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Pleckstrin Homology (PH) Domains in Signal Transduction

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Abstract A diverse array of molecules involved in signal transduction have recently been recognised as containing a new homology domain, the pleckstrin homology (PH) domain. These include kinases (both serine/threonine and tyrosine specific), all currently known mammalian phospholipase Cs, GTPases, GTPase-activating proteins, GTPase-exchange factors, "adapter" proteins, cytoskeletal proteins, and kinase substrates. This has sparked a new surge of research into elucidating its structure and function. The NMR solution structure of the PH domains of β-spectrin and pleckstrin (the N-terminal domain) both display a core consisting of seven anti-parallel β-sheet strands. The carboxy terminus is folded into a long α-helix. The molecule is electrostatically polarised and contains a pocket which may be involved in the binding of a ligand. The PH domains overall topological relatedness to the retinoid binding protein family of molecules would suggest a lipid ligand could bind to this pocket. The prime function of the PH domain still remains to be elucidated. However, it has been shown to be important in signal transduction, most probably by mediating protein-protein interactions. An extended PH domain of the β-adrenergic receptor kinase (βARK), as well as that of several other molecules, can bind to βγ subunits of the heterotrimeric G-proteins. The possibility that the PH domain, which is found in so many signalling molecules, being generally involved in βγ binding is provocative of implicating these proteins in G-protein signal transduction. While the PH domain and the βγ binding site appear to be concomitant in βARK, detailed analysis indicates that the PH domain is not generally a βγ binding domain. Thus, the race is on to find the ligands of each PH domain and determine a common nature to their interaction.

Key words: βARK, kinase, phospholipase, G-proteins, pleckstrin homology

The recent recognition of a new homology domain [Haslam et al., 1993; Mayer et al., 1993; Musacchio et al., 1993; Shaw, 1993] present in a diverse array of molecules involved in signal transduction has sparked a new surge of research into elucidating its structure and function. This domain (~100 amino acids long) is called the "pleckstrin homology" (PH) domain [Haslam et al., 1993] (see Fig. 1). It was originally identified as an internal repeat, present at the amino and carboxy termini, of pleckstrin, a 47 kDa protein which is the major protein kinase-C substrate in activated platelets [Tyers et al., 1988]. This domain has been proposed to be analogous to the previously characterised SH2 and SH3 domains, in that it mediates protein-protein interactions.

The family of proteins containing PH domains consists of over 30 distinct members, not counting species homologues and highly related isoforms [Musacchio et al., 1993]. Many of these molecules can be clustered into groups of functionally related proteins. A significant number of proteins that participate in the regulation of small GTP binding proteins contain a PH domain. These include ras-GAP, ras-GRF, Bcr, SOS, Bem3, Dbl, Vav, and cdc24. Interestingly, ras-GRF contains two intact PH domains. It is also present in a number of protein kinases, both serine/threonine specific (RAC α/β, βARK1/2, and Nrk A/B) and tyrosine specific (Btk, Tec-A, and tlk). All of the known mammalian phospholipase Cs (PLC-β, -δ, and -γ) contain a PH domain at their amino terminus [Parker et al., 1994]. PLC-γ also contains an additional split PH domain, in which subdomains I–III are separated form subdomains IV–VI by two SH2 domains and one SH3 domain. Two other signalling molecules contain a PH domain, the SH3 binding protein 3BP2 and the growth factor receptor binding protein GRB7. IRS-1 is a 131 kDa protein which becomes rapidly and multiply tyrosine phosphory-

