Seeing two domains

Conventional protein kinase Cs have two conserved regulatory domains, C1 and C2, shared by many other membrane-interacting proteins. The structures of a C1 and a C2 domain provide insights into how they function.

Protein kinase Cs are a club of kinases that transduce the plethora of signals promoting lipid hydrolysis [1]. Membership in this continually growing — and, by recent appearances, not very exclusive — club requires the presence of a lipid-regulated region and a kinase domain. For most members of the club, binding of the lipid second messenger, diacylglycerol, to the amino-terminal half of the protein allows catalysis to proceed by removing an autoinhibitory pseudosubstrate domain from the active site. How ligand interactions in the regulatory half of the molecule effect this change in the catalytic domain has come one step closer to being answered with two recent reports solving the structure of each of the two main pieces of the regulatory region [2,3].

The cloning of the first known protein kinase Cs in the mid-1980s revealed that they have four highly conserved regions, C1–C4 [4]. The C1 and C2 domains are in the amino-terminal regulatory half of the protein, and are separated by a proteolytically labile variable region from the carboxy-terminal catalytic half of the molecule that contains the C3 and C4 domains (Fig. 1). Each domain in the regulatory half appears to be a functional module, and many other membrane-interacting proteins have one or the other. The C1 domain is found in diacylglycerol kinase, the c-Raf protein kinase, n-chimaerin, Unc-13 and Vav, among others [5], and the C2 domain is present in cytosolic phospholipase A2, phospholipase Cγ and the GTPase-activating protein, GAP [6], as well as in a transmembrane protein, synaptotagmin [7].

The function of each domain has been established by biochemical and molecular biological studies: the C1 domain binds diacylglycerol or phorbol esters (potent functional analogues of diacylglycerol); the C2 domain binds Ca^{2+} and acidic phospholipids; and the C3 and C4 domains comprise the kinase module [1]. Biochemical data are consistent with the following model for the regulation of the native enzyme. Protein kinase C is normally in the cytosol, with a low affinity for membranes. Generation of diacylglycerol causes a dramatic increase in the enzyme's affinity for membranes, resulting in its 'translocation', typically to the plasma membrane (phorbol esters are a remarkable two orders of magnitude more potent than diacylglycerol in causing the kinase to associate with membranes). *In vitro*, this large increase in affinity requires both the lipid second messenger and phosphatidylserine [8]. *In vivo*, additional interactions with proteins that bind protein kinase C may target the enzyme to specific intracellular membrane locations [9].

The high-affinity binding to diacylglycerol and phosphatidylserine causes a conformational change that removes the pseudosubstrate domain (dark blue in Fig. 1) from the active site, allowing substrate binding and catalysis. In addition to lipid, conventional protein kinase Cs (the α, βI, βII and γ isotypes) are also regulated by Ca^{2+}; the cation increases the enzyme's affinity for acidic lipids. Elucidation of the first protein kinase structure (that of protein kinase A [10]) proved invaluable in modeling how protein kinase C's basic pseudosubstrate is maintained in the active site [11]. But insight into how the binding of lipid and Ca^{2+} to the regulatory domain of the kinase breaks these contacts has awaited information on the structure of its regulatory domains.

