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Minireview

Modular PH and C2 domains in membrane attachment and other functions

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Abstract The pleckstrin homology and C2 domains are modular protein structures involved in mediating intermolecular interactions. Although they represent distinct domains, there are several parallels regarding their function and type of interactions in which they participate. Both domains are stable structural entities that incorporate variable regions which, in different proteins, can be adapted to perform a specific function through binding to membrane phospholipids or specific protein ligands. A number of recent examples illustrate the function of some of these domains in regulated membrane attachment, with an important role in many cellular signalling pathways.

1. Introduction

The concept that modular domains act as mediators of distinct intermolecular interactions has been central to our understanding of a variety of cellular processes and, in particular, cellular signalling. Two such domains, the pleckstrin homology (PH) and the C2 domain, were originally identified in pleckstrin and protein kinase C (PKC), respectively. Subsequently, they have been identified in a large number of other proteins [1,2]. During the past 5 years, both domains have been the focus of extensive studies and frequent reviews (e.g. [3–7]). Here, we describe how recent structural studies on these domains together with the identification of a range of lipid and protein ligands by in vitro and cellular studies have provided new insights into the possible functions of these domains. Many examples have been provided from studies of phospholipases (phospholipases C (PLCs) and cytoplasmic phospholipase A2 ($cPLA_2$)) that incorporate both of these domains.

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Abbreviations: PH, pleckstrin homology domain; PTB, phosphotyrosine binding domain; DH, Dbl homology domain; CBR, calcium binding region; GFP, green fluorescent protein; PAF, platelet activating factor; $Ins(1,4,5)P_3$, inositol (1,4,5)-trisphosphate; $Ins(1,3,4,5)P_4$, inositol (1,3,4,5)-tetrakisphosphate; $PtdIns(4,5)P_2$, phosphatidylinositol (4,5)-bisphosphate; $PtdIns(3,4,5)P_3$, phosphatidylinositol (3,4,5)-trisphosphate; PKC, protein kinase C; PLC, phospholipase C; GAP, GTPase activating protein; β-ARK, β-adrenergic receptor kinase; Btk, Bruton's tyrosine kinase; PKB/Akt, protein kinase B, product of the Akt gene; PI-3 kinase, phosphoinositide 3 kinase; ARNO, ARF nucleotide binding site opener; Sos, son of sevenless; SynI C2A/B, synaptotagmin I first (C2A) and second (C2B) C2 domains; $cPLA_2$, cytoplasmic phospholipase A2; PDK1/2, 3-phosphoinositide-dependent protein kinase 1 and 2

2. Structure and structural basis for ligand binding in PH and C2 domains

A number of structures of PH domains (recently reviewed in [3]) and several structures of C2 domains [8–11] have been solved. In both cases, despite a low sequence similarity, the structure of each domain represents a well-defined and conserved structural entity (Fig. 1). The structure of all PH domains is an electrostatically polarized antiparallel β-sheet sandwich, one containing four β-stands and the other three, closed at one end with a C-terminal α-helix (Fig. 1, left panel). Variable loops connecting the β-strands (shown in magenta) participate in the formation of a ligand binding surface. The general structure of the C2 domains is an eight-stranded antiparallel β-sandwich, made up of two four-stranded β-sheets, connected by variable loops (Fig. 1, right panel). Loops at one end of the domain (shown in magenta) vary significantly in sequence and length and, like the loop region of the PH domain, contain residues that are involved in ligand binding. Variability of these loop regions allows these stable structures to incorporate specific features and interact with different ligands or to coordinate the same ligand in several different ways. Interactions with the best-characterized ligands, the headgroup of inositol lipids for PH domains and calcium ions for C2 domains, are shown in Fig. 1. Inositol-lipid binding by the PH domains and calcium-dependent phospholipid binding by the C2 domains can both mediate interactions with cellular membranes. However, other ligands and functions for ligand binding have been suggested for these modular structures. These are generally less well understood.