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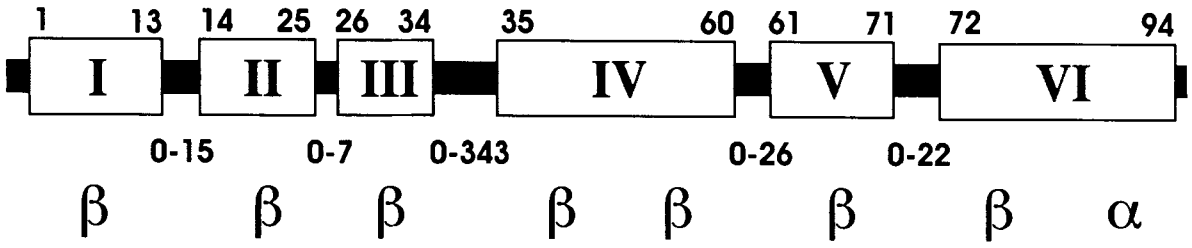
A



B

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HUMORFA5_1 LVMEGHLFKRASNAFKTWSRRWFTIQS.NQLVYQKKY........KDPVTVVVDDLRLCTVKLCPDSER..
HUMORFV_1  IVMEGYLFKRASNAFKTWNRRWFSIQN.NQLVYQKKF........KDNPTVVVEDLRLCTVKHCEDIER..
HUMORF3_1  PIKMGWLKKQRSIV.KNWQQRYFVLRA.QQLYYYKDEEDT.....KPQGCMYLPGCTIKEIATNPEEAG..
SWH1       PTYKGYLKKWTNFA.QGYKLRWFILSSDGKLSYYIDQ.........ADTKNACRGSLNMSSCSLHLDSSE..
R01H10     ATVCYKSKTYSAK..ILLHGNVLCVEESGGAVTSA..........CVPLIFLLERVVLKKLEENSDTA...
1bc        KNAAGRLKEYQA...VLLTDILVFLQEKDQKYIFASLD.......QKSTVISLKKLIVREVAHEEKG....
ABR         LLCAKLKKTSAGKHQQYDCKWYIPLA.DLVFPSPEESEAS-13-MKMKISALKSEIQKEKANKGQS-22-

PH-con     VIKEGYLKKKGsg..ksWKRRYFVLRD.aGLSYYKDSdey.....RPKGLIDLENIQIVEVEDgkkg....
                                       o

HUMORFA5_1 RFCFEVVSTSK....SCLLQADSERLLQLWVSAVQSSI
HUMORFV_1  RFCFEVVSPTK....SCMLQADSEKLRQAWIKAVQTSI
HUMORF3_1  KFVFEIIPASWD-7-SYVLMASSQAEMEEWVKFLRRVA
SWH1       KLKFEIIGGNNG.VIRWHLKGNHPIETNRWVWAIQGAI
R01H10     KFTFLLTFTDPA..ENLEISVESEELREQWMIKLATCS
1bc        LFLISMGMTDPE...MVEVHASSKEERNSWIQIIQDTI
ABR        TIPFRIHNRNGK...SYLFLLSSDYERSEWAIQKLQKK

PH-con     kHCFEIVTKDGS...sLILQAESEEEREEWVAALRRAI
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Fig. 1. **A:** Pleckstrin homology (PH) subdomain structure. The relative size of subdomains I to VI are shown schematically. Above is indicated the number of amino acids in the core consensus of each subdomain. Below is indicated the size of insertions found in various PH domains between the subdomains. Also shown is the secondary structural elements of each subdomain. β, β-sheet; α, α-helix. **B:** Alignment of novel PH domains with the consensus sequence. The consensus sequence is separated into subdomains. The open circle indicates the conserved residue in subdomain II mutated in the Btk gene which gives rise to the XID phenotype. The asterisk indicates the only totally conserved residue, the tryptophan of subdomain VI. The consensus sequence was derived as previously reported [Musacchio et al., 1993]. Gaps are represented by dots. Numbers indicate the size of insertions removed for clarity.

lated by the insulin receptor complex following ligand binding. This molecule has a PH domain at its extreme amino-terminus, away from the phosphorylation sites.

Other molecules not obviously involved in signal transduction also contain PH domains. A human homologue of yeast SEC7, an oxysterol binding protein, dynamin (a GTP binding and microtubule binding protein), *C. elegans* UNC-104 (a kinesin-like molecule), and β-spectrin (a cytoskeletal protein) all have a PH domain [Musacchio et al., 1993].

