Minireview

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C_2-domains, Structure and Function of a Universal Ca^{2+}-binding Domain*

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A vast amount of protein sequence data accumulated over recent years has revealed that protein modules are widespread in nature. Many intracellular and extracellular proteins consist, in part or fully, of combinations of protein modules. C_2-domains, together with SH2, PTB, PH, SH3, WW, and PDZ domains, are typical examples of intracellular protein modules. These modules form independently folding domains of 80–160 residues with characteristic binding properties; C_2-domains bind Ca^{2+} and phospholipids, SH2 and PTB domains phosphotyrosine-containing sequences, PH domains phosphatidylinositol phosphates, SH3 and WW domains proline-rich sequences, and PDZ domains C-terminal sequences. C_2-domains are unique among these modules because phospholipid binding to many C_2-domains is regulated by Ca^{2+}. For this reason, C_2-domains are sometimes referred to as Ca^{2+}-dependent lipid binding domains. However, C_2-domains are not obligatory Ca^{2+}- and phospholipid-binding modules. C_2-domains have diverged evolutionarily into Ca^{2+}-dependent and Ca^{2+}-independent forms that interact with multiple targets. Thus, although most C_2-domains are probably Ca^{2+}-binding domains, they represent a family of versatile protein modules with diverse functions.

C_2-domains comprise approximately 130 residues and were first identified in protein kinase C (1). Close to 100 C_2-domain sequences are listed in the current data banks. Although reviews of several C_2-domain proteins have been published (2–11), recent results on the structure and interactions of C_2-domains by x-ray crystallography and NMR spectroscopy offer a new opportunity to rationalize the properties of C_2-domains in structural terms. In this minireview, we will attempt to use this opportunity and correlate the functional properties of C_2-domains with their structures.

Proteins Containing C_2-domains

Most proteins with C_2-domains function in signal transduction or membrane traffic. The first category includes proteins involved in the generation of lipid second messengers (*e.g.* $cPLA_2$[1] (12), PLCs (13), and phosphatidylinositol 3-kinases (14)), in protein phosphorylation (*e.g.* PKC (15, 16)), in activation of GTPases (*e.g.* Ras-GAP (17)), and in ubiquitin ligation (*e.g.* Nedd4 (18)). The second category contains synaptotagmins (19, 20), rabphilin-3 (21), RIM (22), and Munc13 (2). In addition to many well characterized proteins, several open reading frames with C_2-domains are reported in GenBank™. For example, open reading frames in *Caenorhabditis elegans* and yeast encode transmembrane proteins with three or four C_2-domains whose biological roles have not been identified. This suggests that additional interesting functions for C_2-domain proteins remain to be discovered.

Much of the current data on the structures and interactions of C_2-domains were derived from studies of PLCδ1, PKC, $cPLA_2$, and, in particular, synaptotagmin I. PLCδ1 hydrolyzes phosphatidylinositol 4,5-bisphosphate to generate the second messengers diacylglycerol and inositol-1,4,5-trisphosphate (5). PKCs represent a family of protein kinases that are regulated by diacylglycerol and other lipids (1, 4). In addition, activation of the classical isoforms of PKC (PKCα, -β, and -γ) depends on Ca^{2+}. $cPLA_2$ hydrolyzes glycerophospholipids to produce arachidonic acid, a precursor of prostaglandins and leukotrienes, which are involved in inflammation (7). In PLCδ1, PKC, and $cPLA_2$, the C_2-domain is believed to play a regulatory role by mediating the Ca^{2+}-dependent recruitment of these enzymes to phospholipid membranes. In contrast, synaptotagmin I functions in membrane traffic. Synaptotagmin I belongs to a family of at least 12 transmembrane proteins containing two C_2-domains, the C_2A- and C_2B-domains. The C_2-domains occupy most of the cytoplasmic region of the synaptotagmins and probably act as Ca^{2+} effector domains. Synaptotagmin I is localized to synaptic vesicles where it is essential for the fast, Ca^{2+}-dependent component of neurotransmitter release (23, 24). Synaptotagmin I is believed to function as the main Ca^{2+} sensor in synaptic vesicle exocytosis by a mechanism involving Ca^{2+} binding to both C_2-domains (see below). The functions of the other synaptotagmins and membrane trafficking proteins with C_2-domains are less well characterized but may be similar.

