

REVIEW ARTICLE

Signal-dependent membrane targeting by pleckstrin homology (PH) domains

Mark A. LEMMON¹ and Kathryn M. FERGUSON

The Johnson Research Foundation and Department of Biochemistry and Biophysics, University of Pennsylvania School of Medicine, 809C Stellar-Chance Laboratories, 422 Curie Blvd., Philadelphia, PA 19104-6059 U.S.A.

Pleckstrin homology (PH) domains are small protein modules of around 120 amino acids found in many proteins involved in cell signalling, cytoskeletal rearrangement and other processes. Although several different protein ligands have been proposed for PH domains, their only clearly demonstrated physiological function to date is to bind membrane phosphoinositides. The PH domain from phospholipase C- δ_1 binds specifically to PtdIns-(4,5) P_2 and its headgroup, and has become a valuable tool for studying cellular PtdIns(4,5) P_2 functions. More recent developments have demonstrated that a subset of PH domains recognizes the products of agonist-stimulated phosphoinositide 3-kinases. Fusion of these PH domains to green fluorescent protein has allowed dramatic demonstrations of their independent ability to drive signal-dependent recruitment of their host proteins to the plasma membrane. We discuss the structural basis for this 3-

phosphoinositide recognition and the role that it plays in cellular signalling. PH domains that bind specifically to phosphoinositides comprise only a minority (perhaps 15%) of those known, raising questions as to the physiological role of the remaining 85% of PH domains. Most (if not all) PH domains bind weakly and non-specifically to phosphoinositides. Studies of dynamin-1 have indicated that oligomerization of its PH domain may be important in driving membrane association. We discuss the possibility that membrane targeting by PH domains with low affinity for phosphoinositides could be driven by alteration of their oligomeric state and thus the avidity of their membrane binding.

Key words: lipid, phosphoinositide, phospholipase, PI 3-kinase, recruitment

INTRODUCTION

The pleckstrin homology (PH) domain was first identified in 1993 as a 100–120-residue stretch of amino-acid-sequence similarity that occurs twice in pleckstrin and is found in numerous proteins involved in cellular signalling [1,2]. It was originally proposed that PH domains, like Src homology domains 2 and 3 (SH2 and SH3), might be involved in protein–protein interactions in cellular signalling [1–4]. Subsequent work has shown that many PH domains direct membrane targeting of their host proteins, but by binding to phosphoinositides rather than proteins in cellular membranes. The development of this view has been the subject of a number of review articles [3–12], and we will focus here on the more recent advances.

The number of PH domains detected in protein sequences now greatly exceeds 100 (<http://smart.EMBL-Heidelberg.de>). However, for only a few of these has the function been convincingly demonstrated. In these cases, the PH domain binds with high affinity and specificity to a phosphoinositide. In some cases PtdIns(4,5) P_2 is the ligand [9–11]. In other cases, which have attracted the most recent attention [5,13], the ligands are the products of agonist-stimulated phosphoinositide 3-kinases (PI 3-kinases) [14,15]. These products, PtdIns(3,4,5) P_3 and PtdIns(3,4) P_2 , are barely detectable in resting mammalian cells, but are produced by one or other class of PI 3-kinase in response to activation of almost all known cell-surface receptors [14–16]. By

binding to these PI 3-kinase products, which are *bona fide* lipid second messengers, a small group of PH domains directly drive agonist-stimulated relocalization of their host protein to the membrane surface. The realization that certain PH domains will do this has enhanced significantly our understanding of PI 3-kinase signalling. Furthermore, the coincidence of this appreciation with development of green fluorescent protein (GFP) as a cellular experimental tool has led to the utilization of PH domains as probes for revealing the spatial and temporal aspects of phosphoinositide metabolism.

It is important to appreciate that only about 15 of the unique PH domains (perhaps 10%) fall into the category of high-affinity phosphoinositide-binding PH domains. Of the remaining majority, nearly all of those studied have been reported to bind phosphoinositides or inositol phosphates, but to do so very weakly and with unimpressive specificity [17–19]. One of the challenges for understanding the function of these PH domains is to determine whether this weak phosphoinositide binding is physiologically relevant and, if so, how. We discuss one possible mechanism through which regulated recruitment of proteins to membranes can be achieved through weak and non-specific lipid-binding modules. This mechanism involves avidity effects afforded by protein (and therefore PH domain) oligomerization. Another possibility is that some PH domains may have protein ligands, or may recognize both phosphoinositides and proteins. Although no clearly physiologically relevant protein target has

Abbreviations used: ARF, ADP-ribosylation factor; β -ARK, β -adrenergic receptor kinase; BCR, B-cell antigen receptor; Btk, Bruton's tyrosine kinase; DGK- δ , diacylglycerol kinase- δ ; Dyn1-PH, dynamin-1 PH domain; EVH1, Ena/VASP homology 1; Fc γ R1b, Fc γ receptor 1b; GAP, GTPase-activating protein; GEF, guanine-nucleotide exchange factor; GFP, green fluorescent protein; GST, glutathione S-transferase; IRS-1, insulin receptor substrate-1; PDK1, phosphoinositide-dependent kinase-1; PH, pleckstrin homology; PI 3-kinase, phosphoinositide 3-kinase; PKB, protein kinase B; PKC, protein kinase C; PLC δ_1 , phospholipase C- δ_1 ; PLC γ_1 , phospholipase C- γ_1 ; PleckN-PH, pleckstrin-1 N-terminal PH domain; PTB, phosphotyrosine binding; RACK1, receptor for activated C-kinase 1; RanBD, Ran-binding domain; RanBP2, Ran-binding protein-2; SHIP, SH2-domain-containing inositol 5'-phosphatase; SH2, Src homology domain-2; SH3, Src homology domain-3; WH1, WASP homology 1; PDB, Protein Data Bank; Sos, Son-of-sevenless; Grp1, general receptor for phosphoinositides-1.

¹ To whom correspondence should be addressed (e-mail mlemmon@mail.med.upenn.edu).

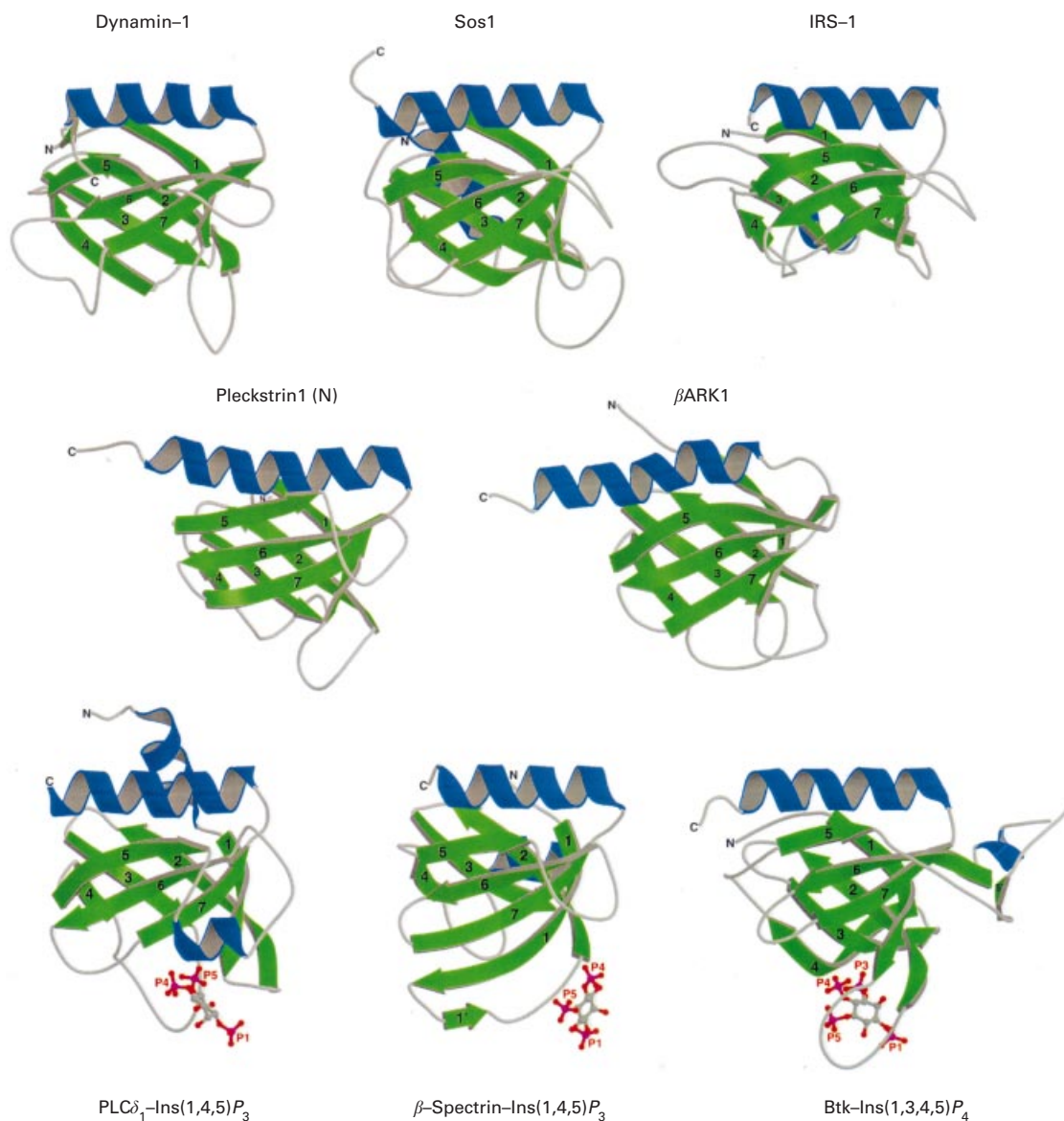


Figure 1 Comparison of the overall fold of all PH domains with known structure

Elements of secondary structure are coloured: blue for α -helices, green for β -strands. The seven β -strands of the PH domain core are labelled 1–7 inclusive, and both N- and C-termini are marked. The orientation of each PH domain is the same, with the C-terminal α -helix at the top of the view, going from right (N-terminal) to left (C-terminal). The structures of the PH domains from pleckstrin1 (N-terminal PH domain) [21] and β ARK1 [27] are NMR-derived structures, while others were determined by X-ray crystallography. The position of the bound Ins(1,4,5) P_3 (phosphate groups red; inositol moiety grey) is shown for the PLC δ_1 and spectrin PH domains, structures of which were determined in complex with this ligand [31,32]. For the Btk PH domain, the position of the bound Ins(1,3,4,5) P_4 in the complex is shown [36]. Co-ordinates for the structures shown were obtained from the Protein Data Bank (PDB), with accession numbers: 1DYN for the dynamin-1 PH domain [29]; 1DBH for the Sos1 PH domain [34]; 1QQG for the IRS-1 PH domain [35]; 1PLS for the N-terminal PH domain from pleckstrin [21]; 1BAK for the β ARK1 PH domain [27]; 1MAI for the PLC δ_1 PH domain in complex with Ins(1,4,5) P_3 [32]; 1BTN for the spectrin PH domain in complex with Ins(1,4,5) P_3 [31]; and 1B55 for the Btk PH domain in complex with Ins(1,3,4,5) P_4 [36]. The representations of the structures were generated with MOLSCRIPT [156] and Raster3D [157].

been described for any PH domain, many reports indicate protein interactions, and there are PH domain-like proteins that clearly do bind to other proteins. We will consider recent advances in this area.

