REVIEW

Pleckstrin Homology Domain as an Inositol Compound Binding Module

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Received December 9, 1997

ABSTRACT—Many of the proteins that participate in cell signalling contain structural modules involved in regulatory interactions between components of signal transduction cascades. One of such modules is the pleckstrin homology (PH) domain, a region of approximately 120 amino acids that can form an electrostatically polarized tertiary structure. Several molecules such as inositol 1,4,5-trisphosphate/phosphatidylinositol 4,5-bisphosphate, the $\beta\gamma$ -subunits of heterotrimeric G proteins and protein kinase C have been proposed as common ligands for the PH domain. Through these potential interactions, the PH domain has been proposed to play a role in membrane recruitment of proteins containing the PH domain, thus targeting them to appropriate cellular compartment or enabling them to interact with other components of the signal transduction pathway. In this review, we mainly focus on membrane targeting through the binding to inositol phosphates/phosphoinositides.

Keywords: Pleckstrin homology domain, Inositol phosphate, Phosphoinositide, Signal transduction

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1. Introduction: What is PH domain?

In 1993, two groups first used the term pleckstrin homology (PH) domain for the homologous region comprising approximately 120 amino acids found in a number of proteins with diverse cellular functions, because the region was repeatedly found in both the N- and C-terminals of pleckstrin, a major protein kinase C substrate in platelets (1, 2). Musacchio et al. (3) then extended the list of proteins containing PH domains to 45 proteins by a better homology search using databases of EMBL nucleotide sequence, Swiss-Prot protein sequence and

PIR protein sequence, and more than 100 proteins are known to date. Because many of these proteins on the list are involved in signal transduction pathways, this led us to expect that the domain is involved in regulatory interactions between components of signal transduction cascades by interacting with a common ligand such as a protein motif or a non-protein molecule, like *src* homology (SH) domains; most SH2 and SH3 domains function as phosphotyrosine binding and proline-rich region binding modules, respectively (4).

Several potential ligands for the PH domain have

Abbreviations used are (in alphabetical order): β ARK, β -adrenergic receptor kinase; Btk, Bruton's tyrosine kinase; InsP_2 , InsP_3 , InsP_4 and InsP_5 , *myo*-inositol bis-, tris-, tetrakis- and pentakisphosphate, respectively, with isomeric positioning of phosphate groups, as denoted in parentheses, and assumed to be the D-enantiomer unless otherwise stated; PH, pleckstrin homology; PLC, phospholipase C; PtdInsP_2 and PtdInsP_3 , phosphatidylinositol bis- and tris-phosphate, respectively, with isomeric positioning of phosphate groups, as denoted in parentheses; SH, *src* homology; Sos, son-of-sevenless.

Fig. 1. Sequence alignments of several PH domains. Amino acids indicated by outline font with thick underline are assumed to be involved in the interaction with Ins(1,4,5)P₃ as assessed by NMR spectroscopy and/or X-ray crystallography. The short sequence in PLC-δ₁ indicated by thin underline is the minimal region for the interaction with Ins(1,4,5)P₃ (7, 8).

been identified including inositol 1,4,5-trisphosphate [Ins(1,4,5)P₃] (5–8) / phosphatidylinositol 4,5-bisphosphate [PtdIns(4,5)P₂] (8–11), the $\beta\gamma$ -subunits of heterotrimeric G proteins (G $\beta\gamma$) (12–14) and protein kinase C (15–17). Although the physiological relevance of the binding of these ligands remains to be elucidated, the PH domain has been proposed to play a role in membrane recruitment of proteins containing the PH domain, thus targeting them to the appropriate cellular compartment or enabling them to interact with other components of the signal transduction pathway. Alternatively, some examples of enzyme activation in response to ligand binding to the PH domain have also been reported without any relation to membrane targeting (18). In this review, we are going to mainly focus on membrane targeting through the binding to inositol phosphates/phosphoinositides because both of them have already been established to function in cell signaling (19) and therefore the proposed functions of inositol phosphates/phosphoinositides might extend the versatility of inositol compounds in cell signaling.