Diverse Ca^{2+}-dependent Properties of C_2-domains

The notion that C_2-domains act as Ca^{2+}-binding motifs arose from the observation that the classical isoforms of PKC, which contain a C_2-domain (PKCα, -β, and -γ), were regulated by Ca^{2+}. In contrast, isoforms apparently lacking a C_2-domain (PKCδ, -ϵ, -η, and -θ) were Ca^{2+}-independent (1). Activation of classical PKCs by Ca^{2+} involves the translocation of PKCs to the membrane by Ca^{2+}-dependent phospholipid binding (4, 25). These observations led to the hypothesis that C_2-domains may mediate Ca^{2+}-dependent phospholipid binding.

This notion was first actually demonstrated for the C_2A-domain of synaptotagmin I. The isolated C_2A-domain was shown to represent an autonomously folding module that binds phospholipids in a Ca^{2+}-dependent manner (26). The C_2A-domain bound all negatively charged phospholipids independent of headgroup structure. Mg^{2+}, Ba^{2+}, and Sr^{2+} were unable to stimulate phospholipid binding. Ca^{2+} acted cooperatively with a Hill coefficient of 3 and an apparent affinity in the low micromolar range. Further studies revealed that the C_2A-domain binds Ca^{2+} directly without phospholipids but with a lower apparent Ca^{2+} affinity (≈0.2 mM compared with ≈5 μM free Ca^{2+}) (27). The C_2A-domain of synaptotagmin I also bound syntaxin 1 as a function of Ca^{2+}, with a low apparent Ca^{2+} affinity resembling that of intrinsic Ca^{2+} binding (28). These data suggested that the functions of C_2-domains may be diverse and include Ca^{2+}-dependent interactions with proteins in addition to phospholipids. Analysis of the C_2A-domains from other synaptotagmins revealed that those from synaptotagmins II, III, V, and VII bind phospholipids and syntaxin 1, but the C_2A-domains of synaptotagmins IV, VI, X, and XI do not (28). Although the C_2A-domains of synaptotagmins I, II, III, V, and VII bind to phospholipids with similar Ca^{2+} affinities, they exhibit distinct cation specificities (29). All of the C_2A-domains from synaptotagmins that bind to phospholipids also interact with syntaxin 1 as a function of Ca^{2+} but with different Ca^{2+} dependences: synaptotagmins I, II, and V require Ca^{2+} concentrations of >0.2 mM whereas synaptotagmins III and VII bind at <1 μM Ca^{2+} (28).

Sequence analyses of the C_2A- and C_2B-domain of synaptotagmin I revealed that they contain evolutionarily conserved differences. The C_2B-domain does not exhibit the same Ca^{2+}-dependent

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[1] The abbreviations used are: $cPLA_2$, cytoplasmic phospholipase A_2; C_2A- and C_2B-domains, first and second C_2-domains of synaptotagmins; PKC, protein kinase C; PLC, phosphoinositide-specific phospholipase C.

phospholipid binding properties as the C_2A-domain. Nevertheless, most C_2B-domains probably bind Ca^{2+} because they contain the requisite Ca^{2+}-binding sequences defined in C_2A-domains (see below), and because the C_2B-domains of synaptotagmins I and II mediate the Ca^{2+}-dependent self-association of synaptotagmins (30). These results led to a model whereby the C_2A- and C_2B-domains of most synaptotagmins are Ca^{2+}-binding domains that are specialized for different Ca^{2+}-dependent activities.

Similar to the C_2A-domains of synaptotagmins, the C_2-domains from $cPLA_2$ (31, 32), PKCβ (27), and Nedd4 (18) bind phospholipids at micromolar Ca^{2+} concentrations. However, the C_2A-domains of synaptotagmins and the C_2-domain of PKCβ preferentially bind to negatively charged phospholipids whereas the C_2-domain from $cPLA_2$ interacts with neutral phospholipids (26, 33). Furthermore, although the C_2-domain from PKCβ is similar to the synaptotagmin C_2A-domains, it does not bind to syntaxin 1 as a function of Ca^{2+}. Therefore, even among C_2-domains that share Ca^{2+}-dependent phospholipid binding, there are functional distinctions that may be important for their biological roles.