What emerges from the large volume of work on PH domains since their naming in 1993 [1,2] is that they are quite diverse, and may fall into several functional classes. The sequence characteristics that led to their initial identification appear to define their overall fold rather than any functional characteristic. It has been argued that the PH-domain fold may represent a particularly

stable structural scaffold that presents ligand-binding loops in a way that can be exploited for multiple functions [20]. With the appearance of this fold in an increasing number of other binding modules, it has been termed the ‘PH superfold’ [5].

PH-DOMAIN STRUCTURE

Basic structure and physical characteristics of PH domains

Determination of the first PH-domain structures preceded understanding of their functions, and NMR [21–28] or crystal

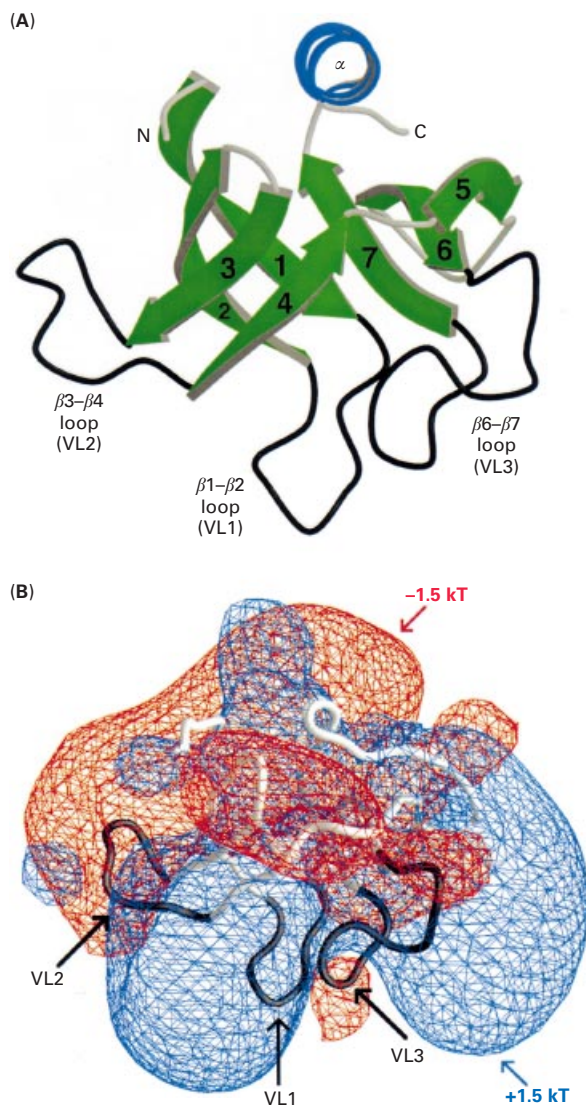


Figure 2 Structural features and electrostatic polarization of the dynamin-1 PH domain

In **(A)** the PH domain from dynamin-1 (1DYN) [29] is shown rotated 90° about a vertical axis compared with the orientation seen in the top left part of Figure 1. The C-terminal α -helix is marked ' α ', and the β -strands are numbered 1–7 inclusive. Strands β 1– β 4 inclusive comprise one β -sheet (left-hand side) and strands β 5 through β 7 (right) comprise the second sheet of the PH domain's β -sandwich. The β 1/ β 2, β 3/ β 4, and β 6/ β 7 connecting loops, found to be the most variable in length and sequence when sequences of multiple PH domains are compared (see the text) are shown in black and are labelled VL1, VL2 and VL3. This Figure was generated with MOLSCRIPT [156] and Raster3D [157]. In **(B)**, the dynamin-1 PH domain is shown in the same orientation as in **(A)**, with the calculated electrostatic potential shown, contoured at -1.5 times kT (red) and $+1.5$ times kT (blue). The backbone of the PH domain is represented by a white 'worm', except in the regions of the variable loops (VL1, VL2 and VL3), which are coloured black. The three variable loops, thought to represent the binding surface of the PH domain, coincide approximately with the positively charged face of the PH domain, which is now known to bind to anionic membrane surfaces (see the text). This Figure was generated with GRASP [158].

[29–36] structures have now been reported for eight different PH domains. As shown in Figure 1, each of these PH domains has essentially the same structure, which is remarkable given that their pairwise sequence identities range from just 7% to a maximum of only around 23% [27]. The core of each PH domain is a β -sandwich of two nearly orthogonal β -sheets (approximately

parallel with the page in Figure 1). One sheet consists of four β -strands (β 1 through β 4), and the other of just three (β 5– β 7 inclusive). Both sheets have the topology of a β -meander, with the strands occurring in the same order along the sheet as they do in the protein sequence. The right-handed twist of the two orthogonally packed β -sheets in the sandwich results in their close contact at only two (close) corners [37], which are to the left and right of each structure in Figure 1. One of these close corners (right) is spanned by strand β 1, which contributes to both sheets of the β -sandwich. The other close corner (left in Figure 1) is completed by a type II β -turn between strands β 4 and β 5. The remaining two corners of the sandwich (top and bottom of the structures in Figure 1) are named 'splayed' corners [37], because the two β -sheets are most distant from one another in these regions. One splayed corner (top in Figure 1 and Figure 2A structures) is capped by the C-terminal amphipathic α -helix found in all PH domains. The second splayed corner (bottom in Figure 1 and Figure 2A structures) is filled in by the side chains from the β 1/ β 2 and β 6/ β 7 connecting loops plus portions of β 4 and/or the β 3/ β 4 connecting loop. These three loops (β 1/ β 2, β 3/ β 4, and β 6/ β 7) were found to be the most variable in length and sequence in early alignments of PH domains [1–4], suggesting, by analogy with immunoglobulin-like domains for example, that they may constitute the ligand-binding site. These loops were termed the 'variable loops', VL1, VL2 and VL3 [29] (Figure 2A). Another early observation was that the PH domains are electrostatically polarized (Figure 2B), with the positively charged face coinciding approximately with the position of the three variable loops [22,29]. These characteristics are precisely those expected for a binding site that interacts with negatively charged membrane surfaces [38]. As this view was being developed, Fesik and co-workers reported that PH domains could bind, albeit weakly, to lipid bilayers containing phosphoinositides [39]. In particular, the N-terminal PH domain from pleckstrin bound to PtdIns-(4,5) P_2 in lipid vesicles or detergent micelles with an apparent K_D of approx. 30 μ M [39]. NMR studies showed that this interaction was mediated by the positively charged face of the PH domain that contains VL1, VL2 and VL3. As we will discuss in more detail below, in all cases for which phosphoinositide binding has been studied (both high-affinity and low-affinity interactions), the polyphosphorylated inositol ring binds to this face of the PH domain [25–27,31,32,36,39].

The PH domain 'superfold'

Several other binding modules have recently been shown to adopt the same core structure as the PH domain, despite an absence of significant sequence similarity [40–46]. These are the phosphotyrosine binding (PTB) domain, the Ran-binding domain (RanBD), and the Ena/VASP homology 1 (EVH1) domain. Examples of these structures are seen in ribbon representation in Figure 3.

PTB domains

PTB domains were the first of these domains to join the PH domain 'superfamily' [40,41]. PTB domains were named for their ability to recognize phosphotyrosine in the context of the amino acid sequence Asn-Pro-Xaa-PTyr [47,48], participating in the recruitment of signal adaptor molecules such as SHC and insulin receptor substrate-1 (IRS-1) to activated cell-surface receptors. However, some PTB domains, notably that from the X11 protein [42], do not require the tyrosine to be phosphorylated for high-affinity recognition. Still others, such as the PTB domain from Numb [43], bind to several different peptide ligands. PTB

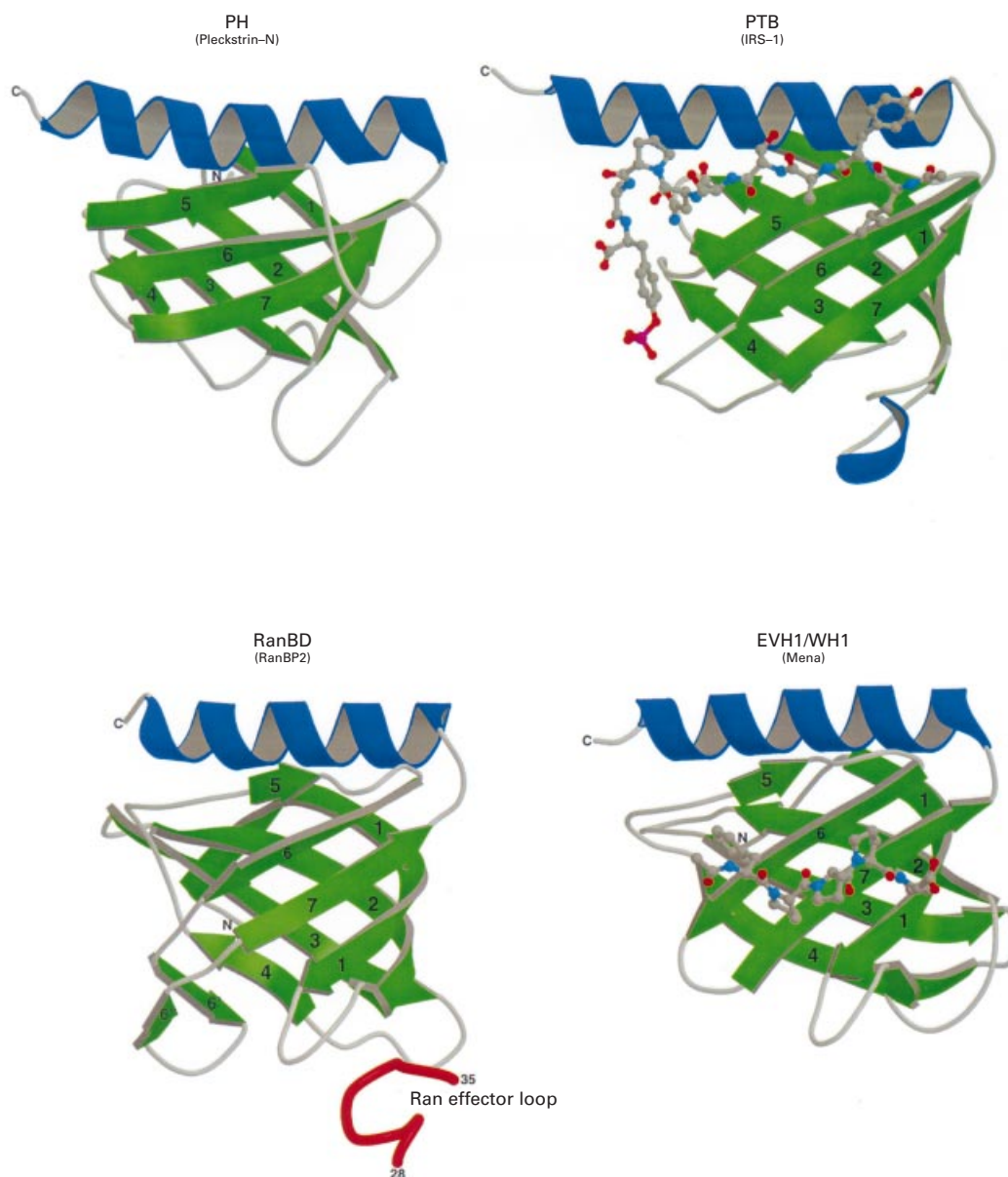


Figure 3 Structural comparison of different modules with the PH domain 'superfold'

