Structural Characterization of the Interaction between a Pleckstrin Homology Domain and Phosphatidylinositol 4,5-Bisphosphate

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ABSTRACT: The pleckstrin homology (PH) domain is a protein module of approximately 100 amino acids that is found in several proteins involved in signal transduction [for a recent review, see Gibson et al. (1994) *Trends Biochem. Sci. 19*, 349−353]. Although the specific function of the PH domain has not yet been elucidated, many of the proteins which contain this domain associate with phospholipid membranes, and PH domains have been shown to bind to phosphatidylinositol 4,5-bisphosphate (PIP_2) [Harlan et al. (1994) *Nature 371*, 168−170] and the $\beta\gamma$ subunits of G-proteins [Touhara et al. (1994) *J. Biol. Chem. 269*, 10217−10220]. We have postulated that pleckstrin homology domains may be important for the translocation of proteins to the membrane by an interaction with the negatively charged head group of phospholipids. Here we show the importance of three conserved lysine residues for binding to PIP_2 by site-directed mutagenesis. These results should aid future site-directed mutagenesis studies in probing the function of PIP_2−PH domain interactions in the various proteins containing this module. In addition, we examine the specificity of this binding and illustrate the importance of charge−charge interactions in PIP_2−PH domain complex formation from binding experiments involving PIP_2 analogs.

The pleckstrin homology (PH)[1] domain was initially identified as an internal N- and C-terminal sequence duplication in pleckstrin, the major protein kinase C (PKC) substrate of platelets (Tyers et al., 1988). Database searches of protein sequences revealed the existence of this domain in a large number of proteins involved in cellular signaling pathways including protein tyrosine kinases, serine/threonine kinases, RasGAPs, RasGRFs (Mayer et al., 1993; Haslam et al., 1993; Musacchio et al., 1993; Shaw, 1993; Gibson et al., 1994), and phospholipase C isoforms (Parker et al., 1994), as well as in cytoskeletal proteins (Haslam et al., 1993; Musacchio et al., 1993; Shaw, 1993; Gibson et al., 1994). Many of the cellular signaling proteins which contain the PH domain also contain SH2 and SH3 domains. On the basis of these observations, it has been suggested that, like SH2 