2. Structure of PH domains and the binding to Ins(1,4,5)P₃/PtdIns(4,5)P₂

Similarity in the secondary structures of PH domains despite low primary sequence similarity is one of the criteria for the PH domain; they comprise seven β -strands plus a C-terminal α -helix, and the loops between these strands, which are variable in length and sequence (Fig. 1). Especially the loop between the 3rd and 4th β -strands ($\beta 3/\beta 4$) sometimes contains an additional α -helix or is

split by a long sequence (see also Fig. 1). Tertiary structural analyses of PH domains derived from several proteins [β -spectrin (20), pleckstrin (11), dynamin (21), phospholipase C- δ_1 (PLC- δ_1 , Ref. 22), Bruton's tyrosine kinase (Btk, Ref. 23) and mouse Son-of-sevenless 1 (mSos1, Ref. 24)] as determined by NMR spectroscopy or X-ray crystallography revealed a common structure; each domain is a β -sandwich formed by two nearly orthogonal antiparallel β -sheets comprising four and three β -strands, respectively, and the C-terminal α -helix caps one end of the β -sheets. As a result of this folding, a positively-charged face is formed by three variable loops ($\beta 1/\beta 2$, $\beta 3/\beta 4$ and $\beta 6/\beta 7$ or alternatively $\beta 1/\beta 2$, $\beta 3/\beta 4$ and $\beta 5/\beta 6$; for a schematic representation, see Fig. 2).

In 1991, we isolated two Ins(1,4,5)P₃ binding proteins with molecular masses of 130 kDa and 85 kDa from rat brain, and partial amino acid sequence determinations revealed that the former (p130) is a novel protein, while the latter is PLC- δ_1 (5). Subsequent studies using a PLC- δ_1 clone clarified that the site involved in the binding was the amino acid sequence of 30 to 43 of PLC- δ_1 , which was mapped in $\beta 1/\beta 2$ of the PH domain (7, 8; also see Fig. 1). Almost at the same time, Rebecchi and his colleagues independently reported that the first 60 amino acids of PLC- δ_1 which comprises the N-terminal part of the PH domain, were responsible for the binding to liposomes containing PtdIns(4,5)P₂ (9, 10). Thus, we as well as Rebecchi's group were apparently the first to show that the PH domain of PLC- δ_1 binds Ins(1,4,5)P₃ and/or PtdIns(4,5)P₂. However, these studies had originally not been done in relation to the involvement of the PH

Fig. 2. Schematic representation of secondary structures of PH domain. Two nearly orthogonal antiparallel β -sheets, comprising four and three β -strands, form a β -sandwich. Numbers in open arrows indicate the β -strands from the N-terminal, and the C-terminal α -helix is omitted from the figure. The loops between $\beta 1/\beta 2$, $\beta 3/\beta 4$ and $\beta 6/\beta 7$ (A) or alternatively, $\beta 1/\beta 2$, $\beta 3/\beta 4$ and $\beta 5/\beta 6$ (B) form the electrostatically-polarized face to accommodate inositol phosphates, which is represented with the shaded area.

domain. On the contrary, Harlan et al. sought ligands for PH domains; and in 1994, they found that the PH domains derived from several proteins such as pleckstrin, β -adrenergic receptor kinase (β ARK), *ras*-GTPase-activating protein and T cell-specific-kinase bound to liposomes containing $\text{PtdIns}(4,5)\text{P}_2$, but not other acidic phospholipids (11). They further identified several amino acids in $\beta 1/\beta 2$ and $\beta 3/\beta 4$ of the N-terminal PH domain of pleckstrin, which showed chemical shift changes of backbone amides in NMR spectroscopy upon addition of $\text{PtdIns}(4,5)\text{P}_2$.

The binding mode of the PH domain from β -spectrin (20) or $\text{PLC-}\delta_1$ (23) was analyzed by X-ray crystallographic studies, in a complex with $\text{Ins}(1,4,5)\text{P}_3$. Several amino acids present in the $\beta 1/\beta 2$ and $\beta 5/\beta 6$ of β -spectrin or $\beta 1/\beta 2$, $\beta 3/\beta 4$ and $\beta 6/\beta 7$ of $\text{PLC-}\delta_1$, respectively, are indeed proved to be involved in the binding through ionic and hydrogen bonding interactions.