The N-terminal PH domain from pleckstrin [21] is compared in a MOLSCRIPT representation [156] with domains that, despite no apparent sequence similarity, have been found to adopt the same fold. Elements of secondary structure are coloured as in Figures 1 and 2(A), and the N- and C-termini are marked where visible. Strands $\beta 1$ – $\beta 7$ inclusive are numbered in each domain (note the additional strands $\beta 6'$ and $\beta 6''$ in RanBD). The PTB (phosphotyrosine-binding) domain from IRS-1 [41] (co-ordinates kindly supplied by Michael Eck and Steven Shoelson) is shown with a bound phosphotyrosine-containing peptide corresponding to its target on the insulin receptor. This peptide lies largely in the cleft between $\beta 5$ and the C-terminal α -helix. The N-terminal 30 amino acids of the RanBD1 from RanBP2 have been removed for clarity. The PDB accession number for RanBD is 1RRP [44]. The position of the Ran effector loop in the complex between RanBD and Ran is shown (in red), corresponding in location approximately to the inositol phosphate-binding site of PH domains. Finally the EVH1/WH1 domain from Mena [45] (PDB accession number 1EVH) is shown with its bound polyproline peptide, which stretches across the $\beta 5$ – $\beta 6$ sheet. This Figure was generated with MOLSCRIPT [156] and Raster3D [157].

domains share the electrostatic polarization seen in PH domains, and in some cases (the PTB domains from SHC and from IRS-1) have been reported to bind weakly and non-specifically to phosphoinositides [40,49]. The peptide ligand of PTB domains binds primarily within a cleft between strand $\beta 5$ and the C-terminal α -helix. It has been argued that the SHC and IRS-1 PTB domains recognize the phosphotyrosine moiety of their peptide ligands in a manner that is analogous to inositol phosphate binding by PH domains [20]. Indeed, phosphoinositides and phosphopeptides have been reported to compete with one another

for binding to the SHC PTB domain [18], lending some support to this idea.

Ran-binding domain

Another surprising occurrence of the PH domain fold was seen when the crystal structure of the first Ran-binding domain (RanBD1) from Ran-binding protein-2 (RanBP2) was determined [44]. RanBD1 does not share significant sequence similarity with PH domains, yet its core structure overlays with the Btk PH

domain structure [33], with a root-mean-square deviation (100 C α atoms) of just 0.14 nm (1.4 Å). In the complex formed between RanBD1 and Ran-guanosine 5'-[β,γ -imido]triphosphate, several of the contacts between the two proteins, especially those with the Ran effector loop (red in Figure 3), involve regions of RanBD1 that correspond in location to the inositol phosphate-binding site of PH domains (involving the variable loops). There have been no reports of inositol phosphate or phosphoinositide binding by Ran-binding proteins. However, the recent finding that inositol phosphates play a role in controlling mRNA export from the nucleus [50] makes this a very intriguing possibility.

EVH1/WH1 domain

A third class of protein module that has been shown by structural studies to adopt the PH-domain fold is the EVH1 (Enabled/WASP homology 1) or WH1 (WASP homology 1) domain [45,46]. It had previously been noted that the EVH1/WH1 domain shares sequence similarity with that of RanBD [51], and there has been some disagreement over whether the N-terminus of N-WASP contains a PH domain [52,53] or a WH1 domain (which overlap, but do not coincide) [54]. The structures show that the EVH1/WH1 domain defines the appropriate boundaries, but that it bears remarkable structural resemblance to PH domains (Figure 3). The EVH1/WH1 domain binds to polyproline-containing sequences. In the crystal structure of an EVH1/WH1 domain in complex with a polyproline peptide, the binding site is seen to stretch across the surface of the β -sheet formed by strands $\beta 5$ – $\beta 7$ inclusive [45]. The region corresponding to the inositol phosphate/phosphoinositide-binding site of PH domains is unoccupied in this structure. Interestingly, the N-terminal portion of N-WASP, which contains this PH-domain-like EVH1/WH1 domain, has been implicated in PtdIns(4,5) P_2 binding by the whole protein [52]. The possibility has been raised that EVH1/WH1 domains may be capable of binding both a protein and a phosphoinositide ligand – in this case simultaneously.

The basic PH-domain β -sandwich fold has now been seen in several guises, in both proteins that clearly bind with high affinity to phosphoinositides and those that bind to protein ligands (Figure 3). It is likely that these occurrences reflect the adaptability of the basic fold to multiple functions by creating a stable structural scaffold that can bear loops that have quite different recognition properties [5]. There is no reason *a priori* to expect that the different domains containing the PH domain fold will share functional similarity, although it has been argued (or seen) in several cases.

PH DOMAINS AS PHOSPHOINOSITIDE-BINDING MODULES

Nearly every PH domain (identified by sequence homology) studied to date binds phosphoinositides or inositol phosphates to some extent [17–19]. To our knowledge, the only exceptions in the literature are the PH domains from the Golgi-associated evelctins [55]. In spite of the apparent conservation of this characteristic among PH domains, the physiological relevance of inositol phosphate/phosphoinositide binding is far from clear in the majority of cases. Indeed, since phosphoinositides are highly negatively charged, vesicles or surfaces that bear them are very good cation-exchangers, and the possibility for 'artifactual' binding is high. Isolated PH domains are known to bind strongly under nominally physiological conditions to the sulphopropyl columns used in their purification (e.g. [29]), yet the functional

groups of these cation-exchangers are not considered likely to be physiological ligands.

These concerns have been negated in some cases by clear demonstration of stereospecific and high-affinity binding of PH domains to phosphoinositides and inositol phosphates that are observed physiologically [17,18]. However, this has still only been achieved for a handful of PH domains. The majority bind the acidic phospholipid ligands only with low affinity and with poor specificity [17]. It remains one of the primary challenges in understanding the role of PH domains to determine whether these weak and promiscuous interactions are important *in vivo*. We will separate our discussion of this into two sections. In the first, PH domains that bind specifically to inositol phosphate/phosphoinositide ligands will be considered. In the second, the possible roles of low-affinity, promiscuous, interactions will be discussed.

High-affinity recognition of specific phosphoinositides by PH domains

The PH domain at the N-terminus of phospholipase C- δ_1 (PLC δ_1) was the first shown to recognize a specific phosphoinositide ligand, and was actually identified as a binding site for Ins-(1,4,5) P_3 and PtdIns(4,5) P_2 before PH domains were discovered [56,57]. The isolated 120-amino-acid PH domain binds strongly and specifically to both PtdIns(4,5) P_2 and its soluble headgroup, Ins(1,4,5) P_3 [58,59], and is sufficient to target its host protein to the surface of the plasma membrane in mammalian cells [60]. The precise role of the PH domain in the regulation of PLC δ_1 *in vivo* is not clear. However, by tethering PLC δ_1 to membranes that contain its substrate [PtdIns(4,5) P_2], the PH domain allows processive hydrolysis by the enzyme of substrate molecules in a membrane, without a requirement for it to dissociate from the membrane surface (and rebind) between catalytic cycles [57]. Excess Ins(1,4,5) P_3 can abolish this processivity *in vitro* by competing with PtdIns(4,5) P_2 for binding to the PH domain [61]. In fact, titration-calorimetry experiments indicate that the PLC δ_1 PH domain binds 8-fold more strongly to Ins(1,4,5) P_3 than to PtdIns(4,5) P_2 [59]. This fact has been made use of in studies (with a fusion of the PLC δ_1 PH domain to GFP) of the patterns of Ins(1,4,5) P_3 production in single cells [62].

Crystallographic studies of intact PLC δ_1 indicate that its N-terminal PH domain (disordered in the crystal structure) is attached to the rest of the molecule via a flexible linker, consistent with the role of the PH domain as a membrane tether for the enzyme [63,64]. Ferguson et al. [32] have determined the X-ray crystal structure of the isolated PLC δ_1 PH domain in complex with Ins(1,4,5) P_3 , to which it binds with a K_D of 210 nM and high stereospecificity. Ins(1,4,5) P_3 binds to the surface of the PH domain that is defined by the variable loops 1–3 inclusive (Figure 1), making direct hydrogen bonds with side chains of amino acids in VL1 and VL2 (Figure 4). The 4- and 5-phosphates participate in a number of hydrogen-bonding interactions that appear to clamp this lipid headgroup into a binding site (Figure 4). In particular, two lysine side chains (from Lys³⁰ and Lys³⁷) are both able to form hydrogen bonds simultaneously with the 4- and 5-phosphates of Ins(1,4,5) P_3 , and a critical arginine side chain (Arg⁴⁰) is hydrogen-bonded to the 5-phosphate group. The 1-phosphate (P1 in Figure 4) participates in only one hydrogen bond (to the indole nitrogen of Trp³⁶) and is substantially solvent-accessible. Attachment of a diacylglycerol moiety to the 1-phosphate on Ins(1,4,5) P_3 while bound to the PLC δ_1 PH domain [thus generating PtdIns(4,5) P_2] would not cause any steric problems, suggesting that this structure also represents a reasonable model for the PtdIns(4,5) P_2 -bound PH domain.