To complicate matters, some C_2-domains that are Ca^{2+}-regulated simultaneously bind other molecules in a Ca^{2+}-independent manner. For example, the C_2B-domain of synaptotagmin I interacts with AP-2 (34), inositol polyphosphates (35), β-SNAP (36), and Ca^{2+} channels (37). Finally, many C_2-domains may not bind Ca^{2+} at all. Several synaptotagmins appear to be unable to bind Ca^{2+}, as may be the case with the C_2-domains of RIM. Interestingly, PKC isoforms that initially were not thought to have a C_2-domain and are Ca^{2+}-independent (PKCδ, -ϵ, -η, and -θ) actually contain a C_2-domain that is located at the N terminus and probably does not bind Ca^{2+} (2, 11, 38). Thus, as a group C_2-domains perform multiple biological functions.

Three-dimensional Structures of C_2-domains

X-ray diffraction analysis of the synaptotagmin I C_2A-domain yielded the first structure of a C_2-domain (39). The structure consists of a compact β-sandwich composed of two four-stranded β-sheets (Fig. 1*A*). Three loops at the top of the domain and four at the bottom connect the eight β-strands. Ca^{2+} binding occurs exclusively at the top three loops (see below). NMR spectroscopy showed that the solution structure of the C_2A-domain is identical to the crystal structure (27).[2]

Determination of the structures of three other C_2-domains (from PKCβ, $cPLA_2$, and PLCδ1) revealed similar designs and interesting differences. PLCδ1 is a modular protein composed of PH-, EF-hand, C_2-, and catalytic domains. X-ray diffraction studies of crystals from PLCδ1 lacking the N-terminal PH domain provided the three-dimensional structure of a C_2-domain in the context of a nearly full-length protein (40, 41). The three-dimensional structure of the PLCδ1 C_2-domain (Fig. 1*B*) is very similar to that of the synaptotagmin I C_2A-domain, with a root mean square deviation of 1.4 Å for 109 equivalent α-carbons. The topology of the β-strands, however, is strikingly different (40). The arrangement of β-strands in the PLCδ1 C_2-domain constitutes a circular permutation of the topology observed in the C_2A-domain of synaptotagmin I (Fig. 1*C*). As a result, strand 1 of the synaptotagmin I C_2A-domain occupies the same position as strand 8 of the PLCδ1 C_2-domain. The N and C termini are at the top of the C_2-domain in synaptotagmin I but at the bottom in PLCδ1 (Fig. 1). The two types of topology are referred to as topology I (synaptotagmin I C_2A-domain) or topology II (PLCδ1 C_2-domain). The crystal structures of the C_2-domains from PKCβ[3] and $cPLA_2$ (42) are also similar to those of synaptotagmin I and PLCδ1 and exhibit topologies I and II, respectively. It is unclear why C_2-domains occur in two topologies. One reason may be that the topology influences the relative orientation of a C_2-domain with respect to its neighboring domains.

There is a high degree of structural homology between C_2-domains in the core β-sandwich and less similarity in the top and bottom loops (Fig. 1). Accordingly, the C_2-domain sequences involved in the core β-sandwich are highly conserved between C_2-domains whereas the sequences of the loops, particularly loop