Several PH containing molecules also possess SH2 and/or SH3 domains, e.g., PLC-γ, Tec, Btk, tlk, GRB7, 3BP2, Vav, and ras-GAP. It is known that SH2 and SH3 domains mediate complex protein-protein interactions involved in signalling cascades transmitted from ligand-bound receptors [Mayer and Baltimore, 1993]. The SH2 domain has been well characterised and binds to phosphorylated tyrosine residues within specific peptide motifs [Marengere et al., 1994]. The SH3 domain is less well characterised but has been shown to bind proline rich motifs [Tu et al.,

1994]. While SH2 and SH3 domains are well conserved with each containing several invariant residues, the PH domain has only one amino acid which is invariant, the tryptophan in subdomain VI. This may indicate a higher diversity of function of the various PH domains compared to that of SH2 and SH3 domains. PH domains are not spatially restricted in the position they occupy in their respective molecules, a feature common with SH2 and SH3 domains. The PH domain also has a long evolutionary history, being found in molecules from yeast through to invertebrates and vertebrates [Musacchio et al., 1993]. However, PH domains have not yet been detected in plant or prokaryote sequences.

Several PH domain containing molecules have been proven to have an oncogenic potential. These include Dbl, Vav, Bcr, and RAC. The serine/threonine protein kinase RAC is the cellular homologue of the *v-akt* oncogene. RAC is one of only five serine/threonine specific kinases demonstrated to have an oncogenic potential, the other four being *raf* [Bonner et al., 1986], *mos* [Leibouitch et al., 1990], *pim*-1 [Zakut-Houri et al., 1987], and *cot* [Miyoshi et al., 1991]. This oncogene contains an intact RAC molecule fused to a partial *gag* sequence. This results in a pronounced change in the subcellular distribution of the RAC protein, presumably by virtue of the myristoylated moiety from the *gag* sequences [Ahmed et al., 1993]. The PH domain present in RAC and those of the other serine/threonine specific kinases (βARK, Nrk) are not highly related and it would appear that these kinases aquired their PH domains idependently of each other.

PH DOMAIN STRUCTURE

The PH domain can be divided into six conserved subdomains that total ~100 amino acids with variable length spaces between these subdomains (see Fig. 1). The most highly conserved residues are either charged or hydrophobic, reminiscent of the SH2 and SH3 domains. The NMR solution structure of the PH domain of β-spectrin [Macias et al., 1994] and that of the amino-terminal PH domain of pleckstrin [Yoon et al., 1994] itself have been solved and their high degree of similarity suggests that all PH domains will also be of a similar structure (Fig. 2).

The secondary structure prediction would suggest that each subdomain consists mainly of β-sheet structure with an α-helix in the last subdomain beginning around the invariant tryptophan [Musacchio et al., 1993]. The solution structure reflects this prediction in that the core