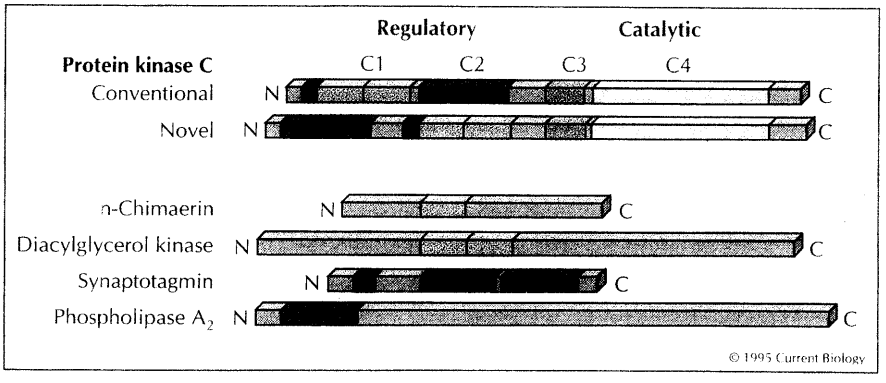

Fig. 1. The primary structures of conventional (Ca^{2+}-dependent) and novel (Ca^{2+}-independent) protein kinase Cs, showing the four domains, C1–C4, that are conserved amongst isozymes. The C1 domain (pink) comprises two cysteine-rich motifs that bind phorbol esters in both conventional and novel protein kinase Cs. This domain is present in a number of other proteins, including n-chimaerin and diacylglycerol kinase. The pseudosubstrate domain (dark blue) precedes the C1 domain in protein kinase C. The C2 domain (turquoise) has been implicated in Ca^{2+}-dependent binding to acidic lipids and is also present in synaptotagmin and phospholipase A2, among others (the purple box in synaptotagmin represents its transmembrane domain). The elucidated structure of a C2 domain [3] suggests why Ca^{2+}-independent isozymes of protein kinase C have a similar domain (stippled turquoise).

The C1 domain comprises a cysteine-rich motif (pink in Fig. 1) that is typically duplicated but present only once in proteins such as atypical protein kinase Cs and n-chimaerin; each motif binds two zinc ions [12]. The recent elucidation of the solution structure of the second cysteine-rich domain in protein kinase C α provided the first glimpse into the fold of this motif: it is an elongated globular domain comprised primarily of β sheet, with two separate metal-binding sites that form structures distinct from those of known zinc-finger proteins [13].

The crystal structure of the second cysteine-rich domain in protein kinase C δ with bound phorbol ester provided the long-awaited answer to how phorbol esters interact with protein kinase C [2]: they interact to form a greasy surface, not surprisingly. The top half of the domain contains two β sheets that are pulled apart to form a cavity, which is lined with water molecules in the absence of ligand. Insertion of phorbol ester displaces the water molecules, with three oxygens on the phorbol moiety replacing broken hydrogen bonds with water (these actually replace interstrand bonds that do not form because the β strands are 'unzipped'). Extensive biochemical analysis identified residues on the phorbol ester molecule that form key contacts with protein kinase C [14], and it is enlightening now to see how each of these atoms forms a specific hydrogen bond with protein kinase C.

Importantly, phorbol ester binding does not significantly alter the conformation of the domain. Rather, a striking change in the surface of the molecule ensues: with phorbol ester bound, the top one-third of the domain becomes an almost contiguous surface of hydrophobic residues. An intriguing possibility is that hydrophobic interactions with the catalytic (or other) domain in the inactive protein are replaced by hydrophobic interactions with the membrane upon phorbol ester binding. Such a possibility would be consistent with the major conformational change in the hinge region (separating the catalytic from the regulatory domain) that accompanies membrane binding [8]. The middle one-third of the elongated domain has a number of basic residues which are potential candidates for interacting with lipid headgroups. The lower half of the molecule contains the two metal-binding sites: one is near the bottom of the structure and involves residues at the amino and carboxyl termini of the domain, and the other is in the middle of the molecule. The importance of the metal-binding sites in maintaining the fold of the domains is apparent from the spatial proximity of residues far removed from each other in the primary sequence.

What about C1 domain-containing proteins that do not detectably bind phorbol esters? All those that do bind phorbol esters have a conserved proline (Pro 241; Fig. 2a) that is absent in some counterparts that do not bind phorbol esters (for example, protein kinase C ζ). The structure provided by Hurley and coworkers [2] reveals that the restricted ϕ angle at this position may prevent the two β sheets from 'zipping-up', thus keeping open the phorbol ester binding site. However, mutating the