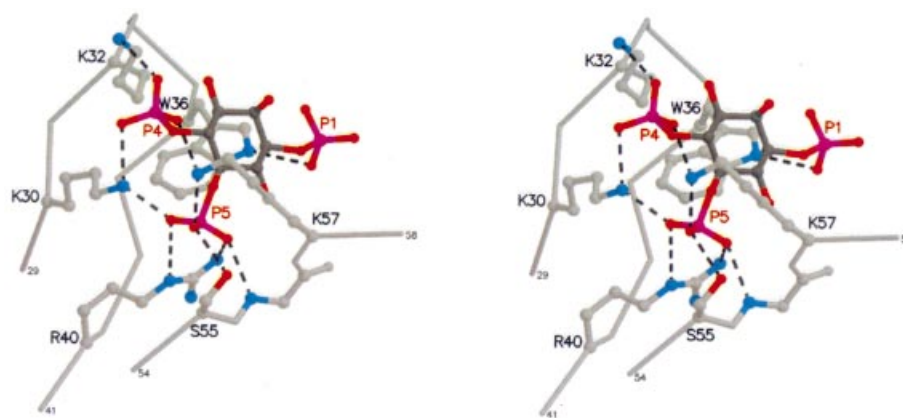


Figure 4 Stereoscopic view of the $\text{Ins}(1,4,5)\text{P}_3$ -binding site of the $\text{PLC}\delta_1$ PH domain

A stereo pair showing the details of the interactions between $\text{Ins}(1,4,5)\text{P}_3$ and the $\text{PLC}\delta_1$ PH domain in the crystal structure of the complex reported by Ferguson et al. [32]. All direct hydrogen bonds between the inositol phosphate ligand and side chains in the PH domain are marked with broken lines. Side chains that do not interact with the bound $\text{Ins}(1,4,5)\text{P}_3$ are excluded from the view. This Figure was generated with MOLSCRIPT [156] and Raster3D [157].

PH domains as targets of PI 3-kinase-derived lipid second messengers

Protein kinase B (PKB)/Akt

Following the realization that some PH domains can bind phosphoinositides, Franke et al. [65] reported the seminal findings that the serine/threonine kinase protein kinase B (PKB, also known as Akt) is a downstream effector of PI 3-kinase signalling, and that mutations in its N-terminal PH domain prevent the response of PKB to PI 3-kinase activation. Recent developments in PKB signalling have been reviewed thoroughly in the *Biochemical Journal* [66,67], and we will not go into details here. The response of PKB to PI 3-kinase results from specific recognition of the 3-phosphoinositides $\text{PtdIns}(3,4,5)\text{P}_3$ and $\text{PtdIns}(3,4)\text{P}_2$ by its PH domain [68–70]. This results in signal-dependent recruitment of PKB to the plasma membrane [71,72]. Although membrane recruitment and phosphoinositide binding have been reported to increase PKB activity directly, its phosphorylation by another kinase, the phosphoinositide-dependent kinase-1 (PDK1), has been shown to be required for full activation *in vivo* [73,74]. PI 3-kinase products activate PKB principally by recruiting the enzyme to membranes at which PDK1 is also found, so that PDK1 may phosphorylate PKB and activate it completely. The presence of PDK1 at the membranes to which PKB is recruited may also be controlled by PI 3-kinase products, since PDK1 has a PH domain at its C-terminus that binds specifically to $\text{PtdIns}(3,4,5)\text{P}_3$ [73–76]. Whether PDK1 is recruited to the plasma membrane in a PI 3-kinase-dependent manner, and how it is regulated by PI 3-kinase products, remain subjects of debate. For a very recent full discussion of PKB activation mechanisms, readers are referred to a splendid *Biochemical Journal* review by Vanhaesebroeck and Alessi [67].

Requirements for PH-domain recruitment to the plasma membrane by 3-phosphoinositide recognition

The requirements for signal-dependent recruitment of a PH domain to the plasma membrane by binding to the products of PI 3-kinases are: (i) that they bind PI 3-kinase products with high affinity; and (ii) that they bind to PI 3-kinase products substantially more tightly than to other phosphoinositides that are

present constitutively in the plasma membrane [e.g. $\text{PtdIns}(4,5)\text{P}_2$ and $\text{PtdIns}4\text{P}$].

On the basis of estimates from Stephens and colleagues [16], the theoretical local concentration of $\text{PtdIns}(3,4,5)\text{P}_3$ at the inner leaflet of the plasma membrane increases 40-fold following stimulation of neutrophils: from a basal level of $5\ \mu\text{M}$ to approx. $200\ \mu\text{M}$. At the same time, $\text{PtdIns}(3,4)\text{P}_2$ concentrations are estimated to increase from $10\text{--}20\ \mu\text{M}$ (basal) to $100\text{--}200\ \mu\text{M}$ (following activation). The local $\text{PtdIns}(4,5)\text{P}_2$ concentration, which is estimated to be approx. $5\ \text{mM}$ prior to stimulation, decreases to about $3.5\ \text{mM}$ following cell activation. Therefore, in order to be capable of driving PI 3-kinase-dependent membrane recruitment, a PH domain must not be attracted to the membrane surface by a local $\text{PtdIns}(4,5)\text{P}_2$ concentration of $5\ \text{mM}$, but must be attracted substantially by a $\text{PtdIns}(3,4,5)\text{P}_3$ concentration of $200\ \mu\text{M}$. For this to be true, the ratio of the affinities of such a PH domain for $\text{PtdIns}(3,4,5)\text{P}_3$ and $\text{PtdIns}(4,5)\text{P}_2$ must be substantially greater than about 25. Furthermore, the K_D for $\text{PtdIns}(4,5)\text{P}_2$ binding by the PH domain must be $10\ \mu\text{M}$ or larger, based on the facts that the $\text{PLC}\delta_1$ PH domain localizes to the plasma membrane of mammalian cells [60,62] and binds $\text{PtdIns}(4,5)\text{P}_2$ with a K_D of $1.7\ \mu\text{M}$ [59], while the N-terminal PH domain from pleckstrin does not localize to the plasma membrane [17] and binds $\text{PtdIns}(4,5)\text{P}_2$ with a K_D of about $30\ \mu\text{M}$ [39]. From these considerations, it can be argued that a PH domain can drive PI 3-kinase-dependent membrane recruitment only if its K_D for $\text{PtdIns}(4,5)\text{P}_2$ binding is larger than $10\ \mu\text{M}$, and its K_D for $\text{PtdIns}(3,4,5)\text{P}_3$ [and/or $\text{PtdIns}(3,4)\text{P}_2$] is substantially smaller than $400\ \text{nM}$. According to quantitative studies of inositol phosphate headgroup binding *in vitro*, these conditions are met for several PH domains, notably those from Bruton's tyrosine kinase (Btk) [36,77,78], $\text{Gap1}^{\text{IP4BP}}$ [79,80], Gap1^{m} [81], general receptor for phosphoinositides-1 (Grp1) [17], DAPP1/PHISH [17] and centaurin- α [82]. Each of these PH domains has also been shown in yeast to be capable of driving PI 3-kinase-dependent membrane recruitment using an *in vivo* assay developed by Skolnik and colleagues [83]. This assay uses cdc25^{ts} yeast, which can only grow at the restrictive temperature if a constitutively active Ras mutant (Ras Q61L) expressed by the yeast is somehow targeted to the plasma membrane. One way of targeting the Ras mutant to the plasma membrane is to fuse it to

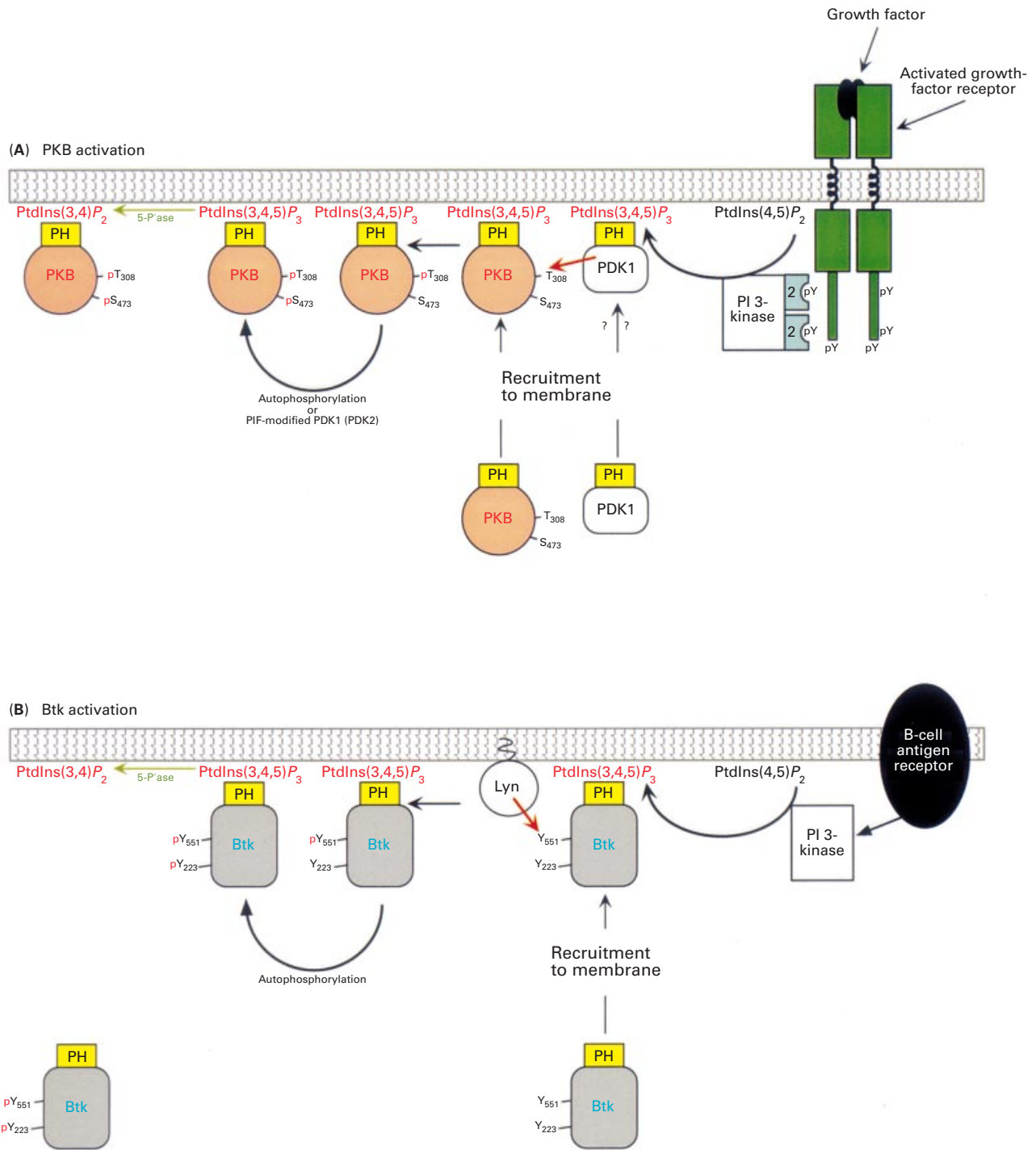


Figure 6 Roles played by PH domains in PI 3-kinase-dependent activation of PKB and Btk

A scheme for activation of PKB by growth-factor-receptor-induced PI 3-kinase activation is shown in (A) (pale blue (cyan) boxes labelled '2' are SH2 domains), and for Btk activation by BCR-induced PI 3-kinase stimulation is shown in (B). Details are provided in the text. Note that there is still disagreement over whether or not PDK1 is recruited to the membrane by PH domain binding to PI 3-kinase products [75,76] and as to whether phosphorylation of S⁴⁷³ in PKB involves phosphorylation by a PDK1-interacting fragment ('PIF')-modified PKB [67] or autophosphorylation [102]. In (B), the Src-family kinase Lyn is constitutively membrane-associated.

acid sequences of these PH domains led to the identification of a consensus sequence motif stretching from strand $\beta 1$ to strand $\beta 2$ that serves as a good predictor of high-affinity binding to PI 3-kinase products (see the legend to Figure 5).

