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REVIEW

The C2 domain calcium-binding motif: Structural and functional diversity

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Abstract

The C2 domain is a Ca^{2+}-binding motif of approximately 130 residues in length originally identified in the Ca^{2+}-dependent isoforms of protein kinase C. Single and multiple copies of C2 domains have been identified in a growing number of eukaryotic signalling proteins that interact with cellular membranes and mediate a broad array of critical intracellular processes, including membrane trafficking, the generation of lipid-second messengers, activation of GTPases, and the control of protein phosphorylation. As a group, C2 domains display the remarkable property of binding a variety of different ligands and substrates, including Ca^{2+}, phospholipids, inositol polyphosphates, and intracellular proteins. Expanding this functional diversity is the fact that not all proteins containing C2 domains are regulated by Ca^{2+}, suggesting that some C2 domains may play a purely structural role or may have lost the ability to bind Ca^{2+}. The present review summarizes the information currently available regarding the structure and function of the C2 domain and provides a novel sequence alignment of 65 C2 domain primary structures. This alignment predicts that C2 domains form two distinct topological folds, illustrated by the recent crystal structures of C2 domains from synaptotagmin I and phosphoinositide-specific phospholipase C-δ1, respectively. The alignment highlights residues that may be critical to the C2 domain fold or required for Ca^{2+} binding and regulation.

Keywords: calcium-dependent phospholipid-binding domain; C2 domain; calcium signaling; cytosolic phospholipase A_2; phospholipase C; protein kinase C; ras-GTPase-activating protein; synaptotagmin

Identification of C2 domains in eukaryotic signaling proteins

The C2 domain was originally identified as the second of four conserved domains (C1 though C4) in the α, β and γ isoforms of mammalian Ca^{2+}-dependent protein kinase C (PKC) (Coussens et al., 1986; Knopf et al., 1986; Ono et al., 1986a; 1986b; Parker et al., 1986). The N-terminal C1 domain of protein kinase C was recognized as the cysteine-rich domain that binds phorbol esters and diacylglycerol, whereas the C-terminal C3 and C4 domains exhibited primary structures homologous to the two lobes of protein kinase A (reviewed by Nishizuka, 1988). Because the kinase activity and phospholipid binding of these 'classical' isoforms of protein kinase C were known to be Ca^{2+} dependent, whereas 'non-classical' ('novel' and 'atypical') isoforms apparently lacking the C2 domain failed to exhibit Ca^{2+} regulation, it was proposed that the C2 domain was responsible for Ca^{2+} regulation of protein kinase C.

Subsequent studies have revealed the presence of homologous C2 domains in other proteins, as summarized in Table 1. Two tandem C2 domains were identified in the cytosolic portion of synaptotagmin, an integral membrane Ca^{2+} sensor found in synaptic vesicles and in secretory granules of endocrine cells (Perin et al., 1990). Because protein kinase C and synaptotagmin shared the feature of binding phosphatidylserine vesicles upon addition of Ca^{2+} (Bazzi and Nelsestuen, 1987, 1990; Brose et al., 1992), it was inferred that the C2 domain was involved in Ca^{2+}-regulated binding to acidic phospholipids.

Independent studies of the Ca^{2+}-dependent cytosolic phospholipase A_2 ($cPLA_2$) revealed the presence of a homologue of the C2 domain at the extreme N-terminus of this enzyme (Clark et al., 1991). In turn, sequence comparisons with cytosolic phospholipase A_2 enabled the identification of C2 domain homologues in the 120-kDa mammalian ras-GTPase-activating protein (ras-GAP) cloned previously (Trahey et al., 1988) and in all three isoforms of phosphoinositide-specific phospholipase C (PLC) (Rhee et al., 1989; Kriz et al., 1990). When a 16-kDa N-terminal fragment of cytosolic phospholipase A_2 containing its C2 domain was liberated from the full-length enzyme by chemical cleavage, it was found to bind to cell membranes as a function of Ca^{2+}, suggesting that this C2 domain behaved as a *Ca*$^{2+}$-dependent *l*ipid-*b*inding (CaLB) domain (Clark et al., 1991), as presumably would the corresponding domains of protein kinase C and synaptotagmin. Independent

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Table 1. *Proteins containing recognized C2 domains*

Protein	Number of C2 domains	Topology[a]
Synaptotagmin I–VIII	2	I
Rabphilin-3A	2	I
Double C2 protein (DOC2)	2	I
Perforin	1	II
UNC-13 I–III	2–3	I and II
Cytosolic phospholipase A_2 ($cPLA_2$)	1	II
Phosphoinositide-specific phospholipase C (PLC-γ I–II)	1	II
Phosphoinositide-specific phospholipase C (PLC-β I–IV)	1	II
Phosphoinositide-specific phospholipase C (PLC-δ I–IV)	1	II
Plant phospholipase D (PLD)	1	II
Yeast phosphatidylserine decarboxylase (PSD2)	1	II
Phosphatidylinositol 3-kinase (PI3K α and β)	1	II
Phosphatidylinositol 3-kinase (VPS34P)	1	II
Phosphatidylinositol 3-kinase (PI3K_68D)	1	I
'Classical' protein kinase C (PKC α, β and γ)	1	I
'Non-classical' protein kinase C (PKC δ, ϵ, η, and θ)	1	II
Yeast protein kinase C (PCK1, 2 and PKC1)	1	II
Protein kinase C-related kinase (PRK1 and 2)	1	II
Yeast cAMP-dependent kinase (SCH9 kinase)	1	II
Ras-GTPase-activating protein (rasGAP)	1	II
Ras-GTPase-activating protein (GAP1 and R-Ras-GAP/GAP1$^{\text{IP4BP}}$)	2	II
Breakpoint-cluster region protein (BCR and ABR)	1	II
BUD2	1	II
RSP5/NEDD-4	1	II

[a] Topology is classified by homology to synaptotagmin I (type I) or PLC-δ1 (type II) as summarized in Figure 3.

folding of the C2 domain was demonstrated by recombinant fragments containing the first C2 domain of synaptotagmin (Davletov and Südhof, 1993; Chapman and Jahn, 1994). These fragments bound phospholipid vesicles in vitro upon addition of Ca^{2+}, confirming that this C2 domain is a CaLB domain. Similarly, recombinant C2 domains from rabphilin-3A, a protein that binds the small GTPase Rab3A during vesicular trafficking (Shirataki et al., 1993), and cytosolic phospholipase A_2 were found to function in vitro as independently folded, Ca^{2+}-regulated phospholipid membrane-binding domains (Yamaguchi et al., 1993; Nalefski et al., 1994).

Most recently, a number of new C2 domains have been identified by sequence comparison, although the ability of these domains to confer Ca^{2+} regulation has not yet been tested in many instances. Included are several neuronal and non-neuronal isoforms of synaptotagmin (Ullrich et al., 1994; Li et al., 1995b), the 100-kDa mammalian Ras GTPase-activating protein (GAP1$^{\text{m}}$) (Maekawa et al., 1994) and its *Drosophila* homologue (GAP1$^{\text{d}}$) (Gaul et al., 1992), a 98-kDa R-Ras GTPase-activating protein similar to GAP1$^{\text{m}}$ (R-Ras-GAP/GAP1$^{\text{IP4BP}}$) (Cullen et al., 1995; Yamamoto et al., 1995), the DOC2 protein of synaptic vesicles (Orita et al., 1995), and a yeast phosphatidylserine decarboxylase (PSD2) (Trotter et al., 1995). Although not initially recognized, C2 domains are present at the N-termini of the non-classical Ca^{2+}-independent protein kinase C isoforms including δ, ϵ, η, and θ as well as novel lipid-dependent protein kinases related to protein kinase C (PRK1 and PRK2) (Sossin and Schwartz, 1993; Ponting and Parker, 1996). Recently, C2 domains have been identified in yeast homologues of protein kinase C (PCK1, PCK2, and PKC1) as well as a yeast cAMP-dependent protein kinase (SCH9 kinase) (Nonaka et al., 1995; Ponting and Parker, 1996). The C2 domain has been identified in the gene product of the breakpoint-cluster region (BCR) (Boguski et al., 1992) and its relative, the product of the active breakpoint cluster region-related gene (ABR) (present report), both of which activate ras-like GTPases (Diekmann et al., 1991; Tan et al., 1993). C2 domains are found in the *unc*-13 gene product, a phorbol ester-binding protein of unknown function whose mutation causes neurological defects in *C. elegans* (Maruyama and Brenner, 1991) and in three mammalian homologues of UNC-13 (Brose et al., 1995). Sequences searches have also led to the discovery of C2 domains in several open-reading frames and in the mammalian gene product NEDD-4 and its homologue in yeast RSP5 (Brose et al., 1995; Hofmann and Bucher, 1995; Pointing and Parker, 1996), which suppresses mutations in the yeast transcription factor SPT3. Finally, sequence alignments have also revealed C2 domains in several other proteins, including catalytic subunits of phosphatidylinositol 3-kinase (PI3Kα and β) (Stephens et al., 1993), the VPS34P form of phosphatidylinositol 3-kinase (Welters et al., 1994), a related phosphatidylinositol 3-kinase from Drosphila (PI3K_68D) (MacDougall et al., 1995), the pore-forming protein perforin, and the yeast GTPase-activating protein BUD2 (Ponting and Parker, 1996).