3. Inositol compounds other than $\text{Ins}(1,4,5)\text{P}_3$ / $\text{PtdIns}(4,5)\text{P}_2$ could also be bound to PH domains

Several inositol compounds other than $\text{Ins}(1,4,5)\text{P}_3$ or $\text{PtdIns}(4,5)\text{P}_2$ could also be nominated as potential ligands for PH domains (Table 1). As a reference for the mutual relationship of many inositol compounds, the metabolic pathway is shown in Fig. 3.

Molecular cloning studies of the gene encoding p130, which has been isolated as an $\text{Ins}(1,4,5)\text{P}_3$ binding protein

Table 1. Putative ligands of PH domains

PH domain	Proposed ligand	Reference
$\text{PLC-}\delta_1$	$\text{Ins}(1,4,5)\text{P}_3$ $\text{PtdIns}(4,5)\text{P}_2$	6, 8 9
p130	$\text{Ins}(1,4,5)\text{P}_3$ $\text{Ins}(1,4,5,6)\text{P}_4$	5, 25, 26 5, 25, 26
RAC/PKB/Akt	$\text{PtdIns}(3,4,5)\text{P}_3$ $\text{PtdIns}(3,4)\text{P}_2$ $\text{Ins}(1,4,5,6)\text{P}_4/\text{Ins}(1,3,4,5,6)\text{P}_5$	31 30 27
Btk	$\text{Ins}(1,3,4,5)\text{P}_4$ $\text{PtdIns}(3,4,5)\text{P}_3$	28, 36 29, 32
Dynamin	$\text{PtdIns}(4,5)\text{P}_2$	29
Sos	$\text{PtdIns}(4,5)\text{P}_2$ $\text{PtdIns}(3,4,5)\text{P}_3$	33 32
Diacylglycerol kinase	$\text{Ins}(1,3,4,5,6)\text{P}_5$	27

together with $\text{PLC-}\delta_1$ as described above (5), revealed that this protein is similar to $\text{PLC-}\delta_1$, but has extra-regions on both the N- and C-terminal (see Fig. 4; Refs. 5, 25). Further studies to locate the binding site of p130 clarified that the PH domain is also responsible, but unlike $\text{PLC-}\delta_1$, the PH domain prefers $\text{Ins}(1,4,5,6)\text{P}_4$ as well as $\text{Ins}(1,4,5)\text{P}_3$ (5, 25, 26). This led us to examine whether other PH domains could bind other inositol phosphates. PH domains from pleckstrin, diacylglycerol kinase or RAC-protein kinase showed the highest affinity to $\text{Ins}(1,3,4,5,6)\text{P}_5$ among the inositol phosphates examined

Fig. 3. Phosphoinositides and inositol phosphates in cells. PP-InsPx indicates the pyrophosphate form of inositol phosphate. Arrows indicate metabolic pathways.

Fig. 4. Comparison of PLC- δ and p130. PH, EF-hand and C2 mean PH domain, EF-hand motif and C2 domain, respectively. X and Y represent the putative catalytic domains of phosphoinositide-specific PLC. Numbers over the box indicate the beginning and end of each domain.

(27).

Fukuda et al. (28) reported that the PH domain of Btk specifically binds to $\text{Ins}(1,3,4,5)\text{P}_4$ and that a point mutation found in human X-linked agammaglobulinemia and murine X-linked immunodeficiency abolished the binding, thus suggesting a causal link to the illnesses. Furthermore, Salim et al. (29) reported that the PH domain derived from dynamin and Btk showed a distinct specific binding to $\text{PtdIns}(4,5)\text{P}_2$ and $\text{PtdIns}(3,4,5)\text{P}_3$, respectively. Moreover, Franke et al. (30) reported that the PH domain of Akt (same as RAC-protein kinase or protein kinase B) specifically recognized $\text{PtdIns}(3,4)\text{P}_2$ but not $\text{PtdIns}(3,4,5)\text{P}_3$, whereas James et al. (31) reported that the same PH domain prefers the latter trisphosphorylated inositides to the former. Very recently, Rameh et al. (32) reported that the PH domains from Btk, Sos and T-lymphoma-invasion-and-metastasis protein select for $\text{PtdIns}(3,4,5)\text{P}_3$; on the contrary, the Sos-PH domain was reported to prefer $\text{PtdIns}(4,5)\text{P}_2$ but not $\text{PtdIns}(3,4,5)\text{P}_3$ (33).