The list of PH domains capable of PI 3-kinase-dependent rescue of *cdc25^{ts}* yeast in the Isakoff assay includes all of those PH domains (listed above) for which binding to soluble inositol phosphates has suggested PI 3-kinase-dependent membrane

recruitment. It also includes several that had not previously been shown to bind PI 3-kinase products. Alongside the sequence listings in Figure 5 are values for the selectivities that have been measured for binding to $\text{PtdIns}(3,4,5)P_3$ [or its soluble headgroup $\text{Ins}(1,3,4,5)P_4$] over $\text{PtdIns}(4,5)P_2$ [or its soluble headgroup $\text{Ins}(1,4,5)P_3$]. In all cases for which measurements have been reported (except Gab1), either the headgroup binding selectivities, the lipid binding selectivities, or both, satisfy the requirements for PI 3-kinase-dependent membrane recruitment based on the estimates of Stephens and colleagues. The only exceptions are the headgroup binding of the PKB PH domain [69], which has been difficult to measure as production of this protein in large quantities has proved difficult, and the lipid-binding studies using the PH domains from centaurin- α [84] and Gab1 [85]. In studies employing the phospholipids themselves, the observed $\text{PtdIns}(3,4,5)P_3/\text{PtdIns}(4,5)P_2$ selectivity may have been underestimated as a result of non-specific binding of both phospholipids to the PH domains under the conditions used. Probably for similar reasons, Rameh et al. [18] reported a $\text{PtdIns}(3,4,5)P_3/\text{PtdIns}(4,5)P_2$ selectivity of just 11-fold for the Btk PH domain, compared with the value of more than 270-fold (over 3000-fold for headgroup binding) reported by Kojima et al. [78]. These comparisons highlight the problems with obtaining reliable values for relative affinities of PH domains for different phosphoinositides. Agreement between studies appears to be most consistent when soluble inositol phosphate binding is considered. As a whole, however, there is general agreement that PH domains involved in PI 3-kinase-dependent recruitment to the membrane surface bind phosphoinositides with K_D values in the 10–250 nM range, and select for $\text{PtdIns}(3,4,5)P_3$ [and/or $\text{PtdIns}(3,4)P_2$] over $\text{PtdIns}(4,5)P_2$ by a factor of 20-fold or more. By indirect immunofluorescence studies or analysis of GFP fusions, all of these PH domains have been shown to be translocated from the cytoplasm to the plasma membrane of mammalian cells upon treatment with growth factors that activate PI 3-kinase [17,72,75,79,82,83,86–93].

Signalling by PH domain recruitment to PI 3-kinase products

As outlined above, arguably the most well-characterized PH domain-mediated signalling pathway involves PKB and PDK1, as is shown schematically in Figure 6(A), and discussed in detail by Vanhaesebroeck and Alessi [67]. A second important signalling pathway that involves PH domain-mediated recruitment to PI 3-kinase products in the plasma membrane is seen in signalling from the B-cell-antigen receptor (BCR), and involves Btk. Btk is a critical component in the pathway leading to maturation of B-cells [94], and mutations in the Btk PH domain are known to cause impaired B-cell development, resulting in agammaglobulinaemia [95,96]. The mutations that correlate with X-linked agammaglobulinaemia in humans are known to decrease the affinity of the Btk PH domain for $\text{PtdIns}(3,4,5)P_3$ and its soluble headgroup $\text{Ins}(1,3,4,5)P_4$ [18,36,77,97], illustrating the importance of this event in BCR signalling. That PI 3-kinase activation is critical in the signalling pathway leading to recruitment of Btk (via its PH domain) to the membrane surface in B-cell maturation was elegantly shown by targeted disruption of the p85 α regulatory subunit of PI 3-kinase in mice [98,99]. Loss of p85 α -mediated PI 3-kinase activation led to essentially the same phenotype as is seen in mice expressing Btk harbouring mutations in its PH domain that impair PI 3-kinase-product binding [98,99]. As depicted schematically in Figure 6(B), membrane recruitment of Btk by its PH domain recognizing PI 3-kinase products [86], brings the protein close to a Src-family kinase (Lyn) present at the membrane [100]. The Src kinase

phosphorylates Btk, which then undergoes autophosphorylation to become completely active [101]. Autophosphorylation of the Src-phosphorylated Btk, if it occurs in *trans*, may be promoted by the increase in local concentration of Btk that results from its restriction to two dimensions upon PH-domain-mediated membrane binding [36]. A similar mechanism was suggested for PKB [68] (Figure 6A). Although currently controversial, it has been argued that PKB activation follows such a two-step pathway. PDK1, playing the same role as Src/Lyn does in Btk activation, phosphorylates membrane-recruited PKB at Thr³⁰⁸. The second phosphorylation event (at Ser⁴⁷³) then occurs through autophosphorylation ([102]; but see [66,67] for alternative views). As suggested for Btk, autophosphorylation of PKB at Ser⁴⁷³ would be enhanced (if it is an intermolecular reaction) by an increased local PKB concentration resulting from PH domain-mediated membrane localization [68]. Thus mechanisms of PKB activation and Btk activation, following their recruitment to PI 3-kinase products, may be very similar indeed.

Details are more sketchy for the role of PH domain recruitment to PI 3-kinase products for the other proteins listed in Figure 5. Grp1, which is a guanine nucleotide exchange factor (GEF) for ADP-ribosylation factor-6 (ARF6), appears to be recruited in a PI 3-kinase-dependent manner by its PH domain to membrane ruffles in which ARF6 is also present [103]. Activation of ARF6 by Grp1-induced GTP \leftrightarrow GDP exchange in this location is likely to induce signal-dependent cytoskeletal rearrangements and membrane structural changes. Centaurin- α is an ARF-GTPase activating protein [82,84] that is recruited to the plasma membrane by binding of one of its PH domains to $\text{PtdIns}(3,4,5)P_3$ [82], but the implications of this for ARF signalling are not yet clear. Gab1 is proposed to act as a docking protein that may facilitate PI 3-kinase signalling [87]. The Gab1 PH domain binds $\text{PtdIns}(3,4,5)P_3$, and, together with a receptor-binding domain, this causes Gab1 to be recruited to the membrane upon cell stimulation [85,87]. Gab1 becomes phosphorylated on tyrosine residues and can itself recruit more PI 3-kinase, thus effectively amplifying the signal. The precise consequences of this are not clear, but Gab1 binding to both PI 3-kinase and its products do appear to be important [85,87]. Details of the signalling pathways in which the remaining 3-phosphoinositide-specific PH domains listed in Figure 5 participate are still less clear, but are the subjects of intense current investigation.

$\text{PtdIns}(3,4,5)P_3$ versus $\text{PtdIns}(3,4)P_2$ as PH domain targets

Most of the PH domains listed in the upper part of Figure 5 recognize $\text{PtdIns}(3,4,5)P_3$ with high affinity and specificity. However, the PKB and DAPP1 PH domains bind almost equally well to both $\text{PtdIns}(3,4,5)P_3$ and $\text{PtdIns}(3,4)P_2$ [17,68,104,105]. Most cellular $\text{PtdIns}(3,4)P_2$ is thought to arise through 5-dephosphorylation of $\text{PtdIns}(3,4,5)P_3$ [16], and, consistent with this, the time course of accumulation of these two lipid second messengers differs. $\text{PtdIns}(3,4,5)P_3$ accumulation in platelets after stimulation is immediate and transient, while $\text{PtdIns}(3,4)P_2$ accumulation is slightly delayed and significantly more sustained [16,68]. A PH domain that recognizes only $\text{PtdIns}(3,4,5)P_3$ (such as those from Grp1 or Btk) will therefore be recruited only transiently to the plasma membrane, whereas one that binds both $\text{PtdIns}(3,4,5)P_3$ and $\text{PtdIns}(3,4)P_2$ (the PKB and DAPP1 PH domains) may have a longer-lived membrane association, and therefore a more sustained activation. Consistent with the ability of the PKB PH domain to recognize both $\text{PtdIns}(3,4)P_2$ and $\text{PtdIns}(3,4,5)P_3$, Franke et al. found that the time course of PKB activation in activated platelets reflects the more sustained

accumulation of PtdIns(3,4) P_2 rather than the transient appearance of PtdIns(3,4,5) P_3 [68].

Recent studies have shown that Btk activation in B-cells is attenuated when the $F_{c\gamma}RIIB$ receptor IIB ($F_{c\gamma}RIIB$) is co-ligated with the BCR [106,107]. This attenuation results from the activation, by recruitment to $F_{c\gamma}RIIB$, of SHIP, an SH2-domain-containing inositol 5'-phosphatase [108]. SHIP dephosphorylates PtdIns(3,4,5) P_3 to PtdIns(3,4) P_2 *in vitro* [108], and the levels of PtdIns(3,4,5) P_3 produced *in vivo* when $F_{c\gamma}RIIB$ is co-ligated with BCR are substantially lower than those observed when BCR is activated alone. Since the Btk PH domain binds only to PtdIns(3,4,5) P_3 , the extent of Btk recruitment to the membrane upon BCR stimulation should be reduced substantially when SHIP is simultaneously activated (via $F_{c\gamma}RIIB$). This inhibitory effect has been clearly observed [106,107]. If SHIP generates PtdIns(3,4) P_2 from PtdIns(3,4,5) P_3 *in vivo* (which has not been clearly demonstrated), it would be anticipated that PKB responses should be inhibited less completely by SHIP than are those of Btk, since the PKB PH domain can bind to both PtdIns(3,4,5) P_3 and PtdIns(3,4) P_2 (the SHIP product). There has been one report that PKB responses (although at a much reduced level) are preserved in the absence of Btk activation when BCR and $F_{c\gamma}RIIB$ are co-ligated [109] (although see [88] for a conflicting result). PtdIns(3,4,5) P_3 accumulation was almost completely abolished compared with that seen when the BCR was activated alone, whereas PtdIns(3,4) P_2 accumulation was only slightly decreased [109].