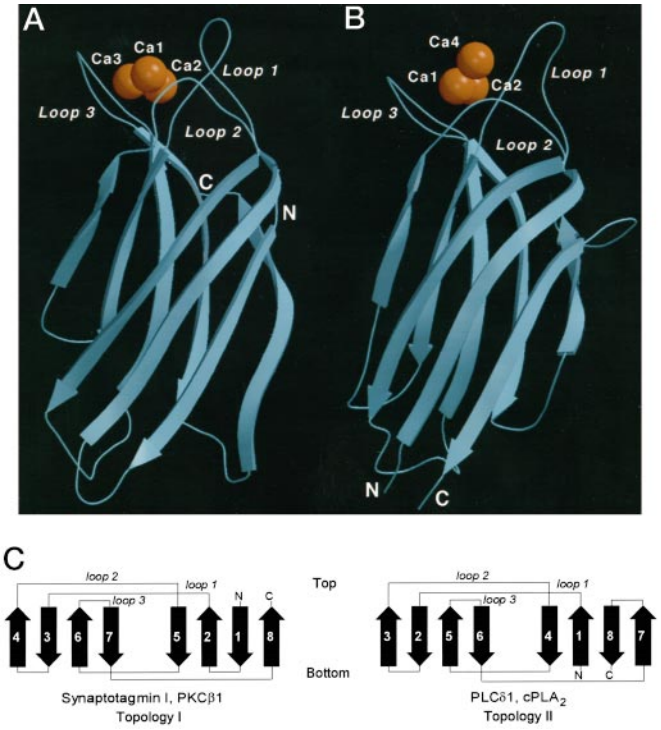

FIG. 1. **Ribbon diagrams of the structures of the C_2A-domain of synaptotagmin I (*A*) and the C_2-domain of PLCδ1 (*B*) and schematic drawing of their β-strand topologies (*C*).** In *A* and *B*, the locations of the N and C termini and of the Ca^{2+}-binding loops are indicated. Each C_2-domain is shown complexed to three Ca^{2+} ions (*orange*) (27, 40, 41, 44).[2,3] The diagrams were prepared with the program MOLSCRIPT (51). In *C*, β-strands in the C_2-domains from synaptotagmin I and PKCβ (*left*) and from PLCδ1 and $cPLA_2$ (*right*) are numbered in the order of the primary sequences. The three Ca^{2+}-binding loops at the top of the C_2-domains are indicated.

1 and the three bottom loops, are not. The high degree of structural identity between the core β-sandwiches of C_2-domains suggests that the β-sandwich represents a scaffold. This scaffold allows the emergence of variable loops at the top and bottom of the domain. As discussed below, the loops are involved in Ca^{2+} binding and may determine the functional specificity of a C_2-domain. The C_2-domain structures provide a framework to interpret the properties of C_2-domains and at the same time allow us to predict the minimum sequences required for a complete, well folded β-sandwich. Thus results from experiments performed with incomplete C_2-domain fragments or with mutants containing deletions in a β-strand should be interpreted with caution since misfolding is likely.

How Do C_2-domains Bind Ca^{2+}?

The Ca^{2+} binding modes of the C_2-domains from synaptotagmin I, PKCβ, PLC-δ1, and $cPLA_2$ were analyzed by x-ray diffraction and NMR spectroscopy (27, 39–44).[2,3] In all C_2-domains, multiple Ca^{2+} ions bind in a cluster exclusively at the top loops (Fig. 1). These loops are widely separated in the primary sequences (Fig. 2). The Ca^{2+}-binding sites are formed primarily by aspartate side chains that serve as bidentate ligands for two or three Ca^{2+} ions.

In the C_2A-domain of synaptotagmin I, loops 1 and 3 contain three Ca^{2+}-binding sites (*Ca1*, *Ca2,* and *Ca3* in Fig. 3). The Ca^{2+}-binding sites are formed by five aspartate side chains, one serine side chain, and three carbonyl groups (27, 39, 44)[2] (Figs. 2 and 3). The presence of three Ca^{2+}-binding sites in the C_2A-domain correlates well with the Hill coefficient of 3 observed in Ca^{2+}-dependent phospholipid binding experiments (26). Ca^{2+} binding to all three sites is necessary for syntaxin 1 and phospholipid binding (44).[4] The coordination spheres of the bound Ca^{2+} ions in the C_2A-domain are incomplete, especially for Ca3. This results in the low apparent intrinsic Ca^{2+} affinity of this site (>1.0 mM). When phospholipids

[2] X. Shao, I. Fernandez, T. C. Südhof, and J. Rizo, submitted for publication.
[3] B. Sutton and S. Sprang, submitted for publication.
[4] X. Zhang, J. Rizo, and T. C. Südhof, submitted for publication.