Further analysis of eukaryotic genomes is predicted to reveal new C2 domains at a rapid pace. Like the ubiquitous EF-hand Ca^{2+}-binding motif of calmodulin and its relatives (reviewed recently by Falke et al., 1994; Chazin, 1995; Linse and Forsén, 1995; Ikura, 1996; Kawasaki and Kretsinger, 1996), the C2 domain is widely distributed in eukaryotes but rare or non-existent in the prokaryotic world, where Ca^{2+} signaling is less widely used as a second messenger. Moreover, not all proteins containing the C2 domain are regulated by Ca^{2+} (see below), just as there exist proteins containing the EF-hand motif that lack Ca^{2+} regulation and in some cases even fail to bind Ca^{2+}.

Structure of the C2 domain: Two distinct topologies

The structures of two C2 domains have been determined by X-ray crystallography. The first C2 domain of synaptotagmin I (termed C_2A) was expressed as a cloned fragment in *E. coli*, and was solved to 1.9 Å resolution (Sutton et al., 1995), as illustrated in Figure 1A. Subsequently, a recombinant fragment of the phosphoinositide-specific phospholipase C-δ1 was solved to 2.4 Å resolution (Essen et al., 1996), thereby providing the structure of its C2 domain (as well as its catalytic domain and a calmodulin-like EF-hand domain). The C2 domains from both proteins are of approximately 130 residues in length, and they differ by a root mean square deviation of only 1.4 Å throughout 109 equivalent α-carbons (Essen et al., 1996). Both structures form an eight-stranded antiparallel β-sandwich consisting of a pair of four-stranded β-sheets. Interestingly, however, these C2 domains represent two distinct topological folds, differing slightly in their β-strand connectivity, as summarized in Figure 1B. Here we term the fold of the original synaptotagmin C_2A domain 'topology I,' while that of the phosphoinositide-specific phospholipase C-δ1 domain is designated 'topology II.' The two topologies are easily interconverted: topology I becomes topology II when its N- and C-termini are fused and new termini are generated by cutting the loop between strands β1 and β2. The key difference between the two topologies is that the

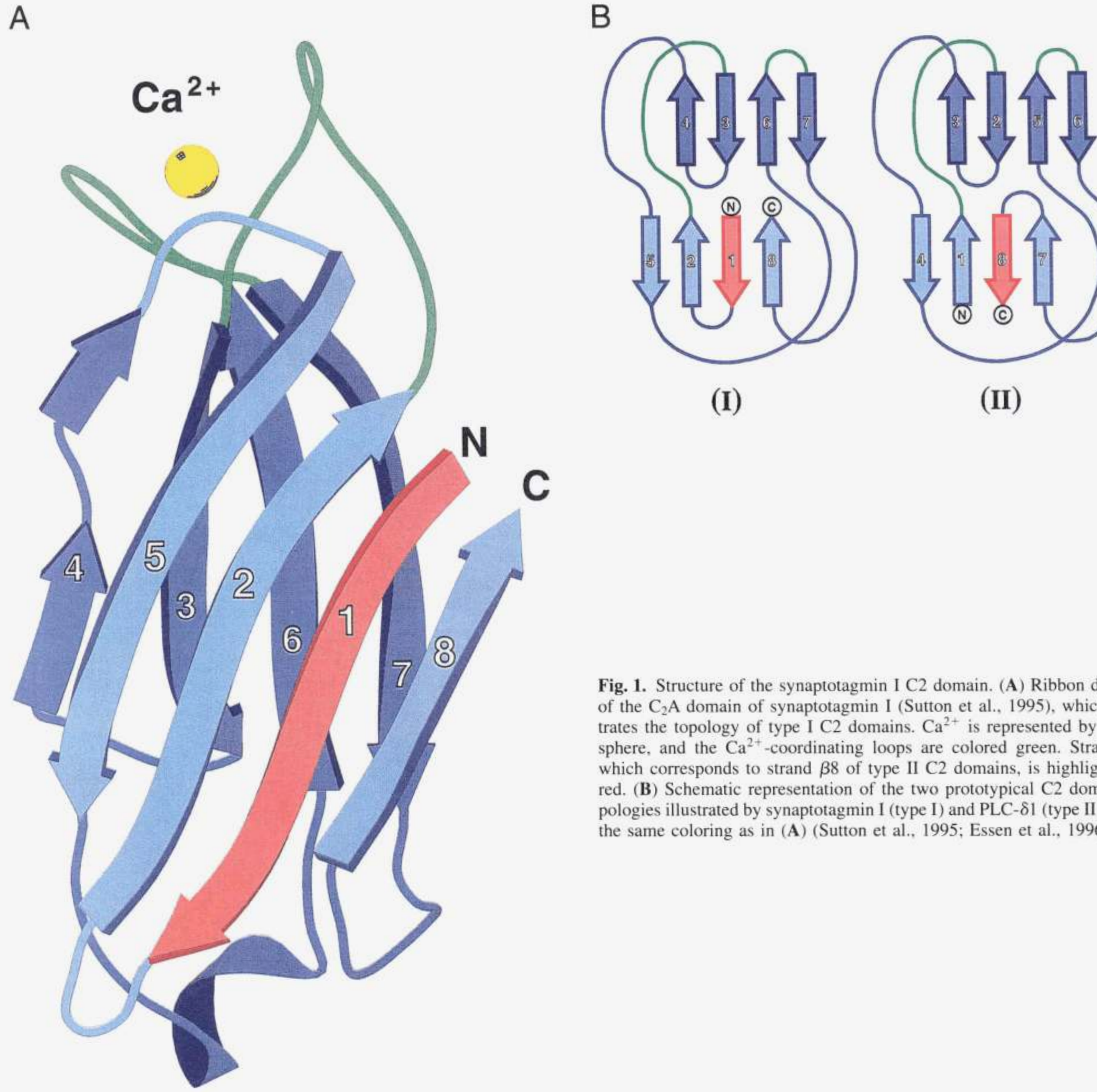

Fig. 1. Structure of the synaptotagmin I C2 domain. (**A**) Ribbon diagram of the C_2A domain of synaptotagmin I (Sutton et al., 1995), which illustrates the topology of type I C2 domains. Ca^{2+} is represented by a gold sphere, and the Ca^{2+}-coordinating loops are colored green. Strand β1, which corresponds to strand β8 of type II C2 domains, is highlighted in red. (**B**) Schematic representation of the two prototypical C2 domain topologies illustrated by synaptotagmin I (type I) and PLC-δ1 (type II), using the same coloring as in (**A**) (Sutton et al., 1995; Essen et al., 1996).

first strand of topology I occupies the same structural position as the eighth β-strand of topology II, which shifts the order of homologous strands in the primary structure (see below).

A single bound Ca^{2+} ion in the crystal structure of the synaptotagmin I domain was identified by soaking crystals of the apo protein in Ca^{2+} and examining a difference electron density map (Sutton et al., 1995). Ca^{2+} binds in a concave depression formed at the edge of the β-sandwich formed by loops $\beta2$–$\beta3$ and $\beta6$–$\beta7$. Metal ion coordination is provided by the main-chain carbonyl oxygen of Phe 231, the well-ordered bidentate side chain of Asp 230, the well-ordered monodentate side chain of Asp 178, the partially ordered monodentate side chain of Asp 232, a water molecule, and perhaps the disordered side chain of Asp 172. That this site represents a physiological site is strongly supported by experiments in which replacement of Asp 178 or Asp 230 with Asn generated proteins incapable of Ca^{2+}-dependent phospholipid binding (Sutton et al., 1995). Interestingly, although the binding of a single Ca^{2+} ion caused little or no change in the structure, it was notable that crystals were destroyed by soaking in higher Ca^{2+} concentrations, suggesting that the binding of additional Ca^{2+} may drive a significant conformational transition.

A variety of independent structural and biochemical approaches have strongly suggested that the fully saturated C2 domain binds at least two metal ions, rather than just one. An NMR study monitoring the Ca^{2+} binding pocket of the synaptotagmin C_2A domain revealed a biphasic titration curve saturated by at least two Ca^{2+} ions, the first exhibiting an apparent dissociation constant of 60 μM, and the second a dissociation constant of 400 μM (Shao et al., 1996). The first ion appeared to bind at the same location observed in the mono-Ca^{2+} crystal structure, while the second bound at an adjacent location in the same site. Analogous NMR evidence also demonstrated the binding of two or more Ca^{2+} ions to the C2 domain of protein kinase C (Shao et al., 1996). Similarly, when crystals of phosphoinositide-specific phospholipase C-δ1 were soaked with the Ca^{2+} analogue La^{3+}, two metal ions bound in approximately the same site observed in synaptotagmin (Essen et al., 1996; Grobler et al., 1996). Further indirect evidence for the binding of multiple Ca^{2+} ions has been provided by the steep, apparently cooperative dependence of membrane binding on the Ca^{2+} concentration by the first C2 domains of synaptotagmins (Davletov and Südhof, 1993; Li et al., 1995a).