These results clearly indicate that PH domains derived from many kinds of proteins could bind various types of inositol compounds with a specificity, although there is some discrepancy in spite being the same PH domain as to the reported preference of inositol phosphates/phosphoinositides (Table 1). This may be partly caused by different assay conditions; one group used whole protein molecules, but the others used isolated PH domains. Furthermore, one group used soluble inositol phosphates or soluble analogues of phosphoinositides, but the others used phosphoinositides embedded in liposomes (see also next section). We think that now is the time for many research groups to show their own results, and therefore we believe there would be a convergence of the results soon, employing similar assay conditions.

The specificity of inositol compound binding by PH domains indicate that the precise structure of the putative pocket for accommodating inositol compounds is different among the type of PH domains, although over-all tertiary structures of PH domains analyzed to date were

much the same in spite of low sequence similarity.

4. Inositol phosphates vs phosphoinositides

Harlan et al. (11) first described that the N-terminal PH domain of pleckstrin could accommodate the acyl moieties of $\text{PtdIns}(4,5)\text{P}_2$ between two orthogonal β -sheets in addition to the positively-charged face formed by loops between β -strands for accommodating phosphate moieties; therefore, $\text{Ins}(1,4,5)\text{P}_3$, which lacks acyl chains, was reported to be a 30-fold weaker ligand than $\text{PtdIns}(4,5)\text{P}_2$. On the other hand, Gibson et al. (34) argued against the idea pointing out that the PH domains lack a hydrophobic pocket sufficient for accepting the fatty acid moieties of phospholipids, as assessed by NMR spectroscopy and X-ray crystal structural analysis (20–23). However, most of the binding studies with PH domains have been carried out using phosphoinositides embedded in liposomes (11, 29–33). We assume that this is simply because binding to phosphoinositides present in the plasma membrane could readily be translated into the putative physiological significance of PH domains in living cells.

Binding studies using both soluble analogues of $\text{PtdIns}(4,5)\text{P}_2$ or $\text{PtdIns}(3,4,5)\text{P}_3$, glycerophosphoryl- $\text{Ins}(4,5)\text{P}_2$ or dibenzoylglycerophosphoryl- $\text{Ins}(3,4,5)\text{P}_3$, respectively, and the corresponding inositol phosphate, $\text{Ins}(1,4,5)\text{P}_3$ or $\text{Ins}(1,3,4,5)\text{P}_4$ were performed. The results clearly indicate the higher affinity to inositol phosphate than the corresponding soluble analogue of phosphoinositide, i.e., the PLC- δ_1 -PH domain showed the "affinity"¹ of 0.21 μM /1.66 μM (eightfold difference) for $\text{Ins}(1,4,5)\text{P}_3$ / $\text{PtdIns}(4,5)\text{P}_2$, respectively (35). Similar difference in the affinity was observed with p130-pH

¹ Absolute values presented as the "affinity" to inositol compounds could not be compared among those in different reports, because the affinities are quite dependent on assay conditions employed such as pH values or the presence/absence of divalent cations. Furthermore, the values were sometimes simply expressed as IC_{50} values required for displacing isotope-labelled ligands bound to each PH domain.

domain (4.3 nM/100 nM, 25-fold difference; Ref. 26). The affinities of the Btk-PH domain to Ins(1,3,4,5)P₄/PtdIns(3,4,5)P₃ was 15 nM/113 nM (eightfold difference) (36). These results suggest that either a fully ionized 1-phosphate is important and/or the glycerol moiety provides some steric hindrance for the recognition.