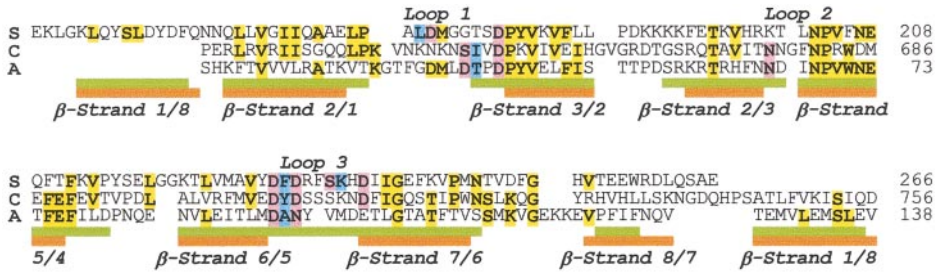

FIG. 2. **Sequences of the C_2-domains from synaptotagmin I (*S*), PLCδ1 (*C*), and cPLA$_2$ (*A*).** Identical residues are shown on a *yellow* background. Aspartate, asparagine, and serine residues that coordinate the Ca^{2+} ions in the different C_2-domains are shown on a *pink* background. Residues whose backbone carbonyl groups coordinate Ca^{2+} ions are shown on a *blue* background. Sequences that are structurally almost identical in the three-dimensional structures are indicated by a *green bar* below the alignment, and locations of β-strands by an *orange bar*. The positions of the three loops involved in Ca^{2+} binding are shown *above* the alignment.

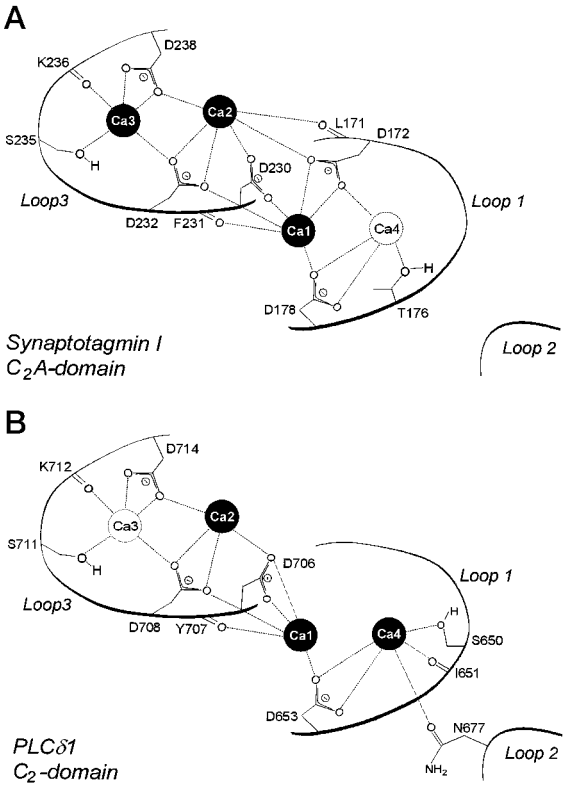

FIG. 3. **Summary of the Ca^{2+}-binding sites in the C_2-domains of synaptotagmin I (*A*) and of PLCδ1 (*B*).** The side chains and carbonyl groups involved in binding are indicated. Sites actually observed in the structures are shown by *solid circles*, and potential additional sites in each C_2-domain by *open circles*. For the PLCδ1 C_2-domain, site Ca2 was only occupied in the La^{3+} complex, and the serine 650 side chain (*S650*) was only involved in metal ion coordination in this complex. Although not observed experimentally, a site could exist in the synaptotagmin I C_2A-domain that is analogous to site Ca4 in PLCδ1, and a site analogous to site Ca3 could be formed by the PLCδ1 C_2-domain.

bind, they probably fill unsatisfied coordination sites on the bound Ca^{2+} ions, resulting in a ≈1000-fold increase in the apparent affinity of the C_2A-domain for Ca^{2+}. The Ca^{2+} binding mode of the C_2-domain of PKCβ, as determined by x-ray crystallography, is very similar to that of the C_2A-domain.[3]