Several reports have also described accumulation of PtdIns(3,4) P_2 without preceding PtdIns(3,4,5) P_3 production, an event that will only target PH domains capable of binding strongly to PtdIns(3,4) P_2 (e.g. those from PKB and DAPP1). Rittenhouse and colleagues have reported that ligation of platelet integrins induces PtdIns(3,4) P_2 generation by a PtdIns3P 4-kinase [110]. Similarly, oxidative stress of mouse fibroblasts appears to cause sustained and selective accumulation of PtdIns(3,4) P_2 , with little apparent involvement of PtdIns(3,4,5) P_3 [111]. In fibroblasts, it has been observed that platelet-derived-growth-factor stimulation causes membrane translocation of both the PKB and Grp1 PH domains [by inducing both PtdIns(3,4,5) P_3 and PtdIns(3,4) P_2 production]. However, in fibroblasts subjected to oxidative stress, only the PH domain of PKB [which binds PtdIns(3,4) P_2 and PtdIns(3,4,5) P_3], and not the PH domain from Grp1 [which binds only PtdIns(3,4,5) P_3], was recruited to the plasma membrane [89]. Although there is still much to be learned, these studies point to PtdIns(3,4) P_2 as a *bona fide* lipid second messenger. While responses that it elicits are likely to overlap those induced by PtdIns(3,4,5) P_3 in many cases, the regulation of PtdIns(3,4) P_2 /PtdIns(3,4,5) P_3 ratios appears to have significant cellular consequences that depend on PH domain specificity.

Structural basis for 3-phosphoinositide binding by PH domains

Baraldi et al. [36] have determined the X-ray crystal structure of the PH domain from Btk in complex with Ins(1,3,4,5) P_4 , the soluble headgroup of PtdIns(3,4,5) P_3 . As described for the PLC δ_1 PH domain, the inositol phosphate ligand binds to the positively charged face of the PH domain that contains variable loops 1–3 inclusive. The location of the Ins(1,3,4,5) P_4 -binding site on the Btk PH domain is very similar to that for Ins(1,4,5) P_3 on the PLC δ_1 PH domain [32], and involves several conserved amino acids. Figure 7 compares stereoscopic views of the two binding sites from similar orientations with respect to the PH domains. The most striking difference between the two complexes is that the inositol phosphate ligand is bound in quite different orientations in the two cases. This is best illustrated by comparing

the position of the 5-phosphate (coloured green) in the two complexes. In the PLC δ -PH-Ins(1,4,5) P_3 complex (Figure 7A), the 5-phosphate is on the left, and quite buried – forming hydrogen bonds with two lysine side chains (Lys³⁰ and Lys⁵⁷) as well as an arginine side chain (Arg⁴⁰). In the Btk-PH-Ins(1,3,4,5) P_4 complex (Figure 7B), the 5-phosphate is on the right-hand side, and interacts with Lys¹⁸ and several backbone amides. The 3-phosphate of Ins(1,3,4,5) P_4 in this complex is in essentially the same location as seen for the 5-phosphate of Ins(1,4,5) P_3 in the PLC δ -PH-Ins(1,4,5) P_3 complex. Whereas the 5-phosphate of Ins(1,4,5) P_3 hydrogen bonds with Lys³⁰ and Arg⁴⁰ of PLC δ -PH (boxed in Figure 7A), the 3-phosphate of Ins(1,3,4,5) P_4 hydrogen bonds with the almost equivalently positioned Lys¹² and Arg²⁸ of Btk-PH (boxed in Figure 7B; these residues align with Lys³⁰ and Arg⁴⁰ of PLC δ -PH – see Figure 5). Arg²⁸ of the Btk PH domain, which forms hydrogen bonds with the critical 3-phosphate of Ins(1,3,4,5) P_4 in this complex, is the residue that was first found to be altered in mutations linked to X-linked agammaglobulinaemia and X-linked immunodeficiency [95,96]. Substitution of a cysteine residue for this residue causes a substantial reduction in PtdIns(3,4,5) P_3 and Ins(1,3,4,5) P_3 binding affinity [18,77,97] and essentially abolishes B-cell maturation [95,96].

It is important to realize, when inspecting Figure 7, that the arrangement of phosphate groups in Ins(1,4,5) P_3 and Ins(1,3,4) P_3 is not spatially equivalent. In Figure 7(A), the 1- and 5-phosphates of Ins(1,4,5) P_3 project into the page, while the 4-phosphate projects out of the page. By contrast, the 1- and 3-phosphates of Ins(1,3,4,5) P_4 in Figure 7(B) (which apparently correspond in location to the 1- and 5-phosphates in Figure 7A when viewed in two dimensions) project *out* of the page, and the 4-phosphate in Figure 7B projects *into* the page. Thus the inositol phosphate has been rotated 180° about the centre of a mirror plane (if the 2-hydroxy group is ignored), resulting in an effective 'inversion' of the phosphate groups (and inositol carbon atoms). It is this difference that allows the DAPP1 and PKB PH domains to recognize Ins(1,3,4) P_3 but not Ins(1,4,5) P_3 , whereas the PLC δ_1 PH domain recognizes Ins(1,4,5) P_3 but not Ins(1,3,4) P_3 .

PH domains that specifically recognize phosphoinositides as experimental tools

Since the realization that certain PH domains can recognize specific phosphoinositides or inositol phosphates, PH domains have become valuable experimental tools for analysing the cellular roles, behaviour and location of these phosphoinositides. Simultaneous developments in the application of GFP variants in subcellular-localization studies have permitted the use of GFP-PH fusion proteins as specific probes for analysis of phosphoinositide production and localization in living cells. For PH domains that recognize PI 3-kinase products, demonstrations of signal-dependent recruitment of GFP-PH fusions have become almost routine [86–93]. These studies have demonstrated that different combinations of 3-phosphoinositides can be generated with different cellular treatments [89]. Moreover, analysis of plasma-membrane translocation of GFP-PH domain fusion proteins has revealed quantitative differences in the PI 3-kinase product generation induced by different growth factors, which may provide an explanation for the distinct signalling outcomes [92]. Studies of a GFP-PKB PH domain fusion protein have shown that an extracellular gradient of chemoattractant induces an even steeper intracellular gradient of signalling molecules, by asymmetric generation of PI 3-kinase products in the cell – strongest at the cell's leading edge [112]. Along similar lines, the PH domain from PLC δ_1 , which is specific for PtdIns(4,5) P_2 and Ins(1,4,5) P_3 , has been used as a GFP fusion protein to monitor

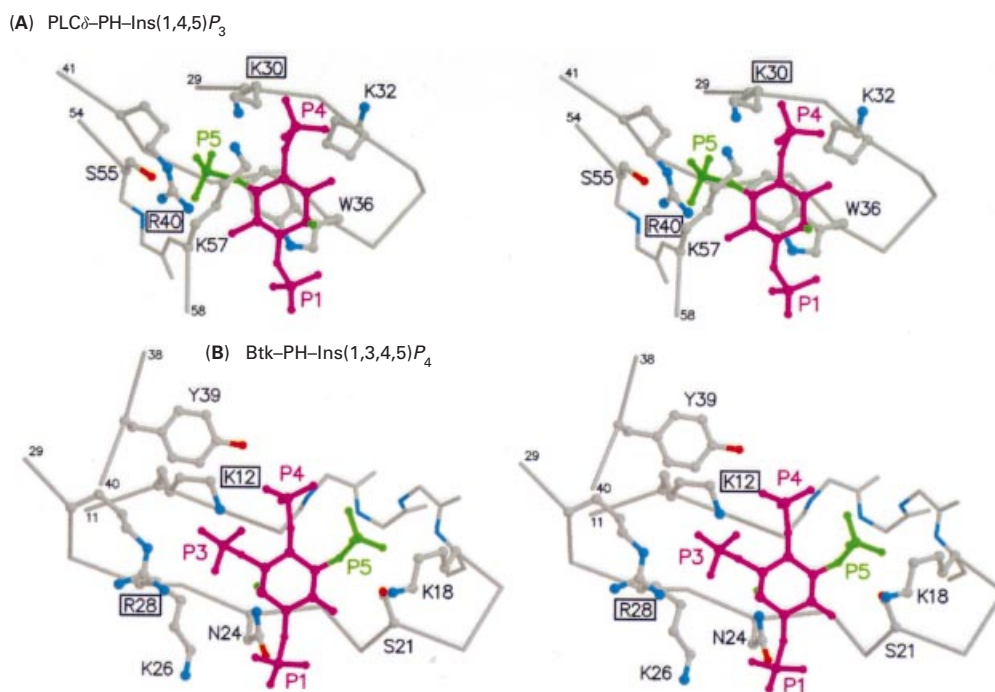


Figure 7 Comparison of high-affinity inositol phosphate-binding sites on PH domains

Stereo views of $\text{Ins}(1,4,5)\text{P}_3$ (**A**) and $\text{Ins}(1,3,4,5)\text{P}_4$ (**B**), coloured magenta, are shown bound to their respective binding sites in the $\text{PLC}\delta_1$ PH domain (**A**) [32] and the Btk PH domain (**B**) [36]. The two binding sites are viewed from essentially the same orientation in each case. The 1-, 4- and 5-phosphates of $\text{Ins}(1,4,5)\text{P}_3$ in the $\text{PLC}\delta$ -PH binding site would overlay with the equivalent phosphates of the Btk-PH-bound $\text{Ins}(1,3,4,5)\text{P}_4$ only if rotated 180° about an axis passing through the carbon-1 and -4 atoms of the inositol moiety. To emphasize this fact, both the 5-phosphate and the 2-hydroxy groups of $\text{Ins}(1,4,5)\text{P}_3$ and $\text{Ins}(1,3,4,5)\text{P}_4$ are coloured green. Only side chains that participate in direct hydrogen bonds with the bound inositol phosphate are shown. In addition, backbone -NH groups are coloured blue if involved in hydrogen bonding with the ligand. As described in the text, and marked in Figure 5, the two critical residues with boxed labels [K^{30} and R^{40} (one-letter amino acid notation) in $\text{PLC}\delta$ -PH; K^{12} and R^{28} in Btk-PH] are similarly positioned in the two PH domains. These basic side chains interact with the 5-phosphate in the $\text{PLC}\delta$ -PH- $\text{Ins}(1,4,5)\text{P}_3$ complex, but with the 3-phosphate in the Btk-PH- $\text{Ins}(1,3,4,5)\text{P}_4$ complex. R^{28} of Btk is the position at which X-linked-agammaglobulinaemia-associated mutations were first identified. This Figure was generated with MOLSCRIPT [156] and Raster3D [157].

diminution of $\text{PtdIns}(4,5)\text{P}_2$ at the plasma membrane upon receptor activation [113,114] and temporal aspects of cellular $\text{Ins}(1,4,5)\text{P}_3$ production (which dissociates $\text{PLC}\delta$ PH from the membrane) [62,114].