On the other hand, the experiments employing phosphoinositides with acyl chains indicate that phosphoinositides showed higher affinity than the corresponding inositol phosphates; Harlan et al. (37) showed the affinity of the N-terminal PH domain of pleckstrin to PtdIns(4,5)P₂ was 51 μ M, about 10 times higher than that to Ins(1,4,5)P₃. Rameh et al. (32) reported that the PH domain of Btk showed about 40 times higher affinity to PtdIns(3,4,5)P₃ than to Ins(1,3,4,5)P₄. These results, together with those obtained using soluble analogues of phosphoinositides as described above, indicate that the acyl chains of phosphoinositides may play an important role in the recognition, as first suggested by Harlan et al. (11). Apparently further studies are needed to clarify the involvement of acyl chains of phosphoinositides in the recognition by PH domains from the structural point of view.

5. Proposed physiological role of PH domains

It has been proposed from the results accumulated in *in vitro* binding experiments that PLC- δ_1 -PH domain plays the role of targeting the enzyme to the inner surface of the plasma membrane through the binding to PtdIns(4,5)P₂, thus permitting the processive mode of PtdIns(4,5)P₂ catalysis (9, 10). An analogous case is proposed in Akt, whose activity in response to growth factor is regulated by the products of PI 3-kinase. The Akt-PH domain was recently reported to bind to the 3-phosphorylated inositides preferentially, especially to PtdIns(3,4)P₂, resulting in the enzyme activation *in vitro* (30). Thus, the role of the PH domain of Akt might promote the recruitment of the enzyme to the plasma membrane, just like the case of PLC- δ_1 , whereas simple binding to PtdIns(3,4,5)P₃/PtdIns(3,4)P₂ does not appear to be sufficient for the enzyme activation (for details about the activation mechanisms of the enzyme, see Ref. 38). These proposals are simply dependent on the *in vitro* results that PH domains could bind to distinct phosphoinositides present in the plasma membrane. However, the proposal for PLC- δ_1 has been verified by showing that the intact enzyme, but not the PH domain-deleted enzyme was localized at the plasma membrane when microinjected into living cells (39); and further results on the point mutation for the PH domain that make it unable to bind inositol compounds favor the proposed role of the PH domain in living cells (40). On the other hand, there is no evidence so far for the membrane localization of Akt through its PH

domain in living cells.

Care should be taken when translating the *in vitro* binding results into the *in vivo* state, because there is a discrepancy as to the preferred inositol compounds reported. It would also be true that the determination of physiologically relevant ligands depends not just on relative binding affinities, but also on relative intracellular abundance of a ligand in cells. Although free concentrations of phosphoinositides are difficult to evaluate, measurements of their total intracellular concentrations have been reported. Total cellular levels of PtdIns(4,5)P₂ and PtdIns(3,4,5)P₃ have been estimated to be 30–160 μ M and less than 0.1 μ M, respectively (41, 42), but the local concentrations are likely to be higher. The concentrations of PtdIns(4,5)P₂ at the inner leaflet of neutrophils were estimated to be 5 mM, decreasing to 3.5 mM upon chemotactic peptide-stimulation (42). The local concentrations of PtdIns(3,4,5)P₃ seem to be much lower, 5 μ M in resting cells and 200 μ M after stimulation (42). These estimates suggest that in order to specifically recognize PtdIns(3,4,5)P₃ inside cells, affinity for this ligand should be considerably higher than for PtdIns(4,5)P₂.

Abundance of different inositol phosphates also vary (1–100 μ M) and is influenced further by agonist stimulation and differences in half-life. For example, type 1-inositol 5-phosphatase acting on Ins(1,4,5)P₃/Ins(1,3,4,5)P₄ is so active that their half-lives are within 10 sec, whereas those of Ins(1,3,4)P₃/Ins(1,3,4,6)P₄, whose concentration increases from 1–4 μ M to 10–60 μ M upon cell-stimulation, is about 10 min (43). Nonetheless, the PH domains of pleckstrin, RAC-protein kinase and diacylglycerol kinase showed preference and high affinity for Ins(1,3,4,5,6)P₅, and this inositol phosphate [as well as Ins(1,2,3,4,5,6)P₆] is assumed to be the most abundant in cells (estimated to be 15–100 μ M, see Ref. 43 for a review). The function of this binding could be to inhibit interactions of the PH domains with phosphoinositides, affecting its cellular localization, or to influence protein function directly by changing activity of the protein.

