Minireview

# Specificity in pleckstrin homology (PH) domain membrane targeting: a role for a phosphoinositide–protein co-operative mechanism

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**Abstract Pleckstrin homology (PH) domains are protein modules found in proteins involved in many cellular processes. The majority of PH domain-containing proteins require membrane association for their function. It has been shown that most PH domains interact directly with the cell membrane by binding to phosphoinositides with a broad range of specificity and affinity. While a highly specific binding of the PH domain to a phosphoinositide can be necessary and sufficient for the correct recruitment of the host protein to the membrane, a weaker and less specific interaction may be necessary but not sufficient, thus probably requiring alternative, co-operative mechanisms. © 2001 Published by Elsevier Science B.V. on behalf of the Federation of European Biochemical Societies.**

*Key words:* Pleckstrin homology domain; Phosphoinositide; Membrane targeting; Signal transduction; Phosphoinositide 3-kinase

## 1. Introduction

The pleckstrin homology (PH) domain is a structural protein module of approximately 100 amino acid residues that was first identified in 1993, occurring twice in pleckstrin, the major protein kinase C substrate in platelets [1,2]. It is found in a large variety of proteins involved in cellular signaling, cytoskeletal organisation, regulation of intracellular membrane transport and modification of membrane phospholipids [3,4]. Several PH domain structures have been solved by nuclear magnetic resonance and X-ray crystallography, showing that, despite their poorly conserved primary structures, they retain a highly conserved three-dimensional organisation (Fig. 1). The core structure is a β-sandwich of two nearly orthogonal β-sheets consisting of three and four strands, respectively. The β-sheets are closely packed, in particular at the so-called close corners, one of which is spanned by strand β1 while the other is completed by a loop connecting strands β4 and β5. There are six loops connecting the β-strands. Three of these (β1/β2, β3/β4 and β6/β7) have been termed 'variable loops', showing hypervariable sequences in early alignments of PH domains. The opposite edge of the structure is capped by the amphipathic C-terminal α-helix. These two latter regions are termed 'splayed corners' and are responsible for the polarisation of the PH domain; one side of the domain, including the variable loops, is a positively charged surface, made of lysines, arginines and histidines, while the opposite face, including the α-helix, is enriched in acidic residues. The majority of PH domain-containing proteins appear to have a functional requirement to be membrane-associated [5] and several studies indicate that PH domains function as membrane adapters or tethers, linking their host proteins to the membrane surface. The identity of the ligands responsible for membrane binding is often controversial.

## 2. PH domain as a protein binding domain

When it was identified, the PH domain was thought to be a protein binding domain. Many different protein ligands were suggested although a general protein target for the PH domain, as shown for the SH2 and SH3 domains, was not identified. The first candidates were the βγ subunits of heterotrimeric G proteins that were shown to be the targets of the PH domains of β-adrenergic receptor kinase (β-ARK) [6] and Bruton's tyrosine kinase (Btk) [7]. In particular, the C-terminal region of the β-ARK PH domain was shown to interact with a region of Gβ, built up from seven repeats of 40 amino acids containing the dipeptide Trp-Asp and known as WD40 repeat [8,9]. This result, together with the observation that the β-ARK PH domain and those of the src-related tyrosine kinase TecIIa and the GTPase dynamin could bind another WD40-containing protein, the Lis1 gene product [10], gave rise to the concept that binding to WD40 motifs was a general feature of PH domains. Further evidence for a protein–protein interaction mediated by the PH domain has been published more recently. By using a yeast two-hybrid system, it has been shown, for instance, that the PH domains of insulin receptor substrate (IRS) 1 and 2 can bind to proteins containing acidic amino acid residues, such as nucleolin [11]. This interaction is likely to be very specific since some other PH

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*Abbreviations:* PH, pleckstrin homology; β-ARK, β-adrenergic receptor kinase; Btk, Bruton's tyrosine kinase; IRS, insulin receptor substrate; PHIP, PH-interacting protein; PtdIns-4,5-$P_2$, phosphatidylinositol 4,5-bisphosphate; PI 3-K, phosphoinositide 3-kinase; PLC, phospholipase C; Grp1, general receptor for 3-phosphoinositides; PtdIns-3,4,5-$P_3$, phosphatidylinositol 3,4,5-trisphosphate; PTB, phosphotyrosine binding; DAG K-δ, diacylglycerol kinase-δ; Gab1, Grb2-associated protein 1; EGF, endothelial growth factor; MBD, Met binding domain