Structural requirements of PH domain interactions with an inositol lipid headgroup have been studied using inositol phosphate as a mimic of the cytoplasm-exposed moiety of the membrane bound phosphoinositides. For example, inositol (1,4,5)-trisphosphate ($Ins(1,4,5)P_3$) has been used to represent the headgroup of phosphatidylinositol (4,5)-bisphosphate ($PtdIns(4,5)P_2$). On the basis of NMR data in the presence and absence of the ligand [12-16] and crystal structures in a complex with $Ins(1,4,5)P_3$ [17,18], the ligand binding site has been determined for a number of the PH domains. In PLCδ1, $Ins(1,4,5)P_3$ is bound between loops β-1/2 and β-3/4 (Fig. 1). A similar position is likely to be occupied by this ligand in several other PH domains (e.g. dynamin, SOS1, pleckstrin and β-adrenergic receptor kinase (β-ARK)) although basic residues in each of these PH domains involved in $Ins(1,4,5)P_3$ binding are not strictly conserved. However, a different part of the β-spectrin PH domain, the region between loops β-1/2 and β-5/6, coordinates binding of $Ins(1,4,5)P_3$. This is unlikely to be artefactual since the same site has been determined by

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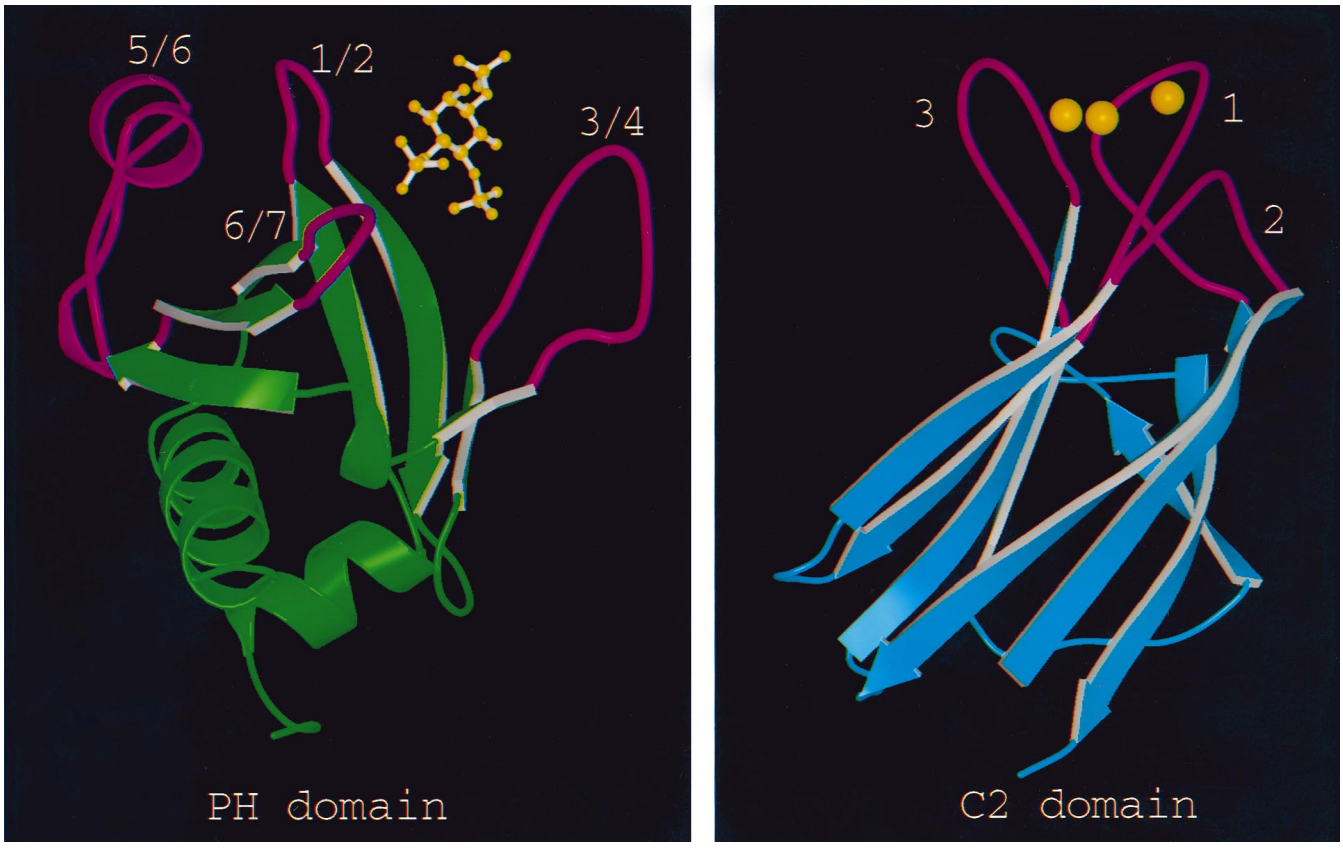