Figure 2 illustrates a side-chain coordination scheme recently proposed for two Ca^{2+} ions bound to a generalized C2 domain of either topology I or II, as extrapolated from the NMR and crystallographic studies of the synaptotagmin C_2A domain (Shao et al., 1996). Each Ca^{2+} ion is liganded by (a) bidentate and monodentate Asp carboxylates separated by 5 ± 1 residues in the proximal loop, (b) a bridging Asp carboxylate lying between the two metal centers, and (c) a more distant Asp carboxylate provided by the distal loop. As observed in the original crystal structure (Sutton et al., 1995), coordinating positions are provided by both the $\beta2$–$\beta3$ and $\beta6$–$\beta7$ loops, or, in topology II, by the $\beta1$–$\beta2$ and $\beta5$–$\beta6$ loops. In the synaptotagmin C_2A site, the proposed coordinating side chains are Asp 172, Asp 178, Asp 230, and Asp 232 for the high-affinity Ca^{2+} ion; and Asp 172, Asp 232, Asp 238, and Asp 230 for the low-affinity ion. Additional coordination may be provided by solvent oxygens, and perhaps by backbone carbonyl oxygens as well.

Certain features of the coordination proposed in Figure 2 are supported by the crystal structure of the phosphoinositide-specific phospholipase C-δ1 site occupied by two Sm^{3+} ions, although significant deviations are observed as well (Grobler et al., 1996). It remains to be seen whether these deviations represent unique features of Sm^{3+} coordination, or protein-specific features of the coordination scheme, or a more accurate representation of a general coordination scheme. Both the synaptotagmin C_2A and phosphoinositide-specific phospholipase C-δ1 coordination models envisage two bound metal ions and include side-chain ligation by residues X(6-8), Y(1), and Y(3) in Figure 2, which are thus likely to represent conserved metal-binding side-chains (Shao et al., 1996; Grobler et al., 1996). By contrast, coordinating residues X(1) and Y(8-10) in Figure 2 have not yet been directly implicated in any protein besides synaptotagmin.

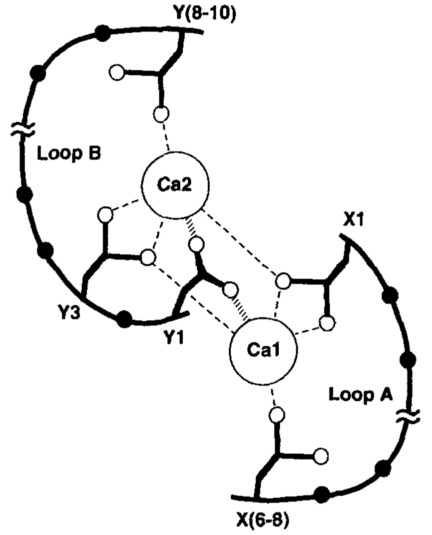

Fig. 2. Model for Ca^{2+}-coordination by the C2 motif. Shown is the proposed coordination environment of two Ca^{2+} ions, as discussed by Rizo and colleagues (Shao et al., 1996).

Why does the C2 domain bind multiple Ca^{2+} ions? One advantage could be the use of positive cooperativity to steepen the Ca^{2+} binding profile, thereby generating a narrower activation threshold as observed for cooperative Ca^{2+} binding to many proteins of the EF-hand class (Linse and Forsén, 1995). Alternatively, multiple Ca^{2+} ions can facilitate protein binding to phospholipids, as observed in annexin V (see below). The Ca^{2+} affinities of proteins containing C2 domains are often enhanced by the presence of target membranes or proteins (Bazzi and Nelsestuen, 1990; Brose et al., 1992; Wijkander and Sundler, 1992). This fact should be noted when evaluating the Ca^{2+} binding affinities and stoichiometries required to trigger the physiological docking interactions.

Sequence alignment of 65 different C2 domains

Figure 3 presents a manual alignment of 65 distinct C2 domains from different proteins or isoforms, guided by (a) patterns of buried positions in the β-strands of the synaptotagmin domain, and (b) similarities between protein isoforms. The alignment procedure avoided introduction of gaps and insertions in the middle of secondary structural elements and sought to align residues at positions that are likely to maintain the C2 fold. For convenience, the alignment is referenced to the residue numbers of the synaptotagmin I C_2A domain, and the positions of β-strands are shown for both this topology I domain and for the topology II domain of phospho-

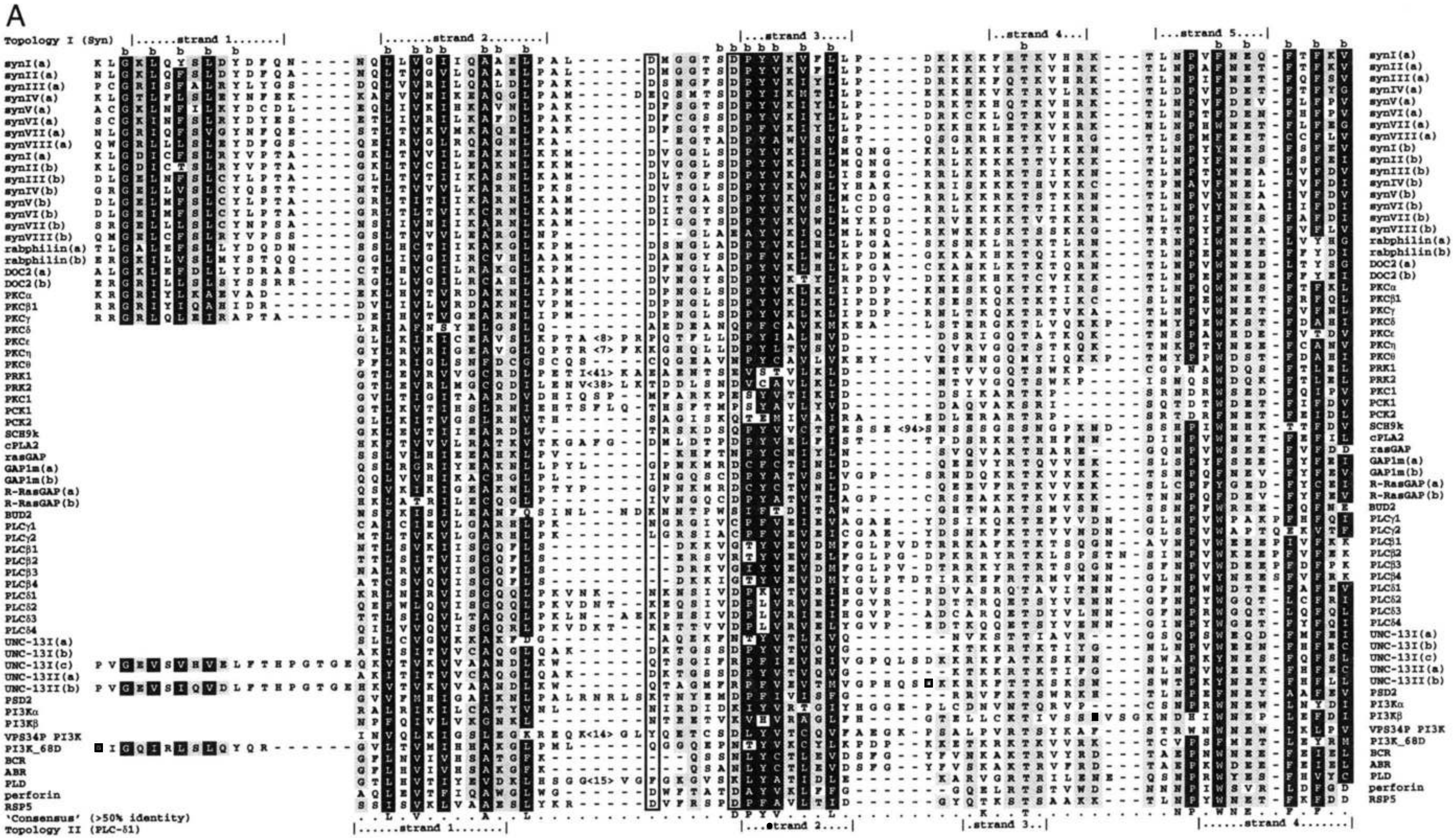

Fig. 3. Sequence alignment of C2 domains. Sequence alignment of 65 published C2 domains from Table 1 using the single-letter code for amino acids. The secondary structures of type I and type II topologies are schematically shown above and below the sequence, respectively, indicating β-strands (brackets) and residues buried (**b**) in synaptotagmin. Note that the sequences of strand 1 in topology I are to be compared to those of strand 8 in topology II domains, which occupy equivalent positions in the tertiary structures (Figs. 1B and 4). The 'consensus' residues present in >50% of the sequences are indicated at the bottom of the alignment. Boxed sequence positions denote side chains proposed to coordinate Ca^{2+} in synaptotagmin. Residues highlighted in black are conserved as non-polar (A, V, M, F, I, L, P, C, or G) or aromatic (F, W, or Y) in at least 80% of the sequences. Highlighted in gray are conserved polar or charged residues (S, T, N, Q, D, E, K, R, H, W, C, or G) found in at least 80% of the sequences. In loop regions where the number of residues is variable and the alignment is questionable, little attempt was made to classify residues. Note that loops contain a significant number of Pro (P) and Gly (G) residues, which have important implications for secondary structure. Dashes in sequences indicate gaps inserted to maximize the alignment; numbers in brackets ⟨ ⟩ indicate intervening residues omitted for clarity. Synaptotagmin is abbreviated as Syn and phosphoinositide-specific phospholipase C as PLC; other protein abbreviations are listed in Table 1. Tandem repeats of C2 domains in a given protein are designated a, b, or c. GenBank accession numbers for the sequences are indicated in parentheses. PLC-δ2 and -δ3 sequences are derived from cDNA cloned by Kriz et al. (1990) and published by Meldrum et al. (1991) and Lee and Rhee (1996), respectively. (*Figure continues on next page.*)

Fig. 3. Continued.