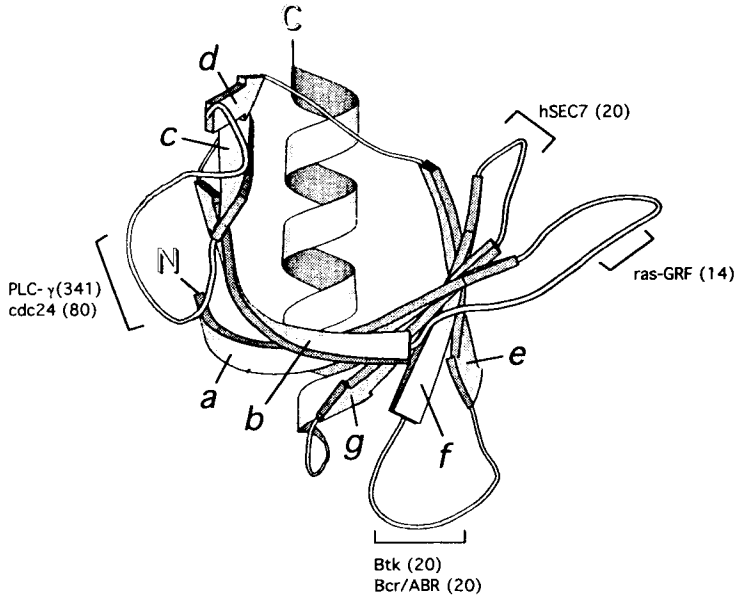

Fig. 2. PH domain tertiary structure. A proposed general PH domain tertiary structure based on the solution structures of the PH domains of β-spectrin [Macias et al., 1994] and pleckstrin [Yoon et al., 1994]. The β-sheet structures (flattened arrows) and α-helical structures (helical ribbons) are shown to depict the core β-barrel potential ligand binding pocket. The C-terminal α-helix containing the invariant tryptophan is placed at the back of the pocket. The size and position of inserts present in the PH domains of PLC-γ, Btk, Bcr, ABR, ras-GRF and hSEC7 are indicated. The seven β-strands are labelled a–e. The N-terminus (ℕ) and C-terminus (ℂ) are indicated. Reproduced from Macias et al. [1994] with permission of *Nature*.

of the domain consists of an anti-parallel β-sheet of seven strands. The carboxy terminus is folded into a long α-helix and is located close to the amino terminus in the tertiary structure. The molecule is electrostatically polarised and contains a pocket which may be involved in the binding of a ligand. This pocket is hydrophobic and contains four conserved lysines near the entrance of the pocket. There is a distant, but significant, relationship to the peptidyl-prolyl-*cis-trans*-isomerase FKBP, in which this pocket is involved in the binding of the macrocyclic compound FK506 [Macias et al., 1994]. The overall topology is also similar to that of the retinoid binding protein family of molecules. These proteins all bind lipophilic molecules and this suggests that the PH domain ligand may be a lipid or lipid containing molecule where the lipid moiety would bind in the hydrophobic β-barrel pocket [Yoon et al., 1994].

The insertions found in the PH domains of hSEC7, Btk, Bcr, ABR, and PLC-γ (internal domain) could all be accommodated in the structure as they are located in external loops, suggesting that these are indeed functional domains.

SOME MORE MEMBERS OF THE PH FAMILY

Some newly identified PH domain-containing molecules are presented in Figure 1B, 3. These were identified by running homology searches with the program FASTA [Pearson and Lipman, 1988] of previously identified PH domains in the data base GP. Three of these, Lbc [Toksoz and Williams, 1994], ARB [Tan et al., 1993], and HUMORF3_1 (Nomura N et al., unpublished) extend the subfamily of small GTP binding protein regulators that contain a PH domain. Lbc is an oncogene which contains an amino-terminal E-F hand (intracellular Ca^{2+} binding site) a central cdc24 (rho-GRF) homology domain, and a carboxy-terminal PH domain. This molecule was identified by its ability to transform NIH-3T3 fibroblasts. ARB is closely related to Bcr (the break point cluster region gene) and has a similar domain structure. These two newly identified PH domain family members, Lbc and ARB, fit into the subfamily of proteins consisting of Vav, Bcr, Dbl, cdc24, SOS, and ras-GRF which all have a PH domain juxtaposed at the carboxy-terminus of a cdc24 (rho-GRF) homology domain. This further suggests an interplay between these two domains. HUMORF3_1 is a randomly sequenced human cDNA which possesses an amino-terminal PH domain followed by a ras-GAP domain. The carboxy-terminal region has no significant homology to any other proteins in the data bases (Swissprot and GP) but contains a high ratio of charged amino acids.