Fig. 1. Structure of the PH (left) and C2 (right) domains from PLCδ1. Structures are ribbon diagrams with inositol trisphosphate (ball and stick) bound to the PH domain and calcium (spheres) to the C2 domain.

crystallographic and NMR studies. The binding site for inositol (1,3,4,5)-tetrakisphosphate (Ins(1,3,4,5)P_4), approximating the headgroup of the phosphoinositide 3 kinase (PI-3 kinase) product phosphatidylinositol (3,4,5)-trisphosphate (PtdIns(3,4,5)P_3), has been structurally defined in the case of Bruton's tyrosine kinase [19], preferentially binding this inositol phosphate and the corresponding inositol lipid. Together with mutational analysis of PtdIns(3,4,5)P_3 binding to the Btk PH domain [20] this has demonstrated that the binding site is analogous to that of PLC δ1. A screen for the PH domains that could specifically bind to lipids produced by PI-3 kinase in *Saccharomyces cerevisiae* [21] identified several PH domains already implicated in these interactions by in vitro studies (e.g. protein kinase B, product of the Akt gene (PKB/Akt) [22,23], Btk [20]) as well as new PH domains. Comparison of the sequences of these proteins suggests that residues which determine specific high affinity binding for the ligand could be conserved and therefore form a structurally similar binding pocket. However, conformation of this hypothesis by structural studies is required.

In addition to binding of inositol lipids or phosphates, the PH domains and the structurally related phosphotyrosine binding (PTB) domains are also involved in protein/protein interactions. Structural studies of the interaction of PTB domains with their protein ligands, exemplified by the interaction of the adaptor protein Shc with a NGF receptor peptide, revealed the involvement of the loop region in phosphotyrosine binding (reviewed in [4]). Recently, a binding domain for small GTPase Ran (RanBD1) has been shown to be closely related to PTB/PH domains, with the loop region involved in interactions with the 'switch region' of Ran [24]. Other protein/protein interactions described for PH domains include binding of Gβγ subunits by β-ARK [25,26] and Gα12 by Btk and Ras GTPase activating protein (GAP) ($GAP1^m$) [27]. However, these interactions are not entirely mediated by the PH domains and are likely to involve adjacent regions.

Structural insights into calcium binding properties of several C2 domains have also been obtained. Comparison of C2 domain/calcium complexes revealed binding of multiple calcium ions in C2 domains from synaptotagmin I C2A (synI C2A) [28], $cPLA_2$ [9], PKCβ [10] and PLCδ1 [29] by acidic residues in two or three of the calcium binding loop regions (designated as CBR1, CBR2 and CBR3 in [9]). Together with mutagenesis and direct binding studies, positions of up to four potential binding sites have been suggested (designated I–IV in [10]). However, not all positions seem to be occupied by calcium in different C2 domains and the residues that could coordinate calcium at a particular position are not present in all structures determined. Calcium binding could lead to the binding of a third ligand (reviewed in [7]), which in case of synI C2A, PKC and $cPLA_2$ has been demonstrated to be a phospholipid. The ternary complex formation may not result from calcium-triggered conformational changes. It is more likely to be formed through stabilization of an 'open' conformation and/or electrostatic changes due to calcium binding to acidic residues within the C2 domain. Clearly, further study is required to investigate the identity of residues and type of interactions that can determine phospholipid binding. Some

information has been obtained from studies of synI C2A [30] and $cPLA_2$ [31,32], demonstrating that CBRs can penetrate the lipid bilayer. In the case of $cPLA_2$, the residues critical for lipid binding have been identified as exposed hydrophobic residues, distinct from residues that coordinate calcium ions.