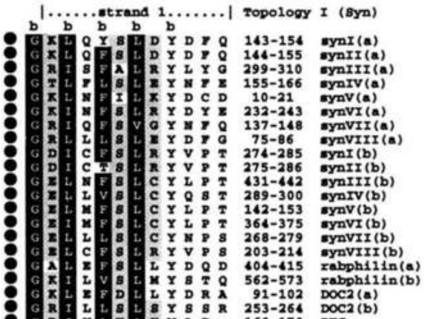

Fig. 4. Sequence alignment of structurally homologous β-strands: strand 1 of topology I and strand 8 of topology II. Shown are the aligned sequences of strand 1 from topology I (designated by closed circles) and strand 8 from topology II (designated by open circles) C2 domains, illustrating the sequence homology between these structurally equivalent elements. Sequences and classification of residues are the same as in Figure 3.

inositide-specific phospholipase C-δ1. Figure 4 confirms the expected homology between strand $\beta 1$ of topology I and strand $\beta 8$ of topology II, which occupy structurally equivalent positions in the tertiary structure (see Fig. 1B).

The illustrated alignment is the first, to our knowledge, to incorporate both topologies of the C2 domain. At present, all recognized C2 domain sequences can be aligned to one topology or the other: a total of 26 of the sequences conform to topology I, while the remaining 39 correspond to topology II. C2 domains located at the extreme N-termini of their parent proteins typically use topology II, while C2 domains at the C-terminus may utilize either topology. Tandem C2 domains may exhibit either topology, but both members of the pair are topologically identical. As illustrated by the dendrogram published by Brose et al. (1995), which compared the relationships between several C2 domain sequences, topology I C2 domains are more closely interrelated than they are related to type II C2 domains. These sequence relationships may reflect common ancestries or primary structural constraints imparted by the two topologies. Nevertheless, these two topologies may play an important functional role: they are likely to confer different orientations of the C2 domain relative to the rest of the protein. In the phosphoinositide-specific PLC-δ1, the presumed Ca^{2+}-dependent lipid-binding surface of the C2 domain is oriented on the same side of the enzyme as its active site (Essen et al., 1996).

The alignment reveals considerable homology between the C2 domains of diverse proteins. Of the approximately 130 total residues in the C2 domain, 26 positions exhibit greater than 50% sequence identity and are hereafter termed 'consensus' residues. Many other positions, termed 'conserved,' exhibit less than 50% identity but display at least 80% side-chain homology, including 28 positions dominated by non-polar or aromatic residues, and 35 positions characterized by polar or charged side chains. Most of the consensus and conserved positions are localized to the eight β-strands, where a clear pattern of alternating polar and nonpolar residues is often apparent except at β-bulge positions and in strands lying at the edges of the two β-sheets (Sutton et al., 1995; Essen et al., 1996). Proline and glycine residues are highly concentrated in the loops between β-strands, where they are likely to facilitate turns. Moreover, large insertions are sometimes observed in the interstrand loops, where they may well impart protein-specific functions.

Significant homology is also observed at positions implicated in Ca^{2+} coordination. Of the five Asp side chains proposed to coordinate Ca^{2+} in synaptotagmin (Sutton et al., 1995; Shao et al., 1996), three lie at consensus Asp positions (Asp 178, Asp 230, and Asp 232), one lies at a conserved polar position (Asp 238), and the remaining one lies at a non-conserved loop position (Asp 172), where 70% of the observed residues are capable of Ca^{2+} coordination. However, 40 of the 65 total sequences, including the majority of those exhibiting topology II, lack Asp or Glu at one or more of the five putative coordinating positions. In such cases, other loop residues capable of Ca^{2+} coordination (Asn, Gln, Ser, Thr) are often found at or near the corresponding sequence position. This variability may arise from minor perturbations of the proposed coordination scheme or from the existence of entirely different coordination schemes. At any rate, it is clear that the Ca^{2+}-binding sites of different C2 domains are specialized, presumably to provide optimized Ca^{2+}-binding parameters, changes in conformation upon Ca^{2+} binding, or docking interactions for different biological functions.

Finally, a comparison of C2 domain sequences for a given protein isolated from different species reveals significantly less divergence than is observed between functionally distinct proteins. For example, the C2 domain of the only known isoform of cytosolic phospholipase A_2 exhibits greater than 50% sequence identity at all positions corresponding to residues 18–141 in organisms ranging from fish through humans (Nalefski et al., 1994). This conservation helps to calibrate the greater sequence divergence found in C2 domains from different proteins (Fig. 3). Such divergence must stem either from a longer period of random evolutionary drift or from specialization for different functions. Interestingly, two of the known exon boundaries in phospholipase A_2 (following Ile 11 and Val 138) are located at the predicted ends of the C2 domain, supporting the contention that the C2 domain is a functional module that has been inserted into a wide array of signaling proteins (Clark et al., 1995). The modular use of the C2 domain, shown in Figure 5, is evident in the crystal structure of residues 133 to 756 from phosphoinositide-specific phospholipase C-δ1, which exhibits three distinct functional modules including the C2 domain (Essen et al., 1996).