Two other closely related (57% identical) randomly sequenced human cDNAs have a PH domain, HUMORFA5_1 and HUMORFV_1 (Nomura N et al., unpublished). Their domain structure is similar but HUMORFA5_1 has an amino-terminal extension of ~100 amino acids. They also posses a Zn^{2+} finger domain and a stretch of five ankyrin repeats. The function of these two proteins has yet to be elucidated.

The yeast SWH1 protein (Schmalix WA and Bandlow W, unpublished) is highly related to the oxysterol binding protein in its carboxy terminal half, especially to the oxysterol binding domain (37% identity). This molecule may represent the yeast homologue of the OSBP. Both proteins contain a PH domain. SWH1 has additionally five amino-terminal ankyrin repeats and an insertion between the PH domain and the oxysterol binding domains compared to the OSBP itself.

Another PH domain is found in the amino-terminal region of an open reading frame of the *C. elegans* cosmid R01H10 of chromosome III [Wilson et al., 1994]. The carboxy-terminal region has no high homology to any other proteins in the data bases (Swissprot and GP). The general domain structure of the new PH domain containing proteins is presented in Figure 3.

PH DOMAIN FUNCTION

A prima face analysis of the molecules containing PH domains does not strongly indicate a single distinct role but does imply a function in signal transduction, possibly by mediating protein-protein interactions. Moreover, the presence of a PH domain juxtaposed to the cdc24 (rho-GRF) homology domain in eight molecules suggests a functional interplay between these two domains.

The most interesting data on PH domain function comes from studies of the carboxy-terminal region of the β-adrenergic receptor kinase (βARK, see Fig. 3), which were completed before it was recognised as containing a PH domain [Koch et al., 1993; Müller et al., 1993; Simonds et al., 1993]. β-ARK is a serine/threonine specific kinase which, to a degree, is homologous