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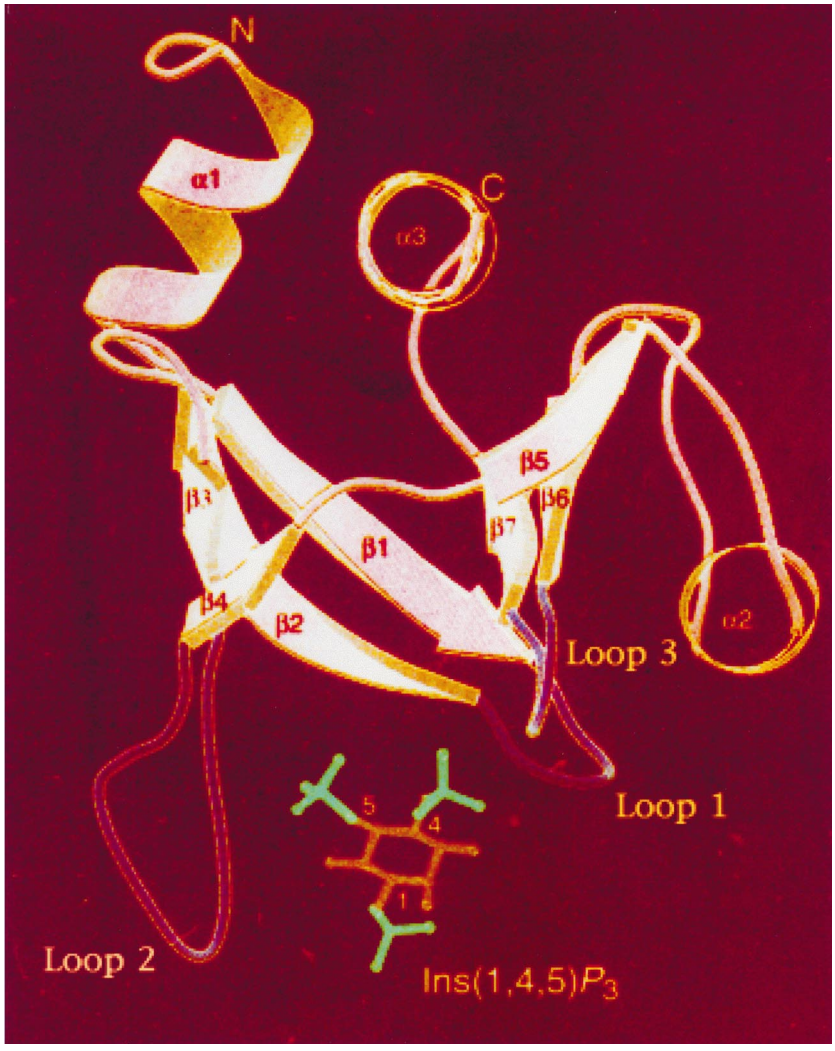


Fig. 1. Ribbon diagram of the phospholipase Cδ PH domain in complex with inositol 3,4,5-trisphosphate.

domains tested do not bind nucleolin; in particular, the PH domain of IRS-1 seems to be more selective since other proteins containing acidic motifs bind to IRS-2 but not IRS-1. These results support the hypothesis that the PH domain in IRS proteins may target the host protein to membranes by binding to acidic peptide motifs or other components in membranes. Further yeast two-hybrid screening has revealed the existence of an interaction between the PH domain of IRS-1 and a novel protein, termed PHIP (PH-interacting protein) [12]. This protein selectively binds to the PH domain of IRS-1 in vitro, associates with IRS-1 in vivo, and may function to link IRS-1 to the insulin receptor. This interaction requires an intact PH fold since mutants of the PH domain that disrupt the PH fold do not bind PHIP.

Several PH domains, as glutathione *S*-transferase fusion proteins or as isolated polypeptides, have been shown to bind to filamentous actin and induce an actin bundle formation. In particular, a short stretch of basic amino acids in the first β-sheet of the Btk PH domain is involved in this interaction although actin binding does not seem to be defined solely by these basic residues [13].