C2 domains are also involved in calcium-dependent and calcium-independent protein/protein interactions (reviewed in [6,7]). In the case of synaptotagmins, a number of protein/protein interactions have been described (see below). Recent studies of some of these interactions using mutagenesis [33] suggested that sites of protein/protein interactions are likely to include CBRs and other parts (e.g. concave region formed by β-strands) of the C2 domain.

3. PH and C2 domains as determinants of regulated membrane interactions

Studies of the PH domain binding to a range of inositol lipids and phosphates now available revealed large differences in the affinity and degree of binding specificity for different PH domains [34,35]. However, these studies do not reflect true binding properties (in particular affinities) of these domains in all cases, since the environment provided by natural membranes is not easy to reconstruct in vitro. Nonetheless, studies using methods where interaction with cellular membranes can be monitored demonstrated that some specific/high affinity ligands in vitro are also likely to be cellular ligands. A number of studies on PH domain-dependent cellular localization have been performed (e.g. [36–38]). In addition, a system has been designed to select PH domains that can bind to the plasma membrane (in the presence or absence of activated PI-3 kinase) based on their ability to rescue the growth of temperature sensitive *S. cerevisiae* [21]. Studies of the PLCδ1 PH domain have demonstrated a high affinity and relative specificity for $PtdIns(4,5)P_2$ and $Ins(1,4,5)P_3$ but can also bind $Ins(1,3,4,5)P_4$ and $PtdIns(3,4,5)P_3$. Since $PtdIns(4,5)P_2$ is present in cells in higher concentrations than $PtdIns(3,4,5)P_3$, it has been regarded as a physiologically important ligand and further studies of membrane binding provided clear evidence that this is indeed the case. In the yeast rescue assay [21], the PLCδ1 PH domain supported growth in the presence of only $PtdIns(4,5)P_2$ in the membrane, i.e. it did not require co-expression with PI-3 kinase. Studies where the wild-type and PLCδ1 with mutations in the PH domain were compared for the ability to bind $Ins(1,4,5)P_3$ in vitro and to interact with membranes of MDCK cells after microinjection demonstrated that the PH domain is essential and sufficient for membrane attachment and that the loss of headgroup binding in vitro correlates with the loss of membrane attachment [39]. Furthermore, using a PLCδ1 PH domain-green fluorescent protein (GFP) fusion protein, it was possible to visualize changes in membrane interactions in non-stimulated and stimulated RBL, MDCK and NIH 3T3 cells ([40–42]), respectively. While in non-stimulated cells, the PH domain was associated with the membrane, stimulation (e.g. stimulation of NIH3T3 cells by angiotensin II [42]) leading to $PtdIns(4,5)P_2$ hydrolysis and a significant reduction of $PtdIns(4,5)P_2$ concentrations (directly measured in [42]) resulted in a detachment of the PLCδ1 PH domain from the membrane. The detachment was transient and was restored following $PtdIns(4,5)P_2$ synthesis (Fig. 2A). These experiments not only demonstrated that $PtdIns(4,5)P_2$ is an in vivo ligand