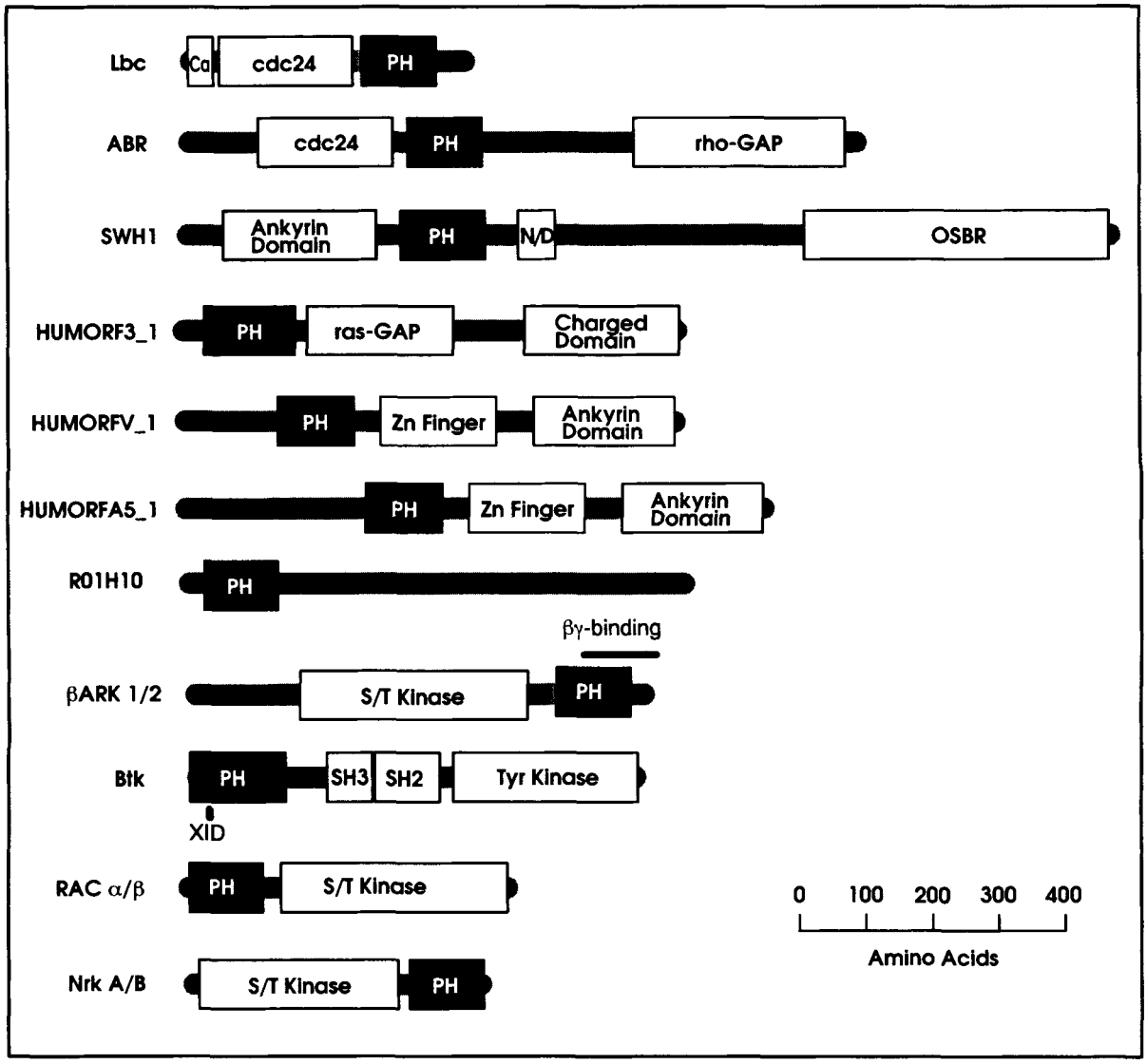

Fig. 3. Domain structure of PH containing proteins. A schematic representation of the domain structures of the novel PH containing proteins Lbc, ABR, SWH1, HUMORF3_1, HUMORFV_1, HUMORFA5_1, and R01H10, as well as those of βARK, RAC, Nrk, and Btk (Tec-A and tlk are schematically identical to Btk) are shown. Unique regions are shown as a grey bar. PH homology domains are shaded black. Other homology domains are indicated as follows: SH2, *src* homology 2 domain; SH3, *src* homology 3 domain; Ca, EF-hand motif for intracellular calcium binding; cdc24, cdc24 homology for nucleotide exchange factor; rho-GAP, homology to GTPase activating proteins of the rho class; Ankyrin domain, region containing five ankyrin-like repeats; N/D, asparagine/aspartic acid rich motif; OSBR, oxysterol binding region; ras-GAP, homology to GTPase activating proteins of the ras class; Charged domain, region high in charged residues; Zn finger, region homologous to Zn fingers; S/T kinase, serine/threonine specific kinase domain; Tyr kinase, tyrosine specific kinase domain. Accession numbers are Lbc, U03634; ABR, U01147; SWH1, X74552; HUMORF3_1, D29642; HUMORFV_1, D26069; HUMORFA5_1, D30758; R01H10, Z31590; βARK, M80776; and Btk, S52906. The scale is indicated. The locations of the βγ binding site of βARK and the XID mutation of Btk are shown. For references, see text.

and analogous to rhodopsine kinase (RK). These two kinases differ, however, at their carboxy-termini, where RK has a four amino acid CAAX farnesyl isoprenylation consensus sequence [Lorenz et al., 1991], while βARK has a 129 amino acid extension which lacks such a farnesylation site. Both kinases are activated upon localisation to the plasma membrane. For RK, membrane localisation is mediated by its farnesylation moiety. In the case of βARK it is the carboxy-terminal region which confers binding to the membrane-bound βγ subunits of the het-

erotrimeric G proteins [Koch et al., 1993]. In this system, the Gα subunit is released from the G protein complex, which is associated with the β-adrenergic receptor (βAR) as a result of agonist activation of the receptor. The βARK then binds to the βγ subunits and phosphorylates and down-regulates the βAR [Simonds et al., 1993]. At first it would appear that the PH domain of βARK is responsible for the βγ binding, and that this could be the function of PH domains in other molecules. Indeed, a 28 amino acid region of the βARK, with the invariant tryptophan of subdomain VI at its amino-terminus, shows binding to βγ subunits [Koch et al., 1993]. However, this peptide contains only the last nine amino acids of the recognised PH domain consensus, and it absolutely requires the additional carboxy-terminal amino acids to confer binding to βγ subunits.