Fig. 2. (a) Amino acids 231–280 in the second cysteine-rich domain of protein kinase C δ. Residues that are conserved in all C1 domains that bind phorbol esters are coloured (cysteines in yellow, histidines in orange, and others in pink). **(b)** Ribbon and surface diagram of the sequence in (a) with bound phorbol-13-acetate (pale yellow), based on the coordinates of Zhang *et al.* [2]. The C12 position of the phorbol ester, which is fatty acylated in bio-active phorbol esters, is indicated. Highlighted in yellow and orange are conserved cysteines and histidines, respectively, of the metal-binding sites. The two zinc ions are represented as green balls.

corresponding residue in protein kinase C ζ to proline, establishing the consensus sequence (highlighted in Fig. 2a), does not convert this isozyme into a phorbol ester-binder [15], suggesting that non-conserved residues are also important for phorbol ester binding.

How the other half of the regulatory region of protein kinase C interacts with its ligands is now also apparent from the elegant work of Sprang and coworkers [3], who solved the crystal structure of the first C2 domain in the transmembrane synaptic vesicle protein, synaptotagmin. Like protein kinase C, most C2 domain-containing proteins bind acidic membranes in a Ca^{2+}-dependent manner. Although synaptotagmin is a transmembrane protein, its C2 domain probably also functions as a Ca^{2+}-regulated, acidic-membrane-targeting domain during the membrane apposition events involved in synaptic vesicle exocytosis.

The 'C2 key' consists of a core of approximately 70 residues that fold into a four-stranded β sheet. Loops at the amino and carboxyl termini form a 'mouth' that is

lined with five aspartate residues (pink in Fig. 3b) that form the Ca^{2+}-binding site. At least three of these aspartates are invariant in the C2 domains of Ca^{2+}-binding proteins. Two features of the mouth are noteworthy. First, the back face of its upper (carboxy-terminal) half has large, hydrophobic residues — particularly striking is the sequence Trp–Asp–Trp–Asp in the protein kinase Cs, in which the aspartates face the inside of the mouth and the tryptophans the back side of it — including an invariant tyrosine (Tyr180 in synaptotagmin; Fig. 3a). Second, immediately behind the mouth is a surface, formed by two β strands, which has a high density of positive charge (orange in Fig. 3). These two observations provide an attractive possible explanation for the allosteric effects of Ca^{2+} on protein kinase C's affinity for acidic lipids: binding of Ca^{2+} might clamp the mouth shut, thus causing the bulky aromatics to stick out and serve as effective membrane anchors. This might then better orient the numerous basic residues on the top face to interact with acidic headgroups. It is interesting that crystal structures of both peripheral and transmembrane proteins have revealed that aromatic residues are often localized at the membrane interface [16] (note that the crystallized C1 domain also has a tryptophan that is likely to be at the membrane interface). Interestingly, equilibration of C2 crystals with 0.1 mM Ca^{2+} did not cause a striking structural change (although apparently exposure to above 0.1 mM $CaCl_2$ caused crystals to crack) [3], which is not surprising given that protein kinase C's affinity for Ca^{2+} appears to be in the millimolar range in the absence of acidic lipids (L.M. Keranen and A.C.N., unpublished observations).

The C2 domain has long been postulated to contain the Ca^{2+}-binding site of protein kinase C because this