A role of inositol phosphates in determining subcellular localization by competing with binding to membrane phosphoinositides has been previously discussed for the PLC- δ_1 -PH domain (5, 44). Based on estimates of total inner-leaflet concentrations of PtdIns(4,5)P₂, it is possible that concentrations of free PtdIns(4,5)P₂, available for interactions with proteins, are also significantly higher than that of Ins(1,4,5)P₃ or other inositol phosphates. In this case, ability to compete could result from higher affinity for inositol phosphates.

There is an example to show a different role of the PH domain, which is observed with p130 (H. Takeuchi et al., manuscript in preparation). The changes in intracellular free Ca²⁺ concentration in COS-1 cells that are stably

overexpressing p130 was diminished in response to bradykinin and epidermal growth factor stimulation, and therefore the dose-response curve shifted to the right, compared to the control cells. On the other hand, the production of Ins(1,4,5)P₃ in response to cellular stimulation was not affected by overexpression of p130. These results indicate that the p130-PH domain binds to cellular Ins(1,4,5)P₃, not to membrane PtdIns(4,5)P₂, thus reducing Ins(1,4,5)P₃ available to the receptor molecules. Therefore, p130-PH domain might function as a negative regulator for Ca²⁺ signaling pathways in cells.

6. PH domain is involved in multidomain binding

There are some cases that the recruitment of proteins containing the PH domain to the plasma membrane through the binding to PtdIns(4,5)P₂ does not appear to be sufficient for the proteins to be targeted to appropriate cellular components or to interact with other components of the signal transduction pathway. Such an example was seen with β ARK. The phosphorylation of β_2 -adrenergic receptor catalyzed by β ARK was only observed when both PtdIns(4,5)P₂ as a ligand for the N-terminal of PH domain and G $\beta\gamma$, which binds to the region encompassing the C-terminal α -helix of PH domain (12), were incorporated into liposomes, together with the receptor and β ARK (45). Neither of each ligand could be omitted, indicating that the coordinated binding of two PH domain ligands appears to be essential for proper targeting to the plasma membrane and functioning. The case observed with the Sos-PH domain might be one of such examples (46); the isolated Sos-PH domain capable of binding PtdIns(4,5)P₂, when microinjected into living cells, localized at the plasma membrane in a serum-dependent manner as expected; however, the mutant PH domain, which fails to bind PtdIns(4,5)P₂, also behaved similarly to the wild-type. The results might indicate that the binding of the PH domain to PtdIns(4,5)P₂ is supplementary to other mechanisms for recruiting the protein to the plasma membrane, such as SH2/SH3 domains of Grb2 interacting with Sos. This might also be the case for PLC- δ_1 because the C2 domain present in the C-terminal of the enzyme has been proposed to bind to phospholipids in a Ca²⁺-dependent manner. Therefore, the coordinate binding of the enzyme to the plasma membrane through the PH domain/PtdIns(4,5)P₂ and C2 domain/phospholipids might be required for processive catalysis (47), although the former interaction alone is likely to be enough for the enzyme to locate at the membrane (39, 40).

7. Perspective

The role of PH domains appears to be recruitment of proteins bearing pH domains to the plasma membrane

through the binding to phosphoinositides. However, soluble inositol phosphates are sometimes more likely ligands for PH domains, the binding of which would prevent the proteins from being recruited to the plasma membrane as well as having proper functions by themselves. Furthermore, we still do not know how much the PH domain is responsible for recruiting by multidomain systems. Moreover, protein kinase C is also reported to bind to PH domains at the site of the N-terminal of PH domains, which might interfere with the binding to inositol phosphates/phosphoinositides (16, 17). In these contexts, further studies are apparently required conclusively establish the physiologically-relevant role of PH domains in cell signaling.

Acknowledgments

Our investigations were financially supported by Grants-in-Aid for Scientific Research from the Ministry of Education, Science, Sports and Culture of Japan (to MH, TK and HY), and by Ono Medical Research Foundation (to MH), The Ryoichi Naito Foundation for Medical Research (to MH) and Fukuoka Cancer Society (to MH).

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