Very recently, an interaction has been described between the PH domain of Etk (a member of the Btk family of tyrosine kinases) and the FERM domain of FAK, which in turn regulates the activation of Etk by extracellular matrix proteins [14]. These data suggest a role for PH domain interactions in integrin signaling.

Despite the above evidence, a unifying physiological role for a protein–protein interaction mediated by the PH domain has not been identified and in the majority of instances such an interaction alone seems unlikely to drive an efficient recruitment of the host protein to the membrane. Taken together, these observations lead us to the conclusion that, in several cases, PH domain binding to a protein may not be sufficient to target the host protein to the plasma membrane but it may be important to stabilise interactions mediated by other components (see below).

## 3. PH domain as a phospholipid binding domain

Most PH domains bind to phosphoinositides or inositol phosphates. The first evidence was provided by Harlan et al. [15] who demonstrated that the PH domains from pleckstrin and several other proteins could bind to lipid vesicles containing the phosphoinositide phosphatidylinositol 4,5-bisphosphate (PtdIns-4,5-$P_2$). Polyphosphoinositides represent diverse membrane targeting sites for PH domains because they include lipids that are produced in response to activation of

Table 1
PH domain classification

| | PH domain | Phosphoinositides | References |
|---|---|---|---|
| Group 1 | Btk | PtdIns-3,4,5-$P_3$ ≫ PIs | [28] |
| | PLC-δ1 | PtdIns-4,5-$P_2$ ≫ PIs | [20,23,24] |
| | Grp1 | PtdIns-3,4,5-$P_3$ ≫ PIs | [20,30] |
| Group 2 | PLC-β1 | PtdIns-3-P > PtdIns-3,4,5-$P_3$, PtdIns-4,5-$P_2$ | [36] |
| | Dbl | PtdIns-4,5-$P_2$, PtdIns-3,4,5-$P_3$ | [39] |
| | Gab1 | PtdIns-3,4,5-$P_3$ > PtdIns-4,5-$P_2$, PtdIns-3,4-$P_2$ | [41] |
| | IRS-1 | PtdIns-3,4,5-$P_3$ > PtdIns-4,5-$P_2$, PtdIns-3,4-$P_2$ | [20] |
| | PLC-γ1 | PtdIns-3,4,5-$P_3$ > PtdIns-3-P, PtdIns-4,5-$P_2$ | [19] |
| Group 3 | Pleckstrin | non-specific | [17,36,49] |
| | Dynamin | non-specific | [21,24,25,36] |
| | DAG K-δ | non-specific | [17,49] |

This classification is based on phosphoinositide binding affinity and selectivity.

cell surface receptors. Examples include the products of the phosphoinositide 3-kinase (PI 3-K), and lipids that are present in cells constitutively and in high abundance, such as the PtdIns-4,5-$P_2$ [16]. When membrane localisation is mediated by PtdIns-4,5-$P_2$, there is a less stringent requirement for high affinity and specificity, since this phosphoinositide is much more abundant than PI 3-K products, even in stimulated cells. Indeed, PH domains bind phosphoinositides with a broad range of specificity and affinity; a clear stereospecificity and high binding affinity has been demonstrated only in a few cases, with the majority of PH domains binding phosphoinositides with low affinity and specificity [17].

## 4. Classification

Several methods have been developed to analyse phospholipid binding specificity of the PH domains [17–20] and different classifications have been proposed [17,21,22]. In the following, we propose a classification based on a comparative analysis of these studies (Table 1). In the first group, we include PH domains that bind with high affinity to a specific phosphoinositide, such as that of phospholipase C (PLC)-δ1 which binds PtdIns-4,5-$P_2$, those of Btk and general receptor for 3-phosphoinositides (Grp1), which bind phosphatidylinositol-3,4,5-trisphosphate (PtdIns-3,4,5-$P_3$). For these the interaction is sufficient to target the host protein to the plasma membrane. The second group includes PH domains that show a low specificity and/or affinity, such as those of the Grb-2-associated protein 1 (Gab1), Dbl, IRS-1, PLC-γ and PLC-β and, more important, that are unlikely to be sufficient to drive a translocation of the host protein to the plasma membrane. Finally, the third group includes PH domains, such as those of pleckstrin, diacylglycerol kinase-δ (DAG K-δ) and dynamin, that bind non-specifically to phosphoinositides.