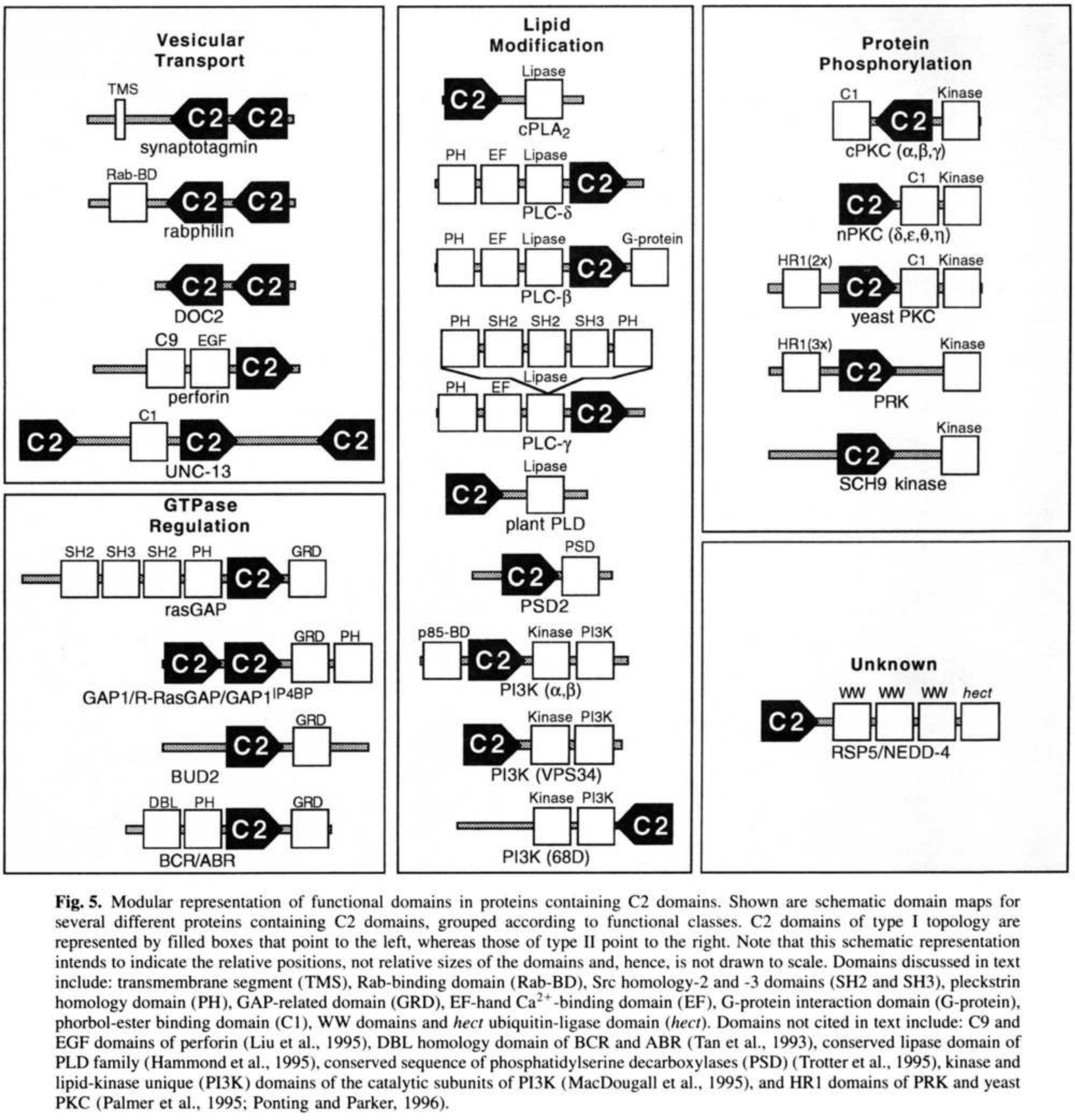

Fig. 5. Modular representation of functional domains in proteins containing C2 domains. Shown are schematic domain maps for several different proteins containing C2 domains, grouped according to functional classes. C2 domains of type I topology are represented by filled boxes that point to the left, whereas those of type II point to the right. Note that this schematic representation intends to indicate the relative positions, not relative sizes of the domains and, hence, is not drawn to scale. Domains discussed in text include: transmembrane segment (TMS), Rab-binding domain (Rab-BD), Src homology-2 and -3 domains (SH2 and SH3), pleckstrin homology domain (PH), GAP-related domain (GRD), EF-hand Ca^{2+}-binding domain (EF), G-protein interaction domain (G-protein), phorbol-ester binding domain (C1), WW domains and *hect* ubiquitin-ligase domain (*hect*). Domains not cited in text include: C9 and EGF domains of perforin (Liu et al., 1995), DBL homology domain of BCR and ABR (Tan et al., 1993), conserved lipase domain of PLD family (Hammond et al., 1995), conserved sequence of phosphatidylserine decarboxylases (PSD) (Trotter et al., 1995), kinase and lipid-kinase unique (PI3K) domains of the catalytic subunits of PI3K (MacDougall et al., 1995), and HR1 domains of PRK and yeast PKC (Palmer et al., 1995; Ponting and Parker, 1996).

Model for Ca^{2+}-dependent membrane binding by the C2 domain

The crystal structures of the synaptotagmin I and phosphoinositide-specific phospholipase C-δ1 C2 domains provide few clues as to how Ca^{2+} induces phospholipid binding by the C2 domain. Two extreme models can be proposed for the Ca^{2+} triggering of phospholipid binding by the C2 domain.

The first model proposes a ternary complex in which the bound Ca^{2+} is coordinated simultaneously by protein and phospholipid residues. The inspiration for this model stems primarily from the structures of secreted phospholipase A_2 ($sPLA_2$) and annexin V, which are not homologous to the C2 domain but nevertheless bind phospholipid in a Ca^{2+}-dependent manner. In Ca^{2+}-bound $sPLA_2$ without lipid, coordination of Ca^{2+} involves five protein oxygens and two water molecules that are replaced by *sn*-3 phosphate and *sn*-2 carbonyl oxygens upon complexation with lipid analogues (Scott et al., 1990; Thunnissen et al., 1990). Comparisons of C2 domains with $sPLA_2$ must be made with caution, however, since the latter enzyme uses Ca^{2+} to hydrolyze lipid substrates, whereas the C2 domain only binds lipid. An alternative scheme is illustrated by a complex between a canonical Ca^{2+}-bound annexin V domain and the phospholipid analogue glycerophosphoserine (GPS), in which two bound Ca^{2+} ions bridge the domain to the phospholipid headgroup (Swairjo et al., 1995). These Ca^{2+}-binding sites, designated the high-affinity AB and low-affinity AB′ sites, are relatively close in proximity. In the absence of lipid, Ca^{2+} is coordinated at the high-affinity AB site via three main chain carbonyl oxygens, a bidendate residue side chain, and two water molecules, one of which is replaced with an *sn*-3 phosphate oxygen upon complexation with GPS. Ca^{2+} is coordinated in the low-affinity AB′ site via a main-chain carbonyl oxygen, a bidendate side chain, and a carboxylate oxygen of the serine head group of GPS. By contrast, binding of the phospholipid analogue glycerophosphoethanolamine, a lower affinity ligand than GPS, results in identical Ca^{2+} coordination except that the Ca^{2+} at the AB′ site makes no contact with the bound phospholipid, suggesting that the second Ca^{2+} may contribute to lipid selectivity (Swairjo et al., 1995).

In the C2 domains of known structure, Ca^{2+}-coordination is likely to involve at least one water molecule (Sutton et al., 1995). In principle, phospholipid could displace the coordinating solvent, providing direct Ca^{2+} coordination by the head group or phosphate oxygens. Consistent with this 'bridging Ca^{2+}' model is the observation that a sulfate ion, which might mimic a lipid phosphate group, is bound in the C2 domain of synaptotagmin adjacent to the bound Ca^{2+} (Sutton et al., 1995).

The second extreme class of model, which is also consistent with the current limited data, proposes that Ca^{2+} binding induces a conformational change in the C2 domain in order to expose functional groups responsible for membrane binding. These functional groups may include hydrophobic residues designed to insert into the interior of the bilayer or charged side chains able to bind specific phospholipid head groups (Newton, 1995a). In the visual signaling protein recoverin, for example, Ca^{2+} binding to multiple EF-hand motifs exposes a sequestered myristoyl group, thereby targeting the protein to membranes (Tanaka et al., 1995). Certain features of this mechanism are observed in annexin V, in which Ca^{2+} binding exposes a buried tryptophan residue that is believed to insert into the hydrophobic region of the bilayer (Concha et al., 1993; Swairjo et al., 1995). The existence of a Ca^{2+}-induced conformational change in the C2 domain has been proposed based on the decreased protease sensitivity of synaptotagmin upon Ca^{2+}-induced binding to membranes (Davletov and Südhof, 1994). In the C2 domain of $cPLA_2$, a Ca^{2+}-triggered conformational change has been detected directly by intrinsic fluorescence (Nalefski and Falke, unpublished results). Finally, the binding of Sm^{3+} to phosphoinositide-specific phospholipase C-δ1 is proposed to 'open the jaws' of the domain, creating a gap large enough to bind a phospholipid head group (Grobler et al., 1996). Overall, although some progress has been made, little is known about the molecular mechanism of C2 domain binding to membranes.

Function of C2 domains in signalling proteins

Much has been learned in recent years about the functional role of the C2 domain in different signalling pathways, although key aspects remain unsolved. Currently, most, if not all, proteins that contain C2 domains are thought to interact with cellular membranes, and, in several instances, the C2 domain has been shown to be directly involved in membrane binding. This multi-talented domain can also, however, mediate protein–protein interactions and small molecule binding, as well as the binding of Ca^{2+} in itself. Proteins known to contain the C2 domain comprise, at present, four functional classes, as summarized in Figure 5: Ca^{2+}-sensors in regulated vesicular transport (the synaptotagmins, rabphilin, DOC2, perforin, and UNC-13), lipid-modifying enzymes ($cPLA_2$, PLC, PLD, PI3K, and PSD2), protein kinases regulated by Ca^{2+} and/or phospholipid (PKC, PKC-like kinases, and SCH9 kinase), and GTPase-activating proteins (ras-GAP, $GAP1^{m}$, R-RasGAP/$GAP1^{IP4BP}$, BCR, ABR, and BUD2). The functions of other C2-containing proteins (e.g., RSP5/NEDD-4) are not yet known.

Regulated vesicle transport

Synaptotagmin

Synaptotagmins are members of a family of ubiquitous integral membrane proteins (Li et al., 1995b) containing an intravesicular N-terminus lacking a signal sequence, a single transmembrane segment (TMS), a linker sequence and two C-terminal cytosolic C2 domains of type I topology (Fig. 3). Four synaptotagmins (I, II, III, and V) are expressed in neural tissue, whereas the remaining four (IV, VI, VII, and VIII) are expressed in other tissues as well (Ullrich et al., 1994; Li et al., 1995b). Synaptotagmins are involved in membrane fusion events (reviewed by Jahn and Südhof, 1994; Bajjalieh and Scheller, 1995; Kelly, 1995; Südhof, 1995), although it is still unclear whether these proteins mediate fusion directly or regulate other factor(s) responsible for fusion. The importance of synaptotagmin is highlighted by observations that fruit flies, nematodes, and mice lacking synaptotagmin alleles display effects on behavior and synaptic function that range from subtle to severe (DiAntonio et al., 1993; Littleton et al., 1993; Nonet et al., 1993; Geppert et al., 1994b).