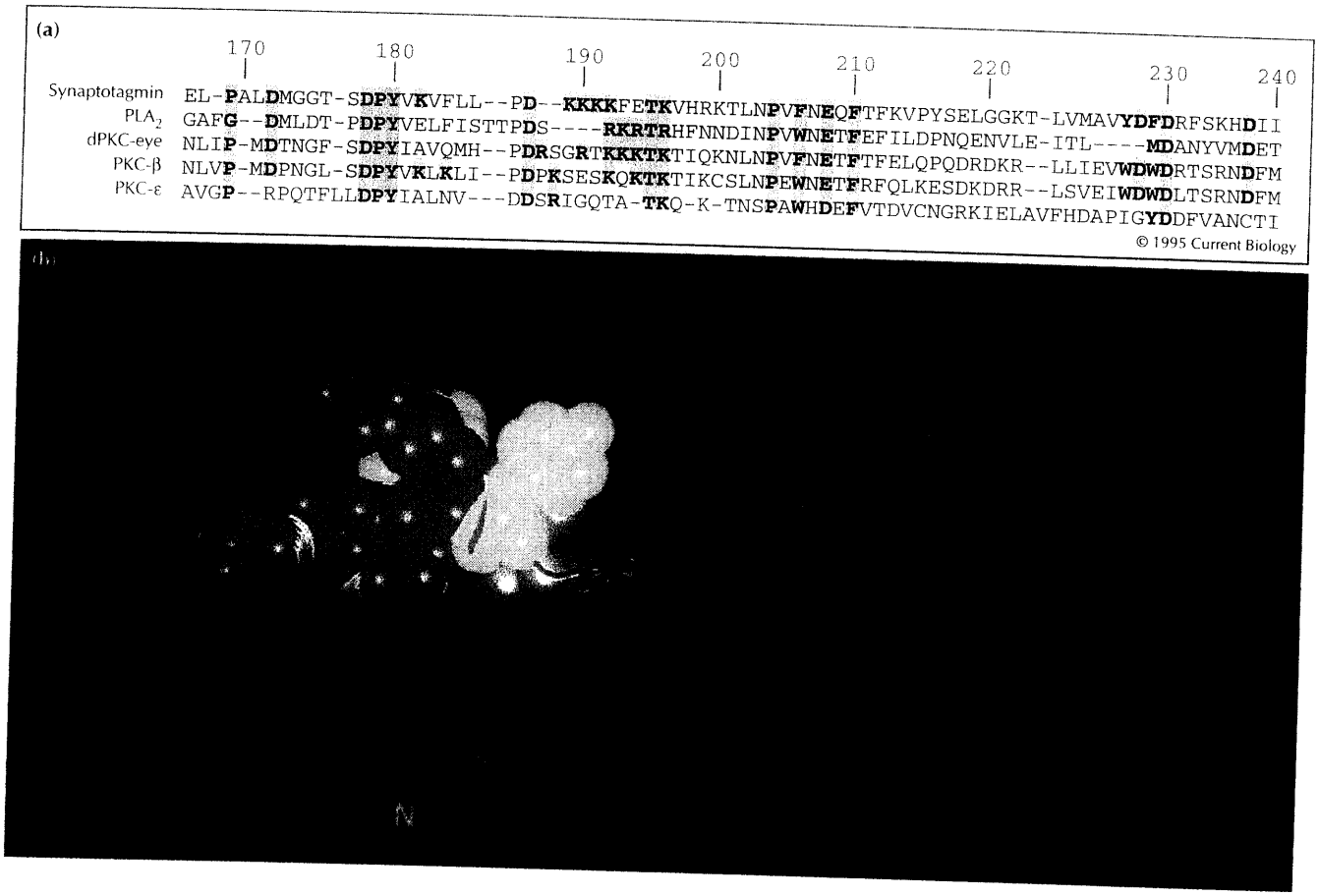

Fig. 3. (a) Amino-acid alignment of the C2 domains of synaptotagmin 1 (residues 167–240; rat [7]); cytosolic phospholipase A2 (residues 33–101; human [6]; two Ca^{2+}-dependent protein kinase Cs, *Drosophila* eye protein kinase C (residues 227–291; [19]) and bovine protein kinase C β (residues 182–256 [4]); and a Ca^{2+}-independent protein kinase C, rabbit protein kinase C ϵ (residues 28–100 [20]). Aspartate residues of the Ca^{2+}-binding site are highlighted in pink; bulky hydrophobic residues on the back-face of the Ca^{2+} site in yellow; the conserved tyrosine of the amino-terminal half of the domain in mauve; basic residues in β strands 3 and 4 and its connecting loop in orange. Other identical or structurally similar residues present amongst C2 domains are highlighted in green. For reference, numbers corresponding to residues in synaptotagmin 1 are indicated. Note that the first conserved aspartate in the C2 domain (172 in synaptotagmin) is an arginine in the non-Ca^{2+}-binding protein kinase C ϵ. **(b)** Ribbon diagram of the 'C2 key' from synaptotagmin. Shown are residues 167–240, encompassing β strands 3–6 from the domain crystallized by Sutton *et. al.* [3] and corresponding to the sequence shown in (a). The five aspartates in the Ca^{2+}-binding site are coloured dark pink; Tyr180 is lighter pink; the two hydrophobic residues on the back side of the Ca^{2+} site (Tyr229 and Phe231) are in yellow; and the basic region on β strands 3 and 4 and connecting loop (residues 182–196) are in orange. The invariant aspartate at position 188 is in the turn between these two strands, and the invariant threonine at position 195 is in the middle of β strand 4 and pointing into the domain (both orange ball-and-sticks). The lower strand is β strand 5 and contains the alternating conserved residues Pro–X–Phe/Trp–X–Glu/Asp–X–Phe (green ball-and-stick) present in almost every C2 domain.