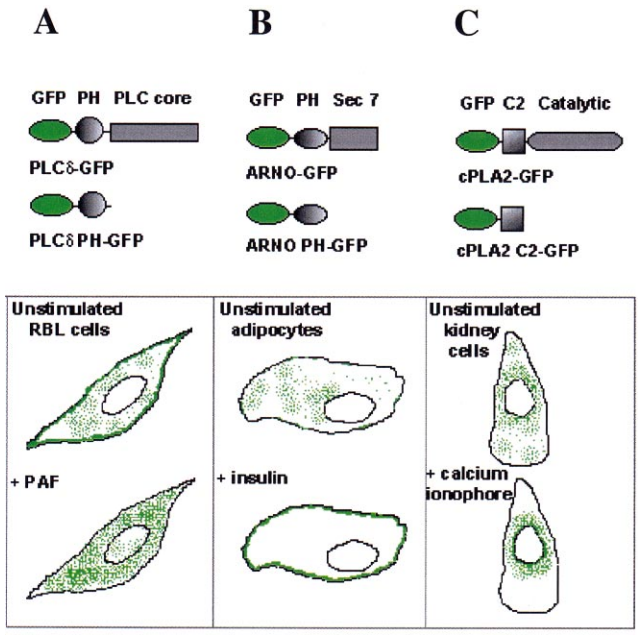

Fig. 2. Examples of regulated membrane interactions mediated by the PH and C2 domains: detachment of PLCδ1 from the plasma membrane following agonist-triggered PIP_2 hydrolysis [40] (A), plasma membrane association of ARNO following stimulation of PI-3 kinase [44] (B) and association of $cPLA_2$ with internal membranes following addition of a calcium ionophore [32] (C).

for the PLCδ1 PH domain but also supported the possibility that changes in the concentration of this ligand can regulate membrane interactions.

Several PH domains can specifically bind products of PI-3 kinase in vitro (e.g. PH domains from Btk [20], PKB/Akt [22,23], ARF nucleotide binding site opener (ARNO) [43]). Some of these PH domains (Btk, PKB/Akt) were analyzed in the yeast rescue assay [21] and found to bind membranes and thus support growth only when PI-3 kinase was introduced into the cells. Studies of cellular localization further confirmed that $PtdIns(3,4,5)P_3$ and $PtdIns(3,4)P_2$ could act as ligands for the PH domains, when synthesized in cells after stimulation. Using GFP fusion constructs of ARNO (a member of the exchange factor family for ARFs), it has been shown that in 3T3 L1 adipocytes, insulin causes a rapid PH domain-dependent translocation from cytoplasm to the plasma membrane [44]. The translocation was prevented by inhibitors of PI-3 kinase (wortmannin and LY294002) and the dominant negative mutant of this enzyme. This suggests that the membrane binding is a consequence of PI-3 kinase activation and the rapid and transient accumulation of its inositol lipid products that can interact with the ARNO PH domain (Fig. 2B). Although correlation of in vitro binding data and cellular studies have been shown for several PH domains, this is not always the case. For example, studies of the Ras exchanger son of sevenless (Sos) demonstrated that the ability of the PH domain to interact with the membrane does not correlate with its ability to bind $PtdIns(4,5)P_2$ [45].

The use of similar experimental approaches supported a role for some C2 domains in calcium-regulated membrane targeting. Well-studied examples include C2 domains from $cPLA_2$ [32], PKCγ [46] and PKCα [47]. Localization studies of $cPLA_2$ or its isolated C2 domain [32] demonstrated that in non-stimulated cells, these proteins are present in the cyto-

plasm. After addition of a calcium ionophore and a consequent increase in the intracellular calcium concentrations, the $cPLA_2$-GFP constructs translocated to internal cellular membranes (endoplasmic reticulum and nuclear membrane) (Fig. 2C). Similar data have been obtained for PKCγ-GFP or isolated PKCγ C2-GFP. In this case, addition of a calcium ionophore or antigen stimulation of RBL cells resulted in translocation to the plasma membrane [46]. Following antigen stimulation, which causes repetitive calcium spikes, it was also possible to demonstrate oscillations of the PKCγ translocation to the membrane which were synchronous with changes in calcium concentrations. These cellular studies therefore confirm previous in vitro observations of calcium-triggered phospholipid binding by C2 domains.

4. Versatile functions of PH and C2 domains

The role of PH and C2 domains in determining the regulated membrane localization, that requires high affinity and specificity of ligand binding, has only been established for some of a vast number of proteins containing these domains. Many PH domains, for example, have low affinities for inositol lipids and it is likely that these weak interactions could contribute to the membrane localization of a protein, mediated by another domain. In the case of pleckstrin, the presence of the PH domain is not sufficient to mediate membrane attachment which also requires phosphorylation on Ser and Thr residues outside this domain [38]. It has recently been suggested that PH domains from PLCβ1 and PLCβ2 can strongly interact with surfaces composed only of neutral lipids and thus mediate non-specific membrane interactions which cannot be regulated by the ligand concentrations [48]. There are also several reports that C2 domains mediate phospholipid binding which is calcium-independent. Examples include the PKC Apl I [49] and PI-3 kinase C2β [50].