The C2 domains present in synaptotagmins are perhaps the best studied biochemically to date. Isolated first (N-terminal) C2 domains from the synaptotagmin I, II, III, V, and VII isoforms have been shown to bind phosphatidylserine vesicles in vitro at low levels (3–6 μM) of Ca^{2+} (Davletov and Südhof, 1993; Ullrich et al., 1994; Li et al., 1995a, 1995b). Not surprisingly, these C2 domains all contain the full complement of Ca^{2+}-coordinating residues implicated by the crystal structure of the C2 domain in synaptotagmin I. The first C2 domains of the IV and VIII isoforms, by contrast, lack at least one of these Ca^{2+}-coordinating residues and fail to bind phospholipid vesicles in vitro (Ullrich et al., 1994; Li et al., 1995b). However, the currently implicated Ca^{2+}-coordinating residues are not sufficient for Ca^{2+}-mediated membrane binding. For example, the first C2 domain of synaptotagmin VI possesses the full complement of potential Ca^{2+}-coordinating residues but fails to bind membranes even in the presence of Ca^{2+} (Ullrich et al., 1994; Li et al., 1995b). Moreover, the second C2 domains of synaptotagmins I and II, which also contain all five potential coordinating residues, bind membranes whether or not Ca^{2+} is present (Damer and Creutz, 1994; Fukuda et al., 1994; MacDougall et al., 1995). However, since fragments of synaptotagmin I containing both C2 domains bind phospholipid vesicles in a Ca^{2+}-dependent manner (Li et al., 1995), Ca^{2+} regulation by the first C2 domain may dominate the Ca^{2+}-independent binding displayed by the second C2 domain or recombinant versions of the isolated latter domain may be non-native. Together, these experiments demonstrate that Ca^{2+}-regulated phospholipid binding is a specialized function of different synaptotagmin isoforms.

Membrane binding by synaptotagmins exhibits phospholipid specificity: these C2 domains bind to synthetic vesicles containing phosphatidylserine or phosphatidylinositol, but not phosphatidylcholine or phosphatidylethanolamine (Davletov and Südhof, 1993; Chapman and Jahn, 1994). Membrane binding also requires a divalent, rather than monovalent cation, where the order of preference is $Ca^{2+} > Sr^{2+} > Ba^{2+}$ (Davletov and Südhof, 1993; Li et al., 1995a). The relative affinities of Mg^{2+}, Na^{+}, and K^{+} cannot be determined, since they all fail to promote phospholipid binding. Sr^{2+} and Ba^{2+} (but not Mg^{2+}) also induce the decreased protease sensitivity of synaptotagmin observed for Ca^{2+} in the presence of phospholipid (Davletov and Südhof, 1994).

The C2 domain can also drive protein–protein interactions, and some synaptotagmin C2 domains have been demonstrated to bind, either constitutively or in a Ca^{2+}-regulated fashion, to other intracellular proteins. First, the second C2 domains of most synaptotagmin isoforms bind in a Ca^{2+}-independent manner with high affinity (dissociation constant of 0.1 to 1.0 nM) to clathrin-AP2 (Zhang et al., 1994; Li et al., 1995b), a protein complex involved in coated pit assembly. Second, synaptotagmin has been shown to interact with syntaxin (Chapman et al., 1995; Li et al., 1995b), a plasma membrane protein critical in the exocytosis of synaptic vesicles (Bennett et al., 1992). The first synaptotagmin C2 domains of isoforms I, II, III, V, and VII (and by deduction the second C2 domain of synaptotagmin VI) bind in a Ca^{2+}-dependent manner to syntaxin (Li et al., 1995b; Kee and Scheller, 1996; Sugita et al., 1996). Point mutations in the Ca^{2+}-binding site of the first synaptotagmin C2 domain that eliminate Ca^{2+}-dependent phospholipid binding also destroy syntaxin binding, suggesting that the same residues may be important for both (Li et al., 1995b). Third, the second C2 domain of synaptotagmin associates with itself in a Ca^{2+}-dependent manner (Chapman et al., 1996; Sugita et al., 1996). Finally, the full-length protein has been reported to bind to intracellular receptors for protein kinase C (see below) (Mochly-Rosen et al., 1992). Thus, C2 domains of synaptotagmins can act as intracellular protein receptors.

Finally, inositol polyphosphates have been observed to bind in low ionic strength buffers to the second C2 domains of synaptotagmin isoforms I, II, and IV, but not III (Fukuda et al., 1994, 1995; Niinobe et al., 1994). Indeed, synaptotagmin II had previously been identified independently as an inositol polyphosphate-binding protein (Niinobe et al., 1994). The inositol polyphosphate-binding site, which does not require Ca^{2+}, has been mapped to a cluster of basic residues corresponding to residues located on the exterior surface of strand $\beta4$ and loop $\beta3$–$\beta4$ in the first C2 domain of synaptotagmin (Fukuda et al., 1995). It follows that synaptotagmin may be, in part, a sensor for cytoplasmic inositol polyphosphate.

Rabphilin-3A

Rabphilin-3A is a 78-kDa cytosolic protein originally purified from bovine brain based on its ability to bind Rab3A, a small ras-related membrane-bound GTPase known to play a role in neurotransmitter release (Geppert et al., 1994a; von Mollard et al., 1994). Rabphilin-3A has been cloned from both brain (Shirataki et al., 1993) and adrenal chromaffin cells (Chung et al., 1995). Rabphilin-3A contains two copies of type I topology C2 domains (Fig. 3), which contain the full complement of Ca^{2+}-coordinating residues, located near its C-terminus. Bacterially expressed fragments containing only C2 domains bind phosphatidylserine vesicles in the presence of low micromolar Ca^{2+} (Yamaguchi et al., 1993). Although membrane binding depends on the concentration of phosphatidylserine in the vesicles, some Ca^{2+}-dependent binding to pure phosphatidylcholine vesicles is observed (Yamaguchi et al., 1993). Finer mapping has shown that Ca^{2+}-dependent phospholipid binding arises from the first, but not second, C2 domain (Fukuda et al., 1994). In contrast to synaptotagmins, neither C2 domain of rabphilin-3A is capable of binding inositol polyphosphates (Fukuda et al., 1994). An N-terminal fragment of rabphilin-3A lacking the C2 domains binds to the GTP-bound form of Rab3A in the absence of Ca^{2+}, mapping this function of the protein to the N-terminal region (Yamaguchi et al., 1993). In addition, GTP-Rab3A and other unidentified receptors have been shown to recruit rabphilin to synaptic vesicles (Shirataki et al., 1994; Stahl et al., 1996).

DOC2

DOC2 is a 44-kDa cytosolic brain protein, highly concentrated in synaptic vesicles, which contains two C2 domains of type I topology (Fig. 3) and resembles rabphilin-3A except that the N-terminal Rab3A-binding domain is replaced by 80 residues of unique sequence (Orita et al., 1995). Bacterially expressed DOC2 binds to phosphatidylserine vesicles, or less tightly to phosphatidylcholine vesicles, as the Ca^{2+} concentration is increased. Maximal phospholipid binding is achieved at low micromolar Ca^{2+} levels, which, together with its expression pattern and subcellular localization, suggests a role for DOC2 in neurotransmitter release. The C2 domains of DOC2 contain all of the putative Ca^{2+}-coordinating residues present in synaptotagmin I.

Perforin

Perforin is a 70-kDa protein found in secretory granules of cytotoxic T cells that binds and inserts into cellular membranes to form pores that result in lysis of target cells (Lichtenheld et al., 1988; Liu et al., 1995). The C2 domain of perforin, recently identified by sequence alignment (Ponting and Parker, 1996), contains all of the predicted Ca^{2+}-coordinating residues implicated by the synaptotagmin I structure and is predicted to form the type II topology (Fig. 3). Because perforin reversibly binds phosphorylcholine in a Ca^{2+}-dependent manner in vitro (Tschopp et al., 1989), its C2 domain might play a role in directing cytolytic granules to target cells.

UNC-13

UNC-13 is a brain-specific protein present in three mammalian isoforms (I, II, and III) (Brose et al., 1995). UNC-13 was originally cloned from *C. elegans*, where mutations resulted in uncoordinated movements (Maruyama and Brenner, 1991). Although the biochemical function of the UNC-13 is not known in detail, it appears to play a role in synaptic transmission. Interestingly, two of the three C2 domains in UNC-13I (a and b) are predicted to utilize the type II topology, whereas a third (c) uses the type I topology (Fig. 3). UNC-13II, which might represent the mammalian homologue of the identified nematode protein, appears to lack the N-terminal C2 domain present in UNC-13I. Although the exact role of the C2 domain in UNC-13 is not known, "middle" C2 domain fragments (corresponding to the C2b domain of UNC-13I) from all three UNC-13 isoforms fused to glutathione S-transferase failed to bind phospholipid vesicles in response to Ca^{2+} in vitro even though these C2 domains contain all five putative Ca^{2+}-coordinating acidic side chains (Brose et al., 1995). Importantly, however, these recombinant C2 domain fragments (residues 590–728 from UNC-13I, residues 857–1095 of UNC-13II and 150–388 of UNC-13III) all appear to lack the proposed strand $\beta8$ of type II C2 domains (Fig. 3). It is possible that the failure to observe Ca^{2+} regulated membrane binding in vitro by these fragments is due to improper folding or failure to form a complete binding interface. Similar constructs lacking the $\beta8$ strand of the $cPLA_2$ C2 domain appear to fold improperly and fail to display Ca^{2+}-dependent phospholipid binding in vitro (Nalefski et al., manuscript in prep.).

