are used as the models of inactive and active forms, respectively. The orientation of the C2 domain and membranes is based on the results of DPC titration to the C2 quad$_{410-526}$ that the surface of the C2 quad$_{410-526}$ where the CSPs were observed upon DPC micelles binding faces to head groups of lipid membrane.
Ca\textsuperscript{2+}-dependent membrane binding of perforin C2

Table 1: Refinement Statistics

| Data Collection       | Apo C2                      | Holo C2                     |
|-----------------------|-----------------------------|-----------------------------|
| Resolution range (Å)  | 36.48-1.611 (1.669-1.611)   | 42.38-2.666 (2.761-2.666)   |
| Space group           | P 2 1 2 1 2                | I 2 2 2                    |
| Unit cell a, b, c (Å) | 37.7, 43.211, 68.077        | 45.009, 59.828, 125.919     |
| α, β, γ (°)           | 90, 90, 90                 | 90, 90, 90                 |
| Total reflections     | 105843 (9865)               | 10189 (930)                 |
| Unique reflections    | 14926 (1443)                | 5115 (477)                  |
| Multiplicity          | 7.1 (6.8)                  | 2.0 (1.9)                   |
| Completens (%)        | 99.87 (98.90)              | 99.65 (96.95)               |
| Mean I/σ(I)           | 10.15 (3.94)               | 21.25 (10.03)               |
| Wilson B-factor       | 11.78                      | 26.68                       |
| CC\textsuperscript{1/2} | 0.979 (0.86)               | 0.997 (0.981)               |
| CC\textsuperscript{*} | 0.995 (0.962)              | 0.999 (0.995)               |
| Rmerge                | 0.2159 (0.5473)            | 0.071 (0.182)               |

| Refinement             |                            |                            |
| Number of molecules in ASU | 1                         | 1                           |
| R-work                 | 0.1661 (0.1929)            | 0.1684 (0.2163)             |
| R-free                 | 0.1852 (0.2491)            | 0.2153 (0.2380)             |
| Number of non-hydrogen atoms | 1120                      | 1033                        |
| macromolecules         | 985                        | 965                         |
| ligands                | 1                         | 5                           |
| water                  | 134                       | 63                          |
| Protein residues       | 122                       | 126                         |
| RMS(bonds)             | 0.011                     | 0.003                       |
| RMS(angles)            | 1.29                      | 0.65                        |
| Ramachandran favored (%) | 98                        | 96                          |
| Ramachandran allowed (%) | 2                        | 4                           |
| Ramachandran outliers (%) | 0                        | 0                           |
| Clashscore             | 1.05                      | 1.08                        |
| Average B-factor       | 17                        | 24                          |
| macromolecules         | 15.5                      | 23.9                        |
| ligands                | 17                        | 22.4                        |
| solvent                | 28.1                      | 24.7                        |
| Mol Probity Score\textsuperscript{*} | 0.09 (100\textsuperspace{th} Percentile) | 1.09 (100\textsuperspace{th} Percentile) |

Statistics for the highest-resolution shell are shown in parentheses.

\textsuperscript{*}Combines the clashscore, rotamer, and Ramachandran evaluations into a single score, normalized to be on the same scale as X-ray resolution.

R free was calculated using 5% of randomly selected reflection, excluded from the refinement.
Figure 2

Ca\(^{2+}\)-dependent membrane binding of perforin C2
Figure 3

Ca\(^{2+}\)-dependent membrane binding of perforin C2
Figure 4

Ca\(^{2+}\)-dependent membrane binding of perforin C2
Figure 5

Ca^{2+}-dependent membrane binding of perforin C2
Figure 6

**Ca$$^{2+}$$-dependent membrane binding of perforin C2**

**A**

**B**

**C**

**D**

**E**

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Figure 7

Ca\(^{2+}\)-dependent membrane binding of perforin C2
Figure 8

Ca^{2+}-dependent membrane binding of perforin C2
Structural Basis for Ca\textsuperscript{2+}-mediated Interaction of the Perforin C2 Domain with Lipid Membranes

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