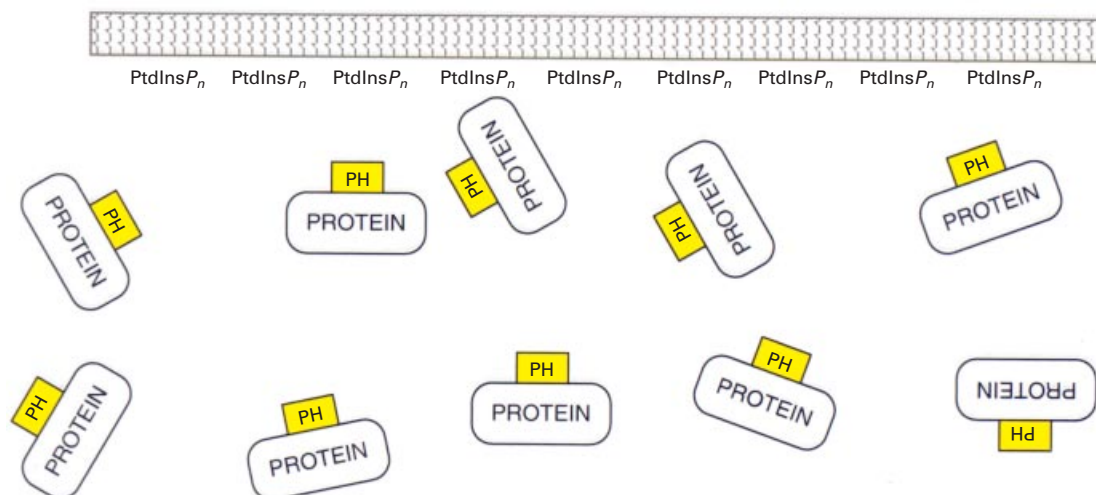
Specific PH domains have also been used to sequester phosphoinositides *in vivo* or *in vitro* in order to determine the role of a certain phosphoinositide in a particular cellular process. For example, the PH domain from $\text{PLC}\delta_1$, but not those from Grp1, dynamin or DAPP1, can inhibit receptor-mediated endocytosis in cell-free assays, implicating $\text{PtdIns}(4,5)\text{P}_2$, but not $\text{PtdIns}(3,4,5)\text{P}_3$, in this process [115]. Studies using the $\text{PLC}\delta_1$ PH domain have implicated $\text{PtdIns}(4,5)\text{P}_2$ in control of exocytosis [116], and overexpression of the $\text{PLC}\delta_1$ PH domain (but not the PKB PH domain) was reported to reduce the strength of interactions between the plasma membrane and the cytoskeleton [117]. Some isolated PH domains, when overexpressed, have also been shown to act as dominant-negative inhibitors of their host proteins. For example, overexpression of the N-terminal PH domain from $\text{PLC}\gamma_1$ inhibits platelet-derived-growth-factor-stimulated PLC activity [118]. The most straightforward interpretation of such studies is that the PH domain inhibits signalling by sequestering a phosphoinositide to which the monitored protein usually responds – PI 3-kinase products in the case of $\text{PLC}\gamma_1$ [118]. However, the situation is often complicated, and these results should not be taken at face value unless there is good reason to exclude other possibilities. The possibility that some PH domains bind to protein targets in addition to phos-

phoinositides remains, and there are many observations to suggest that this is true (see below). One possible example of this is illustrated with the Btk and cytohesin (or Grp1) PH domains. The phosphoinositide-binding properties of these two PH domains appear to be identical [17,18], and both are recruited to the plasma membrane upon activation of Jurkat cells [93]. Despite this similarity, however, overexpression of the cytohesin-1 PH domain, but not that from Btk, inhibits the stimulation of cell adhesion in Jurkat cells [93], arguing that a second PH domain target may be relevant.

PH domains that bind phosphoinositides with low affinity and poor specificity

Most of the PH domains that fail to rescue the *cdc25^{ts}* yeast in the assay described by Isakoff et al. [83] have previously been reported to bind phosphoinositides or inositol phosphates, albeit with low affinity [17–19,25–27,31,39,97,118–123]. Using a qualitative filter-binding assay to compare binding of PH domains to multiple phosphoinositides simultaneously, Kavran et al. showed that most of these low-affinity PH domains are also highly promiscuous in their phosphoinositide binding [17]. The PH domains from β -adrenergic receptor kinase $\beta 1$ ($\beta\text{ARK}1$), spectrin, Ras-GAP, pleckstrin (N-terminal) and diacylglycerol kinase- δ (DGK- δ) all bound to every phosphoinositide tested in this assay, including $\text{PtdIns}5\text{P}$ and $\text{PtdIns}(3,5)\text{P}_2$ [17]. Little, if

(A) Monomers



(B) High-avidity oligomers

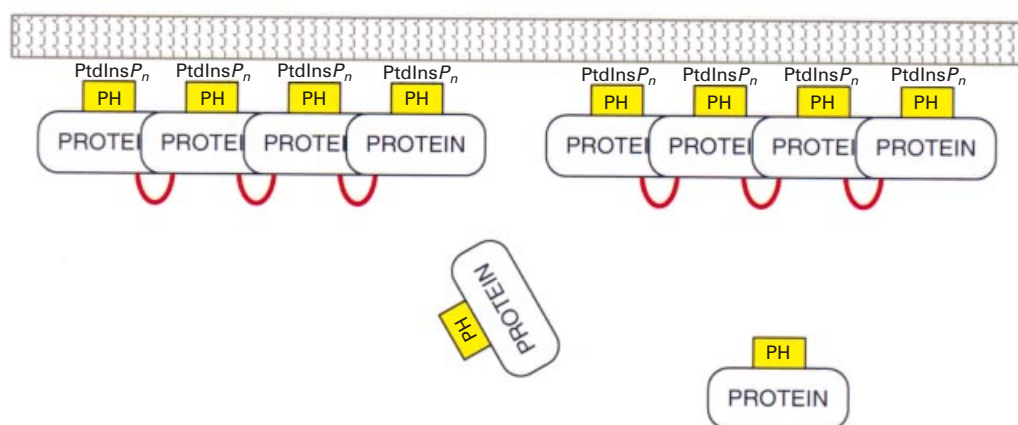


Figure 8 PH domain-mediated membrane recruited by ‘regulated avidity’

A hypothetical situation is depicted, as described in the text. In **(A)**, a protein with a PH domain does not bind to the membrane surface, as its PH domain has only very low affinity (K_D in the millimolar range) for phosphoinositides. The dynamin-1 PH domain is an example. Following some signalling event, the same protein has been induced to oligomerize **(B)**. Protein–protein interactions in **B** are denoted by red loops. In this case, the individual PH domains will co-operate with one another in a high-avidity association of the oligomeric protein with the membrane surface. As described in the text, where K_D for binding of a monomer to the membrane surface is in the millimolar range, by simple addition of energies, the effective K_D for tetramer binding would be in the picomolar range.

any, distinction between the various phosphoinositides by these promiscuous PH domains could be discerned. In several cases, phosphatidylserine was bound just as well as the phosphoinositides, suggesting that the PH domains simply recognize a negatively charged surface and not any particular characteristic of the inositol phosphate headgroup. For the N-terminal PH domain from pleckstrin-1 (PlecN-PH), this promiscuity was confirmed in studies of binding to small unilamellar vesicles containing different combinations of phospholipids [17]. Similarly, Takeuchi et al. [19] have reported promiscuous binding of PlecN-PH and the DGK- δ PH domain to soluble inositol

phosphates. Inositol phosphate binding to these PH domains showed no stereospecificity, and binding strength correlated simply with the number of phosphate groups on the ligand, suggesting that it is driven by non-specific, delocalized, electrostatic attraction between the PH domain and the anionic ligand.

PH domains that bind weakly and non-specifically to phosphoinositides and inositol phosphates all share the same electrostatic sidedness seen in other PH domains. NMR studies of inositol phosphate binding to these PH domains show that the positively charged face contains the ligand-binding site

[25–27,39,97,119], and this has been confirmed by X-ray-crystallographic studies of the spectrin PH in complex with $\text{Ins}(1,4,5)P_3$ (K_D 40 μM) [31], as shown in Figure 1. However, these promiscuous PH domains, which do not (as Ras fusions) rescue *cdc25^{ts}* yeast under any conditions, share few if any of the side chains that comprise the highly specific inositol phosphate-binding sites seen in the PH domains of $\text{PLC}\delta_1$ and Btk (Figure 5; bottom panel). While one or two of the key binding-site residues are frequently conserved, there are nearly always important side chains missing, even from the $\beta 1/\beta 2$ region. Where there is conservation of a side chain that is critical for inositol phosphate binding to the $\text{PLC}\delta_1$ or Btk PH domains, this side chain is almost always among those found to be perturbed upon inositol phosphate binding in NMR studies of promiscuous PH domains [25–27,31,39,97,119]. In these cases we suggest that the inositol phosphate binds to the same region of the PH domain as seen in the crystal structures of high-affinity complexes. However, since there is only a partial binding site, relatively few hydrogen bonds will be made between the bound inositol phosphate and the protein compared with the number seen in the highly specific complexes (compare [31] and [32]). With a smaller number of inositol phosphate–protein interactions, the affinity of binding will be reduced, and there will be fewer constraints on the orientation of the bound inositol phosphate – likely to be manifest as a reduced specificity. Under these circumstances, delocalized electrostatic attraction of the inositol phosphate for the PH domain is likely to be the primary driving force for binding.

Physiological relevance of promiscuous phosphoinositide binding by PH domains

While the physiological functions of PH domains that recognize phosphoinositides with high affinity and specificity are clear in several cases, it is less straightforward to make a case for the physiological importance of the weak and promiscuous phosphoinositide binding seen with other PH domains (which actually constitute the majority). It could be argued that the weak phosphoinositide binding observed for these PH domains *in vitro* is vestigial, and that their physiological ligands are in fact distinct, as-yet-unidentified, entities that bind to these PH domains with high affinity and specificity.