Modification of lipids

Cytosolic phospholipase A_2

The 85-kDa cytosolic phospholipase A_2 ($cPLA_2$) liberates arachidonic acid from the *sn*-2 position of glycerophospholipids to initiate production of leukotrienes and prostaglandins, potent mediators of inflammation (reviewed by Clark et al., 1995). Several experiments have demonstrated that the activation of $cPLA_2$ in vivo arises, at least in part, from Ca^{2+}-induced binding of its single C2 domain to substrate-containing membranes during a cytoplasmic Ca^{2+} flux. First, the isolated C2 domain binds reversibly to membranes in vitro upon addition of low micromolar Ca^{2+} (Nalefski et al., 1994). An engineered $cPLA_2$ lacking its C2 domain ($cPLA_2[\Delta C2]$) fails to bind to membranes in a Ca^{2+}-dependent manner even though it hydrolyzes soluble lysophospholipids at native rates (Nalefski et al., 1994). Additionally, $cPLA_2(\Delta C2)$ fails to display the same subcellular membrane association as the wild-type enzyme (see below) upon cell activation (Schievella et al., 1995). These and other experiments (reviewed by Clark et al., 1995) have led to a two-domain model for $cPLA_2$ in which the N-terminal Ca^{2+}-dependent regulatory C2 domain, residing within residues 1–138, reversibly presents the enzyme to its membrane substrate in the presence of low micromolar Ca^{2+}, whereas the C-terminal Ca^{2+}-independent catalytic domain, beyond residue 138, possesses phospholipase activity.

The C2 domain of $cPLA_2$, unlike those of synaptotagmin, DOC2 and rabphilin-3A, displays a strong preference for Ca^{2+}-stimulated binding to neutral phospholipid vesicles containing phosphatidylcholine or phosphatidylethanolamine rather than acidic phospholipids (Nalefski et al., manuscript in prep.). Thus, the phospholipid specificity of the C2 domain can be varied for different signaling pathways. The divalent cation specificity of the $cPLA_2$ C2 domain, however, shows the same order of preference as the synaptotagmin domains: $Ca^{2+} > Sr^{2+} > Ba^{2+}$; low millimolar Mg^{2+} does not promote liposome binding (Nalefski et al., manuscript in prep.). Moreover, the Ca^{2+} levels required for membrane binding in vitro are similar for the C2 domains of $cPLA_2$, synaptotagmin and rabphilin-3A (i.e., low micromolar), suggesting that Ca^{2+} affinity and specificity are carefully optimized for physiological Ca^{2+} signals. The $cPLA_2$ C2 domain, of type II topology (Fig. 3), contains four of the five acidic side chains implicated in Ca^{2+}-coordination by the synaptotagmin I structure, whereas the fifth is a conservative substitution to Asn.

Although it appears that the C2 domain is responsible for localizing $cPLA_2$ to its membrane substrate upon appearance of Ca^{2+}, it is not yet known whether additional interactions, such as C2 domain binding to a receptor protein, could also play a role in targeting. Specific interactions between $cPLA_2$ and such a receptor might explain why, upon activation of cells with mitogenic stimuli that elicite the release of arachidonic acid, $cPLA_2$ is localized almost exclusively to the endoplasmic reticulum and nuclear envelope (Peters-Golden and McNish, 1993; Glover et al., 1995; Schievella et al., 1995), where some of the downstream enzymes of the arachidonic acid pathway, such as 5-lipoxygenase and cyclooxygenase, are found (Peters-Golden and McNish, 1993; Regier et al., 1993; Brock et al., 1994). Recombinant $cPLA_2$ lacking the C2 domain fails to display this behavior (Schievella et al., 1995). Alternatively, it is possible that accumulated $cPLA_2$ substrate or product helps to trap the enzyme on the appropriate membrane (Ghomashchi et al., 1992).

Phosphoinositide-specific phospholipase C

Phosphoinositide-specific phospholipase C (PLC) liberates inositol (1,4,5)trisphosphate (IP_3) and diacylglycerol (DAG) in response to mitogenic signals that raise intracellular Ca^{2+} levels (reviewed by Berridge and Irvine, 1989; Berridge, 1993). Thus, PLC provides a major branch point in the generation of second messengers: IP_3 is an important mediator of Ca^{2+} channels, whereas DAG activates several PKC isozymes. Three mammalian PI-PLC classes (β, γ, and δ) have been identified (reviewed by Rhee et al., 1989; Kriz et al., 1990), and each contains multiple isoforms composed of modules of EF-hands, pleckstrin homology (PH) domains, X and Y catalytic boxes, and C2 domains (Fig. 5). Thus, this family is likely to be regulated by (a) Ca^{2+} binding to its C2 or EF-hand domains, (b) binding of phosphatidylinositol(4,5)bisphosphate (PIP_2) and IP_3 to the PH domain, and (c) membranes containing the appropriate ligands (Rhee and Choi, 1992; Yagisawa et al., 1994; Ferguson et al., 1995; Lemmon et al., 1996). The different classes may possess additional regulators; for instance, the Src homology-2 (SH2) domains of the PLC-γ isozymes bind to phosphotyrosine on activated receptors, directing PLC-γ to the membrane (reviewed by Pawson and Gish, 1992). Regulation of PLC-β might involve membrane-anchored Gα proteins, which are thought to dock C-terminal to the C2 domain (Park et al., 1992; Wu et al., 1993). In addition, G protein $\beta\gamma$ subunits have been demonstrated to regulate PLC-β isozymes (Camps et al., 1992; Katz et al., 1992; Carozzi et al., 1993); this interaction is mediated via a domain distinct from that which interacts with Gα (Schnabel et al., 1993). Determination of the structure of the PLC-δ1 has led to the "tether and fix" model of PLC membrane binding and activation, in which the PH domain is proposed to "tether" the protein to the membrane, whereas the C2 domain "fixes" the catalytic domain in the appropriate orientation (Essen et al., 1996). Mutants of PLC-δ lacking the C2 domain retain IP_3 binding but fail to hydrolyze membrane substrates (Ellis et al., 1993; Yagisawa et al., 1994).

Isolated C2 domains of PLC, all of which are type II topology (Fig. 3), have not been studied in detail. It is important to define the phospholipid-specificities of the PLC C2 domains, since their catalytic domains are exclusively phosphoinositide-specific at physiological Ca^{2+} levels (see Kriz et al., 1990; Rhee and Choi, 1992) and the PLC PH domain forms a high affinity complex with PIP_2 (Lemmon et al., 1996). Moreover, the PLC-β and PLC-γ isoforms lack the full complement of five Ca^{2+}-coordinating acidic side chains inferred by the synaptotagmin I structure. PLC-γ expressed in COS cells fails to bind cellular membranes in vitro upon addition of Ca^{2+} levels that cause binding of $cPLA_2$ and PKC-β1 (Clark et al., 1995). Clearly, much remains to be learned about the complexities of PLC control.

Phosphatidylserine decarboxylase

Phosphatidylserine decarboxylase (PSD) catalyzes the decarboxylation of phosphatidylserine, generating phosphatidylethanolamine. The yeast PSD2 (Trotter et al., 1995), unlike previously identified PSD, contains a C2 domain of type II topology (Fig. 3). However, the activity of recombinant PSD2 protein is not Ca^{2+} dependent in vitro and the primary structure of the C2 domain lacks one of the five Ca^{2+} coordinating acidic residues inferred by the synaptotagmin I structure. The role of the C2 domain in PSD2 has not been investigated, although it might promote a favorable interaction between the catalytic domain and its membrane substrate as in the case of PLC (Essen et al., 1996).

Phosphatidylinositol 3-kinase

Phosphatidylinositol 3-kinase (PI3K) phosphorylates the 3′-OH of the inositol ring of several phosphoinositides to generate 3-phosphorylated lipids, which have been proposed to act as second messengers in cellular activation (reviewed by Stephens et al., 1993; Parker, 1995). The prototypical mammalian PI3K consists of an 85-kDa regulatory subunit and α or β isoforms of a 110-kDa catalytic subunit, each of which has been cloned and sequenced (Hiles et al., 1992; Hu et al., 1993). The 85-kDa regulatory subunit contains SH2 domains that recognize specific phosphotyrosine residues on activated receptor tyrosine kinases, directing the enzyme to the membrane (reviewed by Pawson and Gish, 1992). A single C2 domain was identified in the mammalian PI3Kα catalytic subunit by sequence alignment (Stephens et al., 1993); a possible C2 domain in the corresponding region of the β isoform is presented in the present report. Potential C2 domains have also been identified in a different form of PI3K, termed VPS34P (Welters et al., 1994), which has been cloned from several species. In yeast, VPS34P has been shown to play a role in vesicular transport (Herman and Emr, 1990), suggesting a similar role for the mammalian enzyme (Volinia et al., 1995). Recently, a family of PI3K has been cloned from *Drosophila*; two members are similar to the aforementioned PI3K, whereas the third (PI3K_68D) represents a novel PI3K that contains a C2 domain at its extreme C-terminus (MacDougall et al., 1995). C2 domains have not been reported in the related novel PI3Kγ isolated recently (Stoyanov et al., 1995).

In general, C2 domains of PI3K exhibit low overall sequence similarity to other C2 domains and also lack several of the putative Ca^{2+}-coordinating residues (Fig. 3). The bacterially expressed C2 domain of a *Drosophila* PI3K, PI3K_68D, failed to bind to phospholipid vesicles in a Ca^{2+}-dependent manner (MacDougall et al., 1995). However, like the second C2 domain of synaptotagmin, the C2 domain of PI3K_68D bound phospholipids in a Ca^{2+}-independent fashion in vitro (MacDougall et al., 1995). Interestingly, the C2 domains of PI3K α/β isoforms and VPS34P are predicted to utilize the type II topology, whereas the C2 domain of PI3K_68D utilizes the type I topology (Fig. 3). The role of the C2 domain in PI3Kα/β and VPS34P remains unknown, although it might promote a favorable orientation for the catalytic domain with respect to the lipid surface, as proposed for PLC (Essen et al., 1996).