The C_2-domain of PLCδ1 shares two of the Ca^{2+}-binding sites of the synaptotagmin I C_2A-domain (Ca1 and Ca2) but contains a distinct third Ca^{2+}-binding site (Ca4) (41, 43) (Fig. 3). Site Ca4 involves one aspartate, one asparagine, and one serine side chain in addition to one backbone carbonyl group. Sites Ca1 and Ca4 were occupied in all complexes of PLCδ1 with Ca^{2+} and Ca^{2+} analogs (La^{3+}, Sm^{3+}, and Ba^{2+}). Site Ca2 was only filled in the La^{3+} complex, but it seems likely that Ca^{2+} also binds to this site at Ca^{2+} concentrations above 1 mM or at lower Ca^{2+} concentrations in the presence of phospholipids (43). In addition, all side chains from site Ca3 in the synaptotagmin I C_2A-domain are conserved in the PLCδ1 C_2-domain and have similar orientations in the structures of both C_2-domains (44).[2] This strongly suggests that Ca^{2+} may also occupy this site and that the PLCδ1 C_2-domain may contain a total of four Ca^{2+}-binding sites. Bound Ca^{2+} ions in the C_2-domain of PLCδ1 have unsatisfied coordination sites, suggesting that in the absence of phospholipids they may also exhibit low apparent affinities. The C_2-domain of cPLA$_2$ has a Ca^{2+} binding mode similar to that of the PLCδ1 C_2-domain but apparently with only sites Ca1 and Ca4 occupied (42).

The Ca^{2+} binding modes summarized above can be used to anticipate the Ca^{2+} binding properties of other C_2-domains. The aspartate residues involved in Ca^{2+} binding in the synaptotagmin I C_2A-domain are conserved in many C_2-domains. Based on this observation, we proposed that the motif formed by these aspartate residues is widespread and named it the C_2-motif (27). Sites Ca1 and Ca2 are probably the most common Ca^{2+}-binding sites in C_2-domains, and additional Ca^{2+}-binding sites are likely to exist in many C_2-domains depending on the side chains present in loops 1–3. Ca^{2+}-dependent C_2-domains thus appear to have been designed to concentrate multiple Ca^{2+} ions in a small region. The Ca^{2+} ions contain unsatisfied coordination sites that remain available for interaction with target molecules.

Mechanisms of C_2-domain Function

The three-dimensional structures of C_2-domains determined so far show no evidence that Ca^{2+} induces a substantial change from one well defined conformation to another well defined conformation. Comparison of the NMR solution structure of the Ca^{2+}-bound form of the synaptotagmin I C_2A-domain with the crystal structure of the Ca^{2+}-free form demonstrated that Ca^{2+} binding involves rotations of some side chains but causes no substantial backbone rearrangements (27).[2] The NMR data indicate that the Ca^{2+}-binding region is flexible in the absence of Ca^{2+} and is stabilized after Ca^{2+} binding. Structural stabilization by Ca^{2+} binding is consistent with decreased B-factors in the crystal structure of the C_2A-domain after partial Ca^{2+} saturation (39) and with the observations that Ca^{2+} causes a large change in denaturation temperature (27) and increases the resistance of the synaptotagmin I C_2A-domain against proteolysis (45). With regard to the PLCδ1 C_2-domain, 10 of 12 x-ray structures obtained in the presence or absence of Ca^{2+} or Ca^{2+} analogs are very similar, suggesting that Ca^{2+} binding does not cause conformational changes (40, 41, 43).

If Ca^{2+} does not induce a major conformational change in C_2-domains, how does Ca^{2+} regulate their function? The structural stabilization induced by Ca^{2+} probably does not account for Ca^{2+} regulation because the conformations compatible with binding to target molecules are also available in the absence of Ca^{2+}. However, Ca^{2+} binding causes a major change in the electrostatic potential of the synaptotagmin I C_2A-domain that may be important for regulating interactions. Analysis by NMR spectroscopy showed that the region around the Ca^{2+}-binding sites of the C_2A-domain is responsible for Ca^{2+}-dependent binding to syntaxin (46). This region contains the cluster of aspartate residues that coordinate Ca^{2+} and a ring of basic amino acids surrounding it. Binding to syntaxin, a negatively charged protein, could therefore be driven by the change in electrostatic potential caused by Ca^{2+} binding and could be mediated by the basic side chains surrounding the Ca^{2+}-binding site. Two exposed hydrophobic side chains in the region and coordination of the unsatisfied Ca^{2+} valences by acidic residues of syntaxin may contribute to the interaction (46).