Glutathione-S-transferase fusions of ras-GRF, ras-GAP, Btk, OSBP, RACβ, β-spectrin, IRS-1, and PLC-γ encompassing their PH domains, but also containing additional amino- and carboxyl-terminal amino acids, have been shown to bind to mixed βγ subunits isolated from brain, but with substantially lower affinities than the βARK carboxy-terminus [Touhara et al., 1994]. The PH domain and the βγ binding domain would appear to overlap but are not equivalent, as the full PH domain does not bind βγ subunits. However, full βγ binding is conferred with PH subdomains IV to VI plus an additional 27 amino acids at the carboxy-terminus [Touhara et al., 1994]. This provocatively implicates that PH domain-containing proteins interact with βγ subunits in vivo. It is likely that the PH domain of βARK aids in the structural placement of the carboxy-terminal extension, which is predicted to form a coiled-coil structure [Simonds et al., 1993], in a position where it can favourably interact with βγ subunits. It is intriguing that the PLC-γ PH domain containing fusion protein showed relatively strong βγ binding [Touhara et al., 1994], since although PLC-β, and under some conditions PLC-δ, are activated by βγ subunits, PLC-γ has not been shown to have any such βγ activation [Parker et al., 1994]. This suggests that βγ subunits may affect PLC-γ in an as yet unknown manner.

The possibility that the PH domain, which is found in so many signalling molecules, is involved in βγ binding provokes the hypothesis that these proteins function in G protein coupled signal transduction. Initially it was thought that only the α subunit of G proteins, of which there are over 16 mammalian isoforms, was the principal signalling subunit [Simon et al., 1991]. However, more recent investigations have revealed that the βγ subunits themselves have signalling properties [Crespo et al., 1994; Müller et al., 1993]. The epitope responsible for the association of the Gα subunit with the βγ subunits has been localised to its amino terminal ~40 amino acids, just before the guanine nucleotide binding site [Simon et al., 1991]. This region does not have any striking similarity to the βγ binding region of βARK. However, Gα subunits are myristoylated and/or prenylated, and this is required for its βγ association. These modifications may increase its affinity for βγ subunits by virtue of their membrane localising properties. Further, a myristoylated random peptide was equally effective as a Gα amino-terminal peptide in binding βγ subunits, suggesting that the lipid modification is more important than the amino acid sequence [Kokame et al., 1992].

It would seem worthwhile to speculate on the mechanism of βARK dissociation from βγ subunits. By analogy with the nucleotide dependent dissociation of the Gα subunit from the heterotrimeric Gαβγ complex, one could suppose that some unknown effector molecule could be involved in the βARK dissociation. When the Gα subunit binds GTP there is an allosteric disruption of its amino-terminal coiled-coil domain and consequently a dissociation from the βγ subunits [Conklin and Bourne, 1993]. Together with the region of the βARK responsible for βγ association (encompassing PH subdomains IV to VI plus the additional carboxy-terminal 27 amino acids), the more amino-terminal PH subdomains I to III may prove to be functional in mediating a regulated dissociation from the βγ subunits. With the structure of the PH domain showing a potential binding pocket for a small molecule or lipid moiety, such a hypothesis may not seem so unreasonable.