However, there are several reasons to expect that this promiscuous binding may have physiological importance. In considering likely general mechanisms for signal-dependent recruitment of proteins to the plasma membrane, two main possibilities come to mind. The first involves the signal-dependent production of a specific lipid molecule that exists only transiently in the plasma membrane and is specifically recognized by the recruited protein. PI 3-kinase products and PH domains that recognize them with high affinity and specificity fulfil these criteria. The second possibility does not require any chemical changes to the membrane, but rather involves alteration of the recruited protein. In this case, the protein to be recruited might be imagined to have an inherent tendency to bind to the membrane surface, but its binding strength is sufficiently weak that the protein remains 99% cytosolic. Following cell stimulation, phosphorylation of this target protein (or some other regulated modification) could cause its oligomerization. The oligomeric protein would now bind efficiently to the plasma membrane because of an increase in the avidity of its membrane association. In this case, the affinity of individual interactions is not changed, but rather membrane recruitment results from regulated increases in avidity. If the monomer can bind to the membrane surface with a K_D of 1 mM ($\Delta G = -4$ kcal/mol) (1 kcal = 4.184 kJ), dimer binding will have an effective K_D in the micromolar range ($\Delta G =$

-8 kcal/mol), trimer binding in the nanomolar range ($\Delta G = -12$ kcal/mol), tetramer binding in the picomolar range ($\Delta G = -16$ kcal/mol) and so on. It is clear that many PH-domain-containing proteins do have an inherent affinity for the membrane surface that is not sufficient to drive their membrane localization under normal circumstances. The isolated PH domains from dynamin-1, pleckstrin and other proteins remain cytosolic when expressed in mammalian cells, with no apparent membrane localization [17,120]. There are numerous precedents for the induction of protein oligomerization by phosphorylation and other means in cellular signalling. We therefore suggest that regulation of the avidity of PH-domain binding to the plasma membrane could play a role in signal-dependent membrane recruitment. In such a mechanism, the temporal specificity of membrane recruitment could be tightly controlled, although spatial specificity would be lost if the individual weak interactions that contribute are all non-specific. High-avidity oligomers could be homomeric or hetero-oligomeric and need not have discrete sizes. Spatial specificity could be achieved, for example, by forming mixed oligomers between PH-domain-containing proteins and proteins containing modules that (alone) bind weakly to phosphorylated proteins. Figure 8 illustrates the basic idea of how regulated avidity could be employed in signal-dependent membrane recruitment of signalling molecules. Although there is not yet a great deal of evidence to support (or refute) this model, recent studies of the PH domain from dynamin suggest that relocation of this protein during receptor-mediated endocytosis involves regulation of the avidity of PH-domain interactions.

Importance of weak phosphoinositide binding by a promiscuous PH domain in dynamin action

Dynamin-1 is a 100 kDa GTPase that is required for the scission of endocytic vesicles from the plasma membrane [124]. $\text{PtdIns}(4,5)P_2$ and other phosphoinositides enhance the GTPase activity of dynamin-1 in a PH-domain-dependent manner [97,125], apparently by promoting its oligomerization [126]. While some groups have reported that the isolated dynamin-1 PH domain (Dyn1-PH) binds strongly to $\text{PtdIns}(4,5)P_2$ [97,119], others have reported otherwise [18,59]. Most recently, Klein et al. showed that Dyn1-PH requires oligomerization for significant association with membrane phosphoinositides [120]. Whereas univalent $\text{Ins}(1,4,5)P_3$ binds to Dyn1-PH with a K_D only in the millimolar range [97,119], lipid vesicles containing many $\text{PtdIns}(4,5)P_2$ molecules can bind strongly to Dyn1-PH, but only when it is in an oligomeric form [120] – e.g. by fusion to dimeric glutathione S-transferase (GST) or by introduction of an intermolecular disulphide bond. The increase in apparent affinity arises from a simple avidity effect in which the energies of multiple PH-domain/headgroup interactions are added. Thus, while monomeric Dyn1-PH binds $\text{Ins}(1,4,5)P_3$ with a K_D of 1.4–4 mM ($\Delta G = -3.9$ to -3.3 kcal/mol) [97,119], dimeric Dyn1-PH binds $\text{PtdIns}(4,5)P_2$ -containing vesicles with an effective K_D of 9 μM (-6.9 kcal/mol) [120]. Since full-length dynamin-1 is a tetramer [127], Klein et al. reasoned that it, too, should be capable of high-avidity, PH-domain-mediated, binding to phosphoinositides in cell membranes [120]. If dynamin-1 can only bind to membranes by virtue of its oligomerization, then over-expression of dynamin-1 containing a PH domain that is defective in phosphoinositide binding should impair membrane targeting of endogenous dynamin-1. Endogenous dynamin and the over-expressed mutant should form mixed oligomers, which will be defective in membrane association. As predicted by this model, several groups have recently shown that a PH-domain-defective

dynamamin-1 mutant is indeed a dominant-negative inhibitor of dynamamin function *in vivo* [128–130].

According to some models of dynamamin function, its oligomerization may be regulated during endocytosis by interactions with other components of the endocytic machinery [131]. If there is a step at which dynamamin-1 oligomerization is tightly regulated, the avidity of its PH-domain-mediated membrane targeting will be regulated similarly tightly. Such ‘avidity regulation’, as described above, could be important in controlling a step in endocytosis at which dynamamin is targeted to the necks of invaginated coated pits prior to vesicle scission. Although this is highly speculative, it illustrates a potentially important property that will be characteristic of PH domains that bind very weakly (and non-specifically) to membranes when monomeric.

Intramolecular co-operation of PH and other domains in driving membrane association

Several reports have suggested that certain PH domains must co-operate with other membrane-targeting modules within the same protein in order to drive membrane association. This will only apply to PH domains that are not capable of independent membrane recruitment (i.e. those from the promiscuous class). Examples include binding of β ARK to membranes that contain the $G\beta\gamma$ subunits of heterotrimeric G-proteins. This binding event requires both direct interaction of the C-terminal tail of β ARK with $G\beta\gamma$ and binding of the β ARK PH domain to PtdIns(4,5) P_2 or other phosphoinositides [132]. Neither one of these interactions alone can drive efficient β ARK recruitment to the membrane. Indeed, the K_D for binding of the β ARK PH domain to the headgroup of PtdIns(4,5) P_2 is only 207 μ M [27]. Similarly, the efficient PI 3-kinase-dependent membrane recruitment of PLC γ_1 to the plasma membrane appears to require co-operation of the enzyme’s PH domain and SH2 domains [118,133]. In another example, membrane targeting of Tiam 1 requires the co-ordinate activity of its N-terminal PH domain and an adjacent-protein interaction domain [134].

POTENTIAL PROTEIN TARGETS OF PH DOMAINS

Since the naming of PH domains in 1993, many different protein ligands have been suggested or reported. However, there are no candidates for consideration as a general protein target for PH domains along the lines of proline-rich sequences or phosphotyrosine-containing sequences that bind SH3 and SH2 domains respectively. Nonetheless, for certain PH domains there are hints that something other than phosphoinositides must be involved in some of their interactions. In the case of the dynamamin-1, there is clear evidence for the importance of PH domain \leftrightarrow phosphoinositide interactions in receptor-mediated endocytosis, as was discussed above. However, Artalejo et al. [135] have shown that the isolated dynamamin-1 PH domain can inhibit another process, termed ‘rapid endocytosis’, in adrenal chromaffin cells. Inhibition of rapid endocytosis does not involve phosphoinositide binding, and it can be abolished by highly specific mutations in the PH domain that do not affect PtdIns(4,5) P_2 binding [120]. Along similar lines, domain-swapping experiments have shown that, despite their apparently very similar phosphoinositide-binding characteristics, the PH domains from β ARK, PLC γ_1 or spectrin cannot functionally replace the PH domain of IRS-1 [136].

The first specific proteins to be proposed as PH-domain targets were the $\beta\gamma$ -subunits of heterotrimeric G-proteins [137]. Several different PH domains, including those from β ARK and Btk [138], were shown as GST fusion proteins to precipitate small quantities of $G\beta\gamma$ subunits from cell lysates. The site of these

interactions has been narrowed down to WD40 repeats of the heterotrimeric G-protein β -subunit [139,140], and appears to involve primarily residues that are at or beyond the very C-terminus of the PH domains with which they have been observed [141]. There is substantial evidence for the physiological relevance of these interactions [142], although the extent to which they can be argued to be properties of PH domains, rather than adjoining sequences, is not clear.

The second main class of protein ligands proposed for PH domains are isoforms of protein kinase C (PKC) and associated proteins. One attractive aspect of this proposal is the fact that many PH-domain-containing proteins are phosphorylated by PKC. Indeed, pleckstrin itself is a major PKC substrate in platelets, and dynamamin is known to go through cycles of phosphorylation by PKC [143]. A GST fusion protein containing the Btk PH domain was reported to bind *in vitro* to a mixture of PKC α , β and γ isoforms from rat brain [144], and Btk was found to co-immunoprecipitate with the β I isoform of PKC. A recent report has suggested that PtdIns(4,5) P_2 can disrupt these interactions [145]. Similarly, the PKB PH domain has been reported to associate with several PKC isoforms, although again the physiological relevance of these interactions has not been demonstrated [146,147]. The PH domain of protein kinase D has also been reported to interact selectively with PKC ϵ [148].

In addition to these reports, results have been presented that suggest a PKC-regulated association of several PH domains with RACK1 – the receptor for activated PKC [149]. $G_{12\alpha}$ [150], a protein called BAP-135 [151] and filamentous actin [152] have all been reported to interact with the PH domain of Btk, and one report has claimed that the PH domain of PKB binds to myosin II [153]. Finally, a 30 kDa phosphoprotein has been reported to interact specifically with the N-terminal PH domain from pleckstrin [154], and yeast two-hybrid screens have identified proteins that contain stretches of acidic amino acids, such as nucleolin, as potential ligands for PH domain from IRS-1 and related proteins [155].

In none of these cases has a clear physiological role for PH-domain binding to a protein target been identified. However, it must be remembered that, for the majority of PH domains, neither has a clear physiological role been ascribed to inositol phosphate or phosphoinositide binding. Just as weak and promiscuous phosphoinositide binding can be argued to play a role in regulated membrane targeting of PH-domain-containing proteins (see above), so could the PH domain \leftrightarrow protein interactions summarized here be of significant importance.

PERSPECTIVES

The quest to identify the functions of PH domains has taught us several important lessons and has provided some valuable technologies. The finding that some PH domains recognize certain phosphoinositides with exquisite specificity – particularly PI 3-kinase products – has taught us a great deal about the regulation of the PH-domain-containing proteins themselves. It has also facilitated studies of PI 3-kinase signalling generally, by providing valuable probes of PI 3-kinase product generation (PH domains as GFP fusion proteins). It is noteworthy that, by binding PtdIns(3,4,5) P_3 and recruiting its host protein to the membrane surface – in the vicinity of an activated receptor – PH domains are more similar in function to SH2 domains (as was first suggested) that might have been appreciated once their targets were found to be phosphoinositides. The PH-domain-mediated membrane recruitment of an ARF GEF like Grp1 to PtdIns(3,4,5) P_3 is highly analogous to the SH2 domain-mediated

recruitment (by Grb2) of the Grb2–Sos (Son-of-sevenless) complex to an activated receptor tyrosine kinase.

It is clear that such specific and independent phosphoinositide targeting is a characteristic of only few PH domains, and it remains a major challenge to unravel the weak and promiscuous phosphoinositide and protein interactions that have been reported for the remaining 100 or so other PH domains. Determining which of these are relevant to the *in vivo* functions of their host proteins, and how these weak interactions can be utilized in the formation of large membrane-localized signalling complexes, will surely provide more interesting lessons in the control of cellular signalling.

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