## 5. Strong interactions between PH domains and phosphoinositides (group 1)

The molecular basis for strong binding of the members of group 1 to a specific phosphoinositide, as well as the physiological function of such an interaction, has been clarified in several cases.

The first PH domain showing a specific phosphoinositide ligand was identified at the N-terminus of PLC-δ1. This PH domain binds strongly and specifically to both PtdIns-4,5-$P_2$ and its soluble headgroup inositol-1,4,5-trisphosphate (Ins-1,4,5-$P_3$) [23,24]. The X-ray crystal structure of the isolated PH domain, complexed with Ins-1,4,5-$P_3$, has shown that binding occurs to the surface of the PH domain defined by the variable loops 1–3 and is stabilised by direct hydrogen bonding between the 4- and 5-phosphates and the side chains of amino acids in these loops [25]. This strong interaction is sufficient to target the host protein to the surface of the plasma membrane in mammalian cells where its substrate, PtdIns-4,5-$P_2$, is located, allowing processive hydrolysis of the substrate molecules.

Analogously, binding of the PH domain of Btk to PtdIns-3,4,5-$P_3$ re-localises this protein from the cytosol to the plasma membrane [26] and this targeting facilitates the phosphorylation and activation of Btk by Src family tyrosine kinases [27]. Btk specifically binds the PtdIns-3,4,5-$P_3$ headgroup; binding to the PtdIns-4,5-$P_2$ headgroup occurs with affinity 100-fold less [28]. This is consistent with a PI 3-K-dependent mechanism for localisation to the plasma membrane. A high-resolution crystallographic structure of the R28C mutant of the Btk PH domain has revealed that the putative phosphoinositide binding site is formed by residues of loop 1 and β-strands 1, 2, 3 and 4 [29].

The PH domain of the Arf exchange factor Grp1 shows a high binding affinity for PtdIns-3,4,5-$P_3$ [20]. Indeed this protein was identified on the basis of its binding to PtdIns-3,4,5-$P_3$ in a cDNA expression library screen [30] and the plasma membrane localisation of its PH domain is strongly enhanced by introduction of a constitutively active PI 3-K catalytic subunit into mammalian cells [17]. Thus this specific, high-affinity binding is likely to be sufficient for signal-dependent membrane recruitment mediated by PI 3-K products. This is confirmed by experiments in intact cells showing that the Grp1–PH domain is both necessary and sufficient to target the full-length protein to the plasma membrane after insulin stimulation [31].

Recently, the crystal structures of Btk PH domain in complex with D-*myo*-inositol-1,3,4,5-tetrakisphosphate (D-*myo*-Ins-1,3,4,5-$P_4$) [32] and of Grp1 PH domain bound to Ins-1,3,4,5-$P_4$ [33,34] have been determined, revealing the details of their specificity and high affinity. In particular, the structural studies of Btk PH domain have shown that the 3-, 4- and 5-phosphates may interact with the side chains of two acidic residues on one side and with the backbone of the β1/β2 loop on the other side. This high number of contacts is sufficient to guarantee a stable interaction and accounts for the high affinity and specificity of the Btk PH domain for Ins-1,3,4,5-$P_4$ over Ins-1,4,5-$P_3$ [32]. Crystal analyses of the Grp1 PH do-

main in an unligated form or in complex with Ins-1,3,4,5-$P_4$ have revealed the presence of a 20-residue insertion within the β6/β7 loop. This insertion, which adopts a twisted β-hairpin structure and extends the β-barrel from seven to nine strands, has been proposed to account for the ability of the Grp1 PH domain to selectively bind PtdIns-3,4,5-$P_3$ but not PtdIns-3,4-$P_2$ or PtdIns-4,5-$P_2$ with high affinity [33]. The high specificity of Grp1 PH domain has been further confirmed by an analysis of its crystal structure, in complex with Ins-1,3,4,5-$P_4$, showing that this PH domain is able to make two unique hydrogen bonds to the 5-phosphate [34]. All these PH domains share a general mechanism for recruitment of proteins to the plasma membrane involving either a constitutive or a signal-dependent presence of a specific lipid molecule in the plasma membrane to which the proteins can bind via their PH domains.

## 6. Weak and promiscuous interactions between PH domains and phosphoinositides (group 2)

Only a limited subset of PH domains can be included in the first group, since the majority of PH domains bind phosphoinositides with a low specificity and/or a low affinity. Low specificity leads essentially to a 'promiscuous' PH domain that is a PH domain that binds several phosphoinositides with similar affinities.