The mode of interaction between synaptotagmin I and syntaxin 1 suggested that synaptotagmin I acts as an electrostatic switch in neurotransmitter release. The binding of phospholipids by the C_2A-domain is also best explained by this model. Mutations in basic and

acidic amino acids that disrupt syntaxin binding (44, 46) also inhibit phospholipid binding.[4] Phospholipid binding correlates with the density of negative charges on the surface of the phospholipid bilayer rather than with a specific chemical structure. Furthermore, binding is inhibited by high salt. These results support the importance of electrostatic interactions for the Ca^{2+}-dependent binding of the synaptotagmin I C_2A-domain to phospholipids (26). Other C_2-domains that bind to negatively charged phospholipids in a Ca^{2+}-dependent manner such as those of classical PKCs may share this mechanism of binding. Substitutions in two of the aspartate residues that bind Ca^{2+} in PKCβ have shown, however, that lipid binding is probably not purely electrostatic (47). It is likely that coordination of the Ca^{2+} ions bound to C_2-domains by the phosphate groups of the lipids may provide a major contribution to the binding energy, which is supported by the observation that the apparent Ca^{2+} affinities are much higher in the presence of phospholipids than in their absence. Insertion of highly exposed hydrophobic side chains into the lipid bilayer may contribute to binding as proposed for the Ca^{2+}-dependent binding of phosphatidylcholine to the C_2-domain of $cPLA_2$ (33). The side chains in the Ca^{2+}-binding loops are likely to influence the preference for types of lipids. Thus, the preference of $cPLA_2$ for neutral rather than negatively charged phospholipids may be because of the presence of two acidic residues in loops 1 and 3, in addition to the Ca^{2+}-binding residues, and to the absence of basic residues.

Evolution of C_2-domains: Example of Synaptotagmins

The differences between C_2-domains in synaptotagmins may give clues about how this domain adapted to diverse functions. In evolution, the C_2-domains of the more than 12 different synaptotagmins developed distinct Ca^{2+} affinities, or in some cases, Ca^{2+} independence (28, 29). Interestingly synaptotagmins IV and XI have a single, evolutionarily conserved amino acid change in the Ca^{2+}-binding residues of the C_2A-motif. These C_2A-domains are unable to bind phospholipids as a function of Ca^{2+} (48). Reversal of this amino acid change restored the ability of synaptotagmins IV and XI to bind phospholipids as a function of Ca^{2+}. Thus all other structural requirements for Ca^{2+}-dependent phospholipid binding were evolutionarily retained in these synaptotagmins, and a single amino acid substitution was selected to abolish Ca^{2+}-dependent phospholipid binding. This finding supports the notion that at least the C_2A-domain in synaptotagmins performs other functions in addition to Ca^{2+}-dependent phospholipid binding.

Conclusion

C_2-domains are remarkable modules present in a wide variety of proteins that can participate in different types of interactions. Two widespread Ca^{2+}-binding motifs defined by structural characteristics are known: EF-hands as the most widely distributed motifs with little structural autonomy; and C_2-domains representing autonomous modules present in probably more than 100 proteins. Although more EF-hands than C_2-domains have been described, the growing number of C_2-domains in the data banks suggests that C_2-domains are universal Ca^{2+}-binding domains. The Ca^{2+}-binding sites formed by C_2-motifs and EF-hands have different architectures and function by distinct mechanisms. The EF-hand is formed by a contiguous helix-turn-helix sequence that binds a single Ca^{2+} ion and usually is a substructure in an α-helical protein domain (49, 50). Multiple EF-hands may be present in a protein. Ca^{2+} binding to EF-hands in contiguous domains often occurs in a concerted manner, causing conformational changes that expose hydrophobic surfaces. In contrast, C_2-domains are autonomously folding modules with a stable β-sheet scaffold. Multiple Ca^{2+} ions bind in a cluster at the tip of the domain in a region formed by loops that are distant in the sequence. The Ca^{2+} binding properties of C_2-domains confer onto them the ability to act as electrostatic switches without requiring large conformational changes. The Ca^{2+} binding mode of C_2-domains may be particularly useful for fast Ca^{2+}-triggered reactions, such as neurotransmitter release. We expect that the number of C_2-domains and the variety of their interactions will continue to grow, with new developments and surprises for years to come.

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