This still leaves the function of the recognised PH domain consensus region, particularly subdomains I to IV with an unknown function. This may be solved by studying the PH domain of Btk (Bruton's tyrosine kinase, see Fig. 3). Btk [Rawlings et al., 1993] belongs to a subfamily (containing in addition Tec and tlk) of *src*-like non-receptor tyrosine specific kinases. These have an amino-terminal PH domain instead of a myristoylation site and they also lack a negative regulatory tyrosine in the carboxy-terminus. The

Btk PH domain differs from that of Tec and tlk by containing a twenty amino acid insertion between subdomains IV and V. This kinase is involved in normal B-cell development and is required for proper B-cell signalling. In X-linked agammaglobulaemia (XLA) Btk is mutated in its kinase domain and this renders it kinase deficient [Vetrie et al., 1993]. The result is the production of few B-cells and severe humoral immunodeficientcy. In X-linked immunodeficient mice (XID) the Btk gene is mutated in a conserved residue of its PH domain [Rawlings et al., 1993]. That being $ARG^{28} \rightarrow CYS^{28}$, this arginine is a conserved residue of subdomain II (70% conservation). This residue is equivalent to VAL^{26} of β-spectrin and MET^{24} of pleckstrin. It is in an exposed position in the middle of the second (b) β-sheet in the solution structures [Yoon et al., 1994; Macias et al., 1994] (see Fig. 2), suggesting the XID mutation causes a major perturbation of the PH domain. This mutation results in the failure of B-cells to become phenotypically and functionally diverse. B-cells from XID mice also do not respond to a variety of activation signals. These results would indicate that the amino-terminal subdomains of the PH domain are important in signal transduction. The coincidence of the presence of a PH domain and the lack of a negative regulatory tyrosine in Btk might indicate that the PH domain may play an analogous role to this tyrosine present in other *src*-like kinases. Alternatively, the lack of a myristoylation consensus sequence may indicate that it has an analogous function in membrane localisation.

The PH domain of β_G-spectrin appears to function in mediating specific protein interactions. A PH domain containing peptide of 273 amino acids, present at the carboxy-terminus of β_G-spectrin, has been shown to mediate part of its isoform specific membrane association [Davis and Bennett, 1994]. Other regions of β_G-spectrin mediate membrane association with different characteristic, such as calcium-calmodulin regulation. The membrane component that this polypeptide is binding to has not been characterised. Neither has it been formally proven that it is the PH domain, present in the carboxy-terminal half of this peptide, which is essential for the observed interaction. But this does suggest that this PH domain plays a role in mediating a protein-membrane association most likely via an integral membrane protein.

The prime function of the PH domain still remains to be elucidated. However, structural studies together with sequence alignments have suggested a number of feasible hypothesis. It has been shown that it is important in signal transduction and in this most likely by mediating protein-protein interactions. Thus the race is on to find the ligands of each PH domain and determine a common nature to their interaction. While the binding of βγ subunits may be the prime function of the extended PH domain of the βARK, there are three PH domains, namely, those of NrkA/B, Lbc and the carboxy-terminal PH domain of pleckstrin, which are most unlikely to be able to bind βγ subunits. This is because these molecules have their PH domain very close to the carboxy-terminus, and thus do not have the extended carboxy-terminus which is required for βγ binding in βARK. Thus the conclusion is that the PH domain and βγ binding domain overlap in some molecules but they are not identical.

FUTURE PROSPECTS

In only 1 year we have gone from the first report [Haslam et al., 1993] of the PH domain in various molecules to the solving of its structure from two proteins [Yoon et al., 1994; Macias et al., 1994]. The main hurdle which remains to be overcome is the identification of the PH domain ligand(s). Owing to the relatively high degree of divergence of the PH domains, compared to the SH2/SH3 domains, the ligand(s) could also prove to be diverse.

The primary sequence of many PH domain containing molecules have been known and available in the databases (e.g., Gene-EMBL and SwissProt) for several years but their identification has only occurred recently. This is principally due to the relaxed nature of the primary sequence conservation of this domain. Consequently, there could be other domains that have a similar low degree of conservation still to be identified.

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