Low affinity essentially means 'weak' PH domains, those that have an inherent affinity for the membrane surface, which is insufficient to drive the membrane localisation of the protein. Many different mechanisms have been suggested that would overcome this. We focused our attention on two hypotheses. The first hypothesis suggests that a weak PH–phosphoinositide interaction could be stabilised by a co-operative binding with a protein within the same PH domain (Fig. 2A). A second possibility is that PH domains may act simultaneously with other domains of the protein to achieve a stable recruitment of the host protein to the plasma membrane (Fig. 2B).

### 6.1. Lipid–protein co-operative mechanism within the same PH domain

This mechanism has been first proposed for the PH domain of β-ARK. In fact, the PH domain of β-ARK has been shown to interact with both Gβγ and PtdIns-4,5-$P_2$ at the carboxy- and amino-termini, respectively [6,15]. The simultaneous interaction of these two PH domain ligands is required for effective membrane association and activation of β-ARK. Neither PH domain binding ligand alone is sufficient to affect this functional activation of the enzyme [35]. The evidence that Gβγ and PtdIns-4,5-$P_2$ bind to different parts of the PH domain suggests a co-operative, and not a competitive, mechanism (Fig. 2A).

We recently suggested a similar putative double interaction for the PH domain of PLC-β1 [36]. The PH domain of this protein binds to phosphatidylinositol 3-phosphate (PtdIns-3-P), while interacting more weakly with PtdIns-4,5-$P_2$ and PtdIns-3,4,5-$P_3$. Accordingly, it undergoes a rapid, but transient, migration to the plasma membrane upon stimulation of cells with serum or lysophosphatidic acid and this is inhibited by pretreatment with PI 3-K inhibitors. These results indicate that the PH domain can target the PLC-β1 to the plasma membrane by binding to the PtdIns-3-P. However, previous studies have already indicated that this PH domain may also bind to the Gβγ subunits, thus providing a further means to

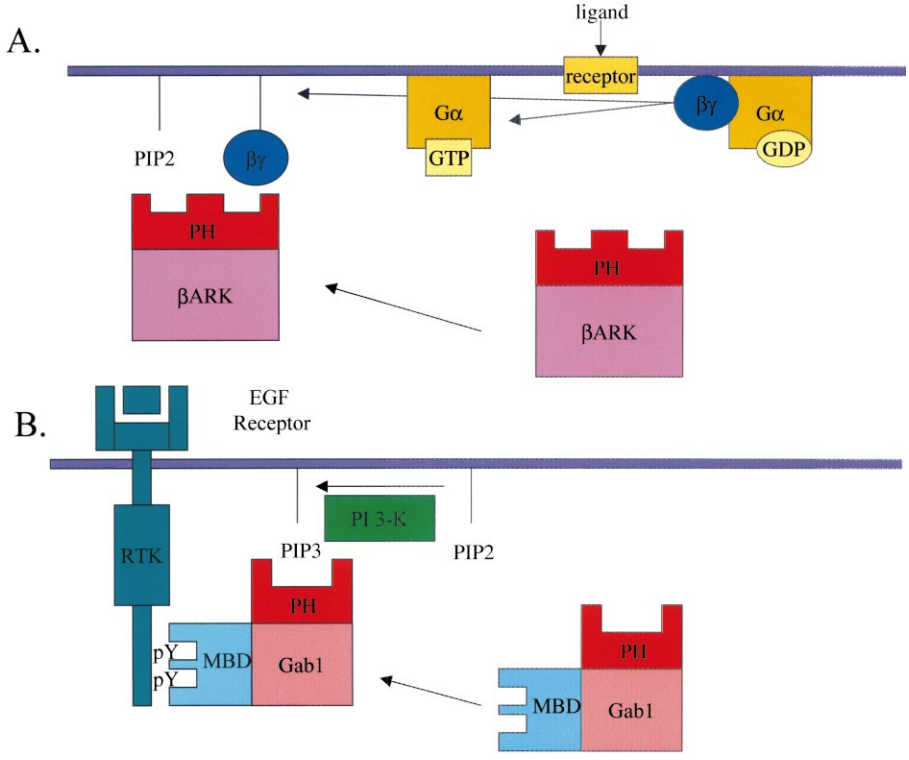


Fig. 2. Model outlining the role of PH domain interactions in membrane recruitment of PH domain-containing protein. A: Lipid–protein co-operative mechanism within the β-ARK PH domain. B: Membrane recruitment of Gab1 through the concerted action of PH and MBD domains.