Phospholipase D

A related group of phosphatidylcholine-specific phospholipase D (PLD) proteins, which liberate choline and phosphatidic acid, has recently been identified in several species (Hammond et al., 1995). These proteins contain a highly conserved domain that might represent a catalytic or cofactor-binding site (Hammond et al., 1995). The plant PLD, cloned from several species including rice, maize and castor bean (Wang et al., 1994), also contains a stretch of sequence similarity at its extreme N-terminus similar to C2 domains (Ponting and Parker, 1996). A truncated version of PLD lacking the N-terminal 30 residues extending into the proposed C2 domain is inactive, in contrast to the full-length polypeptide (Wang et al., 1994). Since the truncated version can be isolated from plants, it has been suggested that these N-terminal 30 residues constitute a leader sequence (Wang et al., 1994); however, we propose that these residues provide an essential element of the C2 domain, namely strand $\beta1$. The plant PLD C2 domain, of type II topology (Fig. 3), lacks four of the five putative Ca^{2+}-coordinating residues.

Protein phosphorylation

Protein kinase C

PKC is a family of related protein kinases, which includes at least 11 different mammalian isoforms (α, $\beta1$, $\beta2$, γ, δ, ϵ, θ, η, ζ, μ, and $\lambda[\iota]$) (reviewed by Nishizuka, 1988, 1992; Dekker and Parker, 1994) and three yeast enzymes (PKC1, PCK1, and PCK2) (Levin et al., 1990; Toda et al., 1993). Much is known about the various functions and regulation of PKC in different cellular signalling pathways, as summarized by Nishizuka (1995); however, the exact role of the C2 domain in PKC is just beginning to emerge (recently reviewed by Newton, 1995a, 1995b). Some non-classical PKC isoforms (ζ, λ, and μ) lack the C2 domain altogether. Interestingly, of the isoforms that possess the C2 domain, the classical isoforms (α, β, and γ) of PKC are predicted to utilize the type I topology, whereas the non-classical mammalian isoforms (δ, ϵ, θ, and η) and the yeast PKC (PKC1, PCK1, and PCK2) use the type II topology (Fig. 3). Moreover, the order of domains differs between the classical (C1, C2, C3–C4) and non-classical (C2, C1, C3–C4) isoforms containing C2 domains (Fig. 5).

Recently the isolated C2 domain of PKC-β has been shown to exhibit Ca^{2+}-induced binding to mixed vesicles containing phosphatidylserine and phosphatidylcholine (Shao et al., 1996). In all classical, Ca^{2+}-regulated PKC isoforms, the C2 domains appear to provide the observed Ca^{2+}-requirement for activation (Nishizuka, 1988) and possess all five Ca^{2+}-coordinating acidic side chains implicated by the synaptotagmin I structure. These C2 domains may act as Ca^{2+}-activated, allosteric molecular switches to turn on kinase activity, as pseudosubstrates for a PKC site that docks to a receptor protein or, by analogy to the binding of synaptotagmin C2 domains to synataxin/clathrin-AP2, as protein receptors (Mochly-Rosen, 1995; Ron and Mochly-Rosen, 1995). The C2 domains of the novel Ca^{2+}-independent isoforms lack at least two of the putative Ca^{2+}-coordinating residues; in these proteins it has been proposed that basic substitutions at one of the coordinating positions substitute for bound Ca^{2+} (Newton, 1995a), thereby enabling constitutive activation of the kinase domain, or docking to a membrane or receptor protein. A putative PKC sequence motif responsible for the phosphatidylserine-binding site is located in the C2 domain of classical isoforms (Igarashi et al., 1995).

Other protein kinases

Recently, sequence alignment (Ponting and Parker, 1996) enabled identification of C2 domains in three proteins cloned previously, the PKC-like lipid-dependent protein kinases from mammals (PRK1 and PRK2) (Mukai and Ono, 1994; Palmer et al., 1995; Palmer and Parker, 1995) and a cAMP-dependent protein kinase from yeast (SCH9 kinase) (Toda et al., 1988). These proteins exhibit overall low sequence similarity to 'conventional' C2 domains, are predicted to form the type II topology, and lack the full complement of predicted Ca^{2+}-coordinating residues (Fig. 3). The role of the C2 domain in these proteins has not been tested but may be similar to that in PKC.

Regulation of GTPases

GTPase-activating proteins

The GTPase-activating proteins (GAPs) are a heterogenous class of proteins related by the presence of the GAP-related domain (GRD) (reviewed by Boguski and McCormick, 1993). C2 domains, of the type II topology (Fig. 3), have been identified in several GAPs including p120-rasGAP, GAP1^{m}, R-Ras-GAP/GAP1^{IP4BP}, BCR, ABR, and BUD2, although none contains a full set of the five putative Ca^{2+}-coordinating acidic residues. Interestingly, the GRD domain and the C2 domain present in the BCR gene product (Heisterkamp et al., 1985) are deleted during formation of the BCR-ABL oncoprotein by translocation in the Philadelphia chromosome, reviewed by Daley and Ben-Neriah (1991). *Drosophila* containing mutant homologues of GAP1^{m} display aberrant eye development (Gaul et al., 1992).

The role of the C2 domain has been tested for p120-rasGAP; bacterially expressed proteins containing a portion of the C2 domains from p120-rasGAP attached to glutathione S-transferase bind phosphatidylserine or phosphatidylinositol, but not phosphatidylcholine, vesicles in the presence of low micromolar Ca^{2+} in vitro (Gawler et al., 1995b). Ca^{2+}-dependent membrane binding was observed using constructs that encoded a truncated C2 domain containing only a portion of the loop between $\beta1$ and $\beta2$ and strands $\beta2$ through $\beta4$ (Gawler et al., 1995a, 1995b). Attachment of a portion of the p120-rasGAP C2 domain to a transformation-defective v-*src* (lacking its own N-terminal myristylation sequence) restored transformation ability and particulate localization in vivo (Gawler et al., 1995b). In vitro, however, recombinant p120-rasGAP, which lacks four of the five putative Ca^{2+}-coordinating acidic side chains, failed to bind cellular membranes in response to Ca^{2+} levels that cause binding of cPLA$_2$ and PKC-$\beta1$ (Clark et al., 1995). Thus, membrane binding by this protein may involve its distinct PH domain, which in other proteins binds inositol polyphosphates (Lemmon et al., 1996). Alternatively, a polybasic stretch of Lys or Arg residues may provide inositol polyphosphate binding, as proposed for the second C2 domain of GAP1^{IP4BP} (Cullen et al., 1995). Clearly, the exact role of the C2 domain in GAPs must be investigated further. As proposed for phosphoinositide-specific PLC (Essen et al., 1996), GAPs may initially be "tethered" to the membrane via PH or SH2 domains present in several GAPs (see Fig. 5), while their C2 domains may "fix" the GAP to the membrane. Such an arrangement could orient the GRD catalytic domain favorably with respect to the ras-like GTPases, which are anchored in the membrane via post-translational modifications.

Unknown functions

RSP5/NEDD-4

RSP5 is a yeast protein of unknown biochemical function that suppresses mutations in the yeast transcription factor SPT3 (Eisenmann et al., 1992). It contains an N-terminal C2 domain of type II topology (Fig. 3), three WW domains (Hofmann and Bucher, 1995; Bork and Sudol, 1996) and a C-terminal *hect* domain, which has been shown to act as a ubiquitin-ligase in vitro (Huibregste et al., 1995). A mammalian homologue of RSP5, termed NEDD-4, has been isolated as a cDNA differentially expressed during neural development (Kumar et al., 1992) and independently as a receptor for proline-rich regions of the amiloride-sensitive epithelial sodium channel (ENaC) (Staub et al., 1996). A model has been proposed in which NEDD-4 suppresses ENaC activity by ubiquitin-mediated receptor degradation upon binding of its C2 domain to channel membranes and docking of its WW domains to proline-rich sequences in the ENaC (Staub et al., 1996). Regulation of the epithelian sodium channel by intracellular Ca^{2+} (reviewed by Garty, 1994) and the experiments described above raise the intriguing possibility that C2 domains may participate in hormonal modulation of sodium transport.

Future Directions

Although much has been learned regarding the structure and function of the C2 motif, several fundamental questions remain unanswered. The stoichiometry of Ca^{2+} binding and the identities of coordinating residues have only been partly defined, and very little is known about the mechanism of Ca^{2+}-triggered membrane binding. Ligands for many of the C2 domains have not yet been identified. Isolated C2 domains will be used to answer many of these questions, but eventually it will be important to study C2 domains in their native, full-length proteins of origin where interesting interactions with other domains may be present. Such full-length systems will also be required to determine the functional basis of the two topologies observed for the C2 domain. In short, the C2 motif will continue to provide tantalizing problems for the forseeable future.

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