It is becoming clear, however, that a view of PH and C2 domains merely as devices for recruitment to the membrane is too simplistic. Evidence obtained from studies of Ser/Thr kinase PKB/Akt suggests that binding to products of activated PI-3 kinase not only mediates membrane interactions but is also likely to trigger critical conformational changes of the protein which allow phosphorylation of Ser and Thr residues by other kinases (PDK1 and PDK2). Phosphorylation of these residues outside the PH domain is critical for activation of PKB/Akt activity (reviewed in [51]). Modulation of enzymatic activity through PH domain-phosphoinositide interactions has also been demonstrated for Vav, a guanine nucleotide exchange factor of Rac [52]. The exchange factor activity is a property of the adjacent DH domain and binding of $PtdIns(4,5)P_2$ to the PH domain inhibits while binding of $PtdIns(3,4,5)P_3$ stimulates the activity of the DH domain. In addition to this direct allosteric effect, binding of $PtdIns(3,4,5)P_3$ also increases the phosphorylation of Vav by tyrosine kinase Lck and in this way contributes towards Vav activation. The structure of the DH/PH region from other exchange factors, Sos [53] and βPIX [54], demonstrated that although the two domains could communicate, the surface area of PH/DH interaction is poorly conserved among different DH/PH proteins. Therefore, the relative disposition of the two domains may not allow a change, caused by the PH domain ligand binding, to affect the function of the DH domain in all DH/PH containing proteins.

Recent studies have also indicated that binding of inositol phosphates to some PH domains may not just reflect their ability to interact with the inositol lipid headgroups but could itself have physiological relevance. The binding affinity of $Ins(1,4,5)P_3$ to the PLCδ1 PH domain (210 nM) is higher than for $PtdIns(4,5)P_2$ (1.7 μM) and competition between the ligands could contribute to membrane detachment when $Ins(1,4,5)P_3$ is produced in cells following stimulation [55]. The PH domain of $GAP1^{IP4BP}$, purified as IP_4 binding protein with Ras-GAP activity, has about a 10-fold higher affinity for $Ins(1,3,4,5)P_4$ than for $PtdIns(3,4,5)P_3$ derivatives and shows a much lower binding of other inositol phosphates and inositol lipids (reviewed in [56]). It is possible that inositol-polyphosphate binding to this, and probably some other PH domains, could have important physiological consequences. Function(s) of $Ins(1,3,4,5)P_4$ binding to $GAP1^{IP4BP}$, however, remain(s) unclear. It could be required for enhancement of the GAP activity towards Ras or it functions could be related to IP_4-regulated calcium entry [56].

A number of protein/protein interactions have been reported to be mediated by the PH and C2 domains. Although some of the protein binding partners are membrane components and the interaction can be regarded as indirect membrane binding, functional consequences of these protein/protein interaction are specific and governed by the properties of interacting proteins. Examples of PH/PTB domains as interaction sites with specific proteins have been discussed above. A broad spectrum of protein ligands has been described for the C2 domains where interactions could be constitutive or regulated in a calcium-dependent fashion (reviewed in [6]). Many of these interactions have been described for synaptotagmin isoforms and are believed to be important for their function in regulated vesicle transport (synaptic transmission, exo- and endocytosis), by mediating formation of protein complexes. For example, synaptotagmins can interact with syntaxin, a plasma membrane protein critical in the exocytosis of synaptic vesicles, in a calcium-dependent manner [57,58]. However, the interaction of the C2B domain in most synaptotagmin isoforms with clathrin adaptor protein-2, involved in coated pit assembly, is calcium-independent [57,59]. Protein interactions mediated by C2 domains are not restricted to synaptotagmins. Other examples include rabphilin-3A binding to the cytoskeletal protein β-adducin in the presence of calcium and phospholipid [60] and interaction of C2 domains from PKC with receptors for activated C kinase [61].

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