domain had only been identified in the conventional (Ca^{2+}-regulated) protein kinase Cs. However, Sossin and Schwartz pointed out [17] that novel protein kinase Cs contain an amino-terminal domain with striking similarity to the C2 consensus sequence (Figs 1,3a). The beauty of the synaptotagmin structure is that it unveils the mystery behind this apparent discrepancy: it reveals how Ca^{2+}-independent protein kinase Cs have the C2 structure without binding Ca^{2+}. Figure 3a shows that protein kinase C ε has all the conserved residues of the C2 key, except for a major difference in the conserved aspartates. Specifically, only two of the five aspartates are present, and, remarkably, an arginine is present in the position of one of them (equivalent to Asp172 of synaptotagmin). Could it be that this arginine, on the lower jaw, forms an electrostatic interaction with the aspartate on the upper jaw, thus mimicking the effect of Ca^{2+}? This would be consistent with biochemical data showing that the only difference in the acidic lipid regulation of novel and conventional protein kinase Cs is that the latter require Ca^{2+}. Occupancy of this Ca^{2+} site in conventional protein kinase Cs may convert them to a conformation that novel protein kinase Cs already have.

How do the C1 and C2 domains interact? From biochemical data, we know that each domain is capable of binding its ligand(s) in the absence of ligand binding to the other domain. That is, conventional protein kinase Cs will bind acidic membranes in a Ca^{2+}-dependent manner in the absence of diacylglycerol or phorbol esters [18], and will bind phorbol ester-containing membranes in the absence of acidic lipids or Ca^{2+} (M. Mosior and A.C.N., unpublished observations). Furthermore, Ca^{2+} and acidic lipids interact allosterically but Ca^{2+} does not influence the affinity of protein kinase C for diacylglycerol [18]. This suggests that each domain forms a separate membrane anchor, and that the advantage of having both in the same protein lies in allowing protein kinase C to bind membranes with extraordinarily high affinity, in addition to affording more sensitive regulation. Curiously, protein kinase C binds membranes composed of varying acidic lipids with equal affinity in the absence of diacylglycerol, but binds phosphatidylserine-containing membranes ten-fold better than other acidic membranes if diacylglycerol is present [18]. This suggests that one, or both, of these domains bind(s) its lipid ligand better if the serine headgroup is present.

We have gained invaluable insight into the structure of each domain of protein kinase C and how cofactors/substrate interact with each specific domain. The crystal structure of a C1 domain reveals how phorbol esters bind and how this binding could increase protein kinase C's membrane affinity; the crystal structure of a C2 domain has finally elucidated where Ca^{2+} binds and suggests how this binding might increase the domain's affinity for acidic lipids; and a modeled structure of the kinase domain indicates how the basic pseudosubstrate is maintained in the substrate-binding cavity. How these three domains are assembled, and how each regulates the function of the other, awaits determination of the structure of the native protein kinase C and further biochemistry. In the meantime, we have been treated to a view of how phorbol esters, Ca^{2+}, acidic lipids and substrate bind, or are likely to bind, to their respective domains.

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