anchor the protein to the plasma membrane [37]. Consistent with this observation, it has been shown that the isolated PH domain of PLC-β1, fused to the green fluorescent protein, can be recruited to the plasma membrane also in unstimulated cells if microinjected with the Gβγ subunits [36]. Taken together, these data seem to indicate that localisation and activation of PLC-β1, mediated by PH domain, is regulated by a co-operative mechanism involving PtdIns-3-P as well as Gβγ subunits.

A putative double interaction is likely to occur also in the case of the Dbl PH domain [38]. This PH domain binds to both PtdIns-4,5-$P_2$ and PtdIns-3,4,5-$P_3$, and it has been shown that three positively charged amino acids located in the β1/β2 loop mediate this interaction. It is noteworthy that the wild-type PH domain localises both to the plasma membrane and to actin stress fibres while Dbl mutants, unable to bind the phosphoinositides, fail to localise to the plasma membrane but still co-localise with actin. This is consistent with the observation that this PH domain is necessary and sufficient for the association of Dbl with the Triton X-100-insoluble cytoskeletal components [39]. These findings suggest that PH domain localisation to plasma membrane and actin stress fibres may be mediated by different residues in its PH domain [38].

### 6.2. *'Tandem domain' co-operative binding*

A 'tandem model' has been proposed as potential mechanism that stabilises a weak interaction of PH domain through a co-operation with other domains of the host protein. This model has been proposed for the components of the so-called Grp1 family proteins, ARNO and cytohesin-1 [31]. A similar model has been proposed for the endothelial growth factor (EGF)-induced recruitment of the docking protein Gab1. The Gab1 PH domain has been found to bind PtdIns-3,4,5-$P_3$ and to a lesser extent PtdIns-3-P, PtdIns-3,4-$P_2$ and PtdIns-4,5-$P_2$ [40]. Significantly, the β1/β2 loop of this domain shows strong sequence identity with PH domains of other proteins known to bind PI 3-K products. Considerable evidence supports a critical role for the PH domain and a requirement for PI 3-K in the translocation of Gab1 to the plasma membrane [18,40,41]. The PH domain is likely to act co-operatively with another domain, the so-called Met binding domain (MBD), whose role on binding to the receptor has been previously emphasised [42]. Since the binding of MBD to the EGF receptor is relatively weak, concerted action of both domains may be required to achieve stable membrane recruitment of Gab1 of sufficient duration to enhance its tyrosine phosphorylation and association with downstream signaling molecules (Fig. 2B) [40].

A similar mechanism has been proposed for PLC-γ. It is clear that, upon growth factor stimulation, the SH2 domains of PLC-γ bind to tyrosine-autophosphorylated sites in growth factor receptors, leading to tyrosine phosphorylation and stimulation of PLC-γ activity [43]. In this model, receptor tyrosine kinases function as docking sites, providing one mechanism for targeting the enzyme to the cell surface. Other studies have shown that the PLC-γ PH domain binds to PtdIns-3,4,5-$P_3$ and is targeted to the plasma membrane in a PI 3-K-dependent manner [19]. These data indicate that PLC-γ targeting to the plasma membrane is the result of a synergistic action of the SH2 domains (mediating the association to the receptor) with the PH domain; it could stabilise the interaction by binding to the membrane via PtdIns-3,4,5-$P_3$. In particular, a model was proposed in which plasma-derived growth factor-induced generation of PtdIns-3,4,5-$P_3$ leads to translocation of PLC-γ to the plasma membrane, a step that increases the local concentration of the enzyme in the vicinity of its substrate, PtdIns-4,5-$P_2$, probably leading to more efficient substrate hydrolysis [19,43]. Recent work demonstrates that all determinants required for the association of PLC-γ to the EGF receptor are found within a region unique for the PLC-γ family. Furthermore, the association of PLC-γ to the EGF receptor is unaffected by mutations in the PH domain or inhibition of PI 3-K, thus indicating that the PH domain-mediated translocation does not contribute to the stability of the complex [44]. In addition, PH domain-mediated translocation occurs later, compared to receptor translocation, thus excluding the possibility that it could precede the interaction with the receptor. This finding suggests that the PH domain may not play a role in the initial translocation to the EGF receptor indicating that it could be important for some other steps involved in the stimulation of PLC-γ [44].

Finally, both mechanisms have been proposed for the PH domain of IRS-1. In addition to the specific protein binding discussed above [11,12], the PH domain of IRS-1 has been reported to bind phosphoinositides and the latter property is required for IRS-1 function [20,45,46]. In particular, PtdIns-3,4,5-$P_3$ and PtdIns-4,5-$P_2$ bind with almost the same affinity [45] although a preferential binding to PtdIns-3,4,5-$P_3$ is likely to occur [20]. PtdIns-3,4-$P_2$ and PtdIns-4-P show four- to eight-fold lower affinity [20,45]. It could be hypothesised that a protein and a phosphoinositide can bind to different residues within the same PH domain, thus acting co-operatively to localise the protein and having a specific role in the membrane compartmentalisation of IRS-1. Alternatively, a co-operation with another domain of the protein has been proposed [45]. In IRS-1, PH domain is flanked immediately downstream by a phosphotyrosine binding (PTB) domain. The crystal structure of the region of IRS-1 containing both these domains has been determined, showing that they both adopt the conserved PH fold and they are closely associated such that the front sheet of the PH domain is against the back of the PTB domain. However, despite their similar overall structure and close interaction, mediated by a series of hydrogen bonds, their binding specificities remain quite distinct. Direct assays demonstrate that phosphatidylinositides bind the PH but not the PTB domain that in its turn binds to phosphotyrosines in the motif found near the transmembrane region of the insulin receptor. It is noteworthy that binding to either domain does not alter the binding properties of the other, indicating that the two binding domains can act co-operatively to localise the protein at the membrane [45]. Consistent with this hypothesis, many reports indicate that both the PTB domain and the PH domain are required for efficient tyrosine phosphorylation of IRS-1 in response to insulin stimulation [47,48].

## 7. Non-specific interactions between PH domains and phosphoinositides (group 3)

Several PH domains appear quite non-specific in their phospholipid binding. Studies of binding to small unilamellar vesicles containing different combinations of phospholipids have shown that the N-terminal PH domain of pleckstrin-1

can bind phosphatidylserine as well as phosphoinositides [17]. A comparison of phosphoinositide binding specificity using a dot-blot screen has shown that also the DAG K-δ PH domain gives a significant signal with phosphatidylserine [17]. These data have suggested that these PH domains simply recognise a negatively charged surface and not any particular characteristic of the inositol phosphate headgroup. Consistent with these results, studies of inositol phosphate binding to the pleckstrin-1 and DAG K-δ PH domains have demonstrated that there is no stereospecificity and that affinity correlates most strongly with the number of phosphate groups [49]. The non-specific interaction of the pleckstrin PH domain has also been confirmed by [$^3$H]inositol binding analysed by anion exchange HPLC [36].

Although it has been reported that the isolated PH domain of dynamin-1 specifically interacts with liposomes containing PtdIns-4,5-$P_2$ [28], other studies have been unable to detect significant binding of this PH domain to phosphoinositides [21,24,25,36]. It has been demonstrated that the PH domains from two mammalian dynamin isoforms require their oligomerisation for high-affinity phosphoinositide binding [50]. Since the capability of dynamin to form tetramers and higher-order assemblies is critical for its physiological function, it has been suggested that the PH domain-mediated binding of dynamin to phosphoinositide-containing membranes requires its self-assembly. This hypothesis raises the possibility that alternative mechanisms to those presented in this review may actually exist to overcome the problem of a low affinity and/or specificity of the PH domains.

## 8. Concluding remarks

Although the functional role has been elucidated for a few PH domains, for the majority it remains elusive. The potential synergy of two different ligands, a lipid and a protein, may shed light on the function of PH domain in several proteins that contain this structural domain. This raises the possibility that two different steps may regulate the signaling pathways involving a PH domain protein. This gives a higher specificity to both membrane binding and intracellular targeting mediated by the PH domain. It is an important challenge to identify specific methods that will enable lipid–protein synergistic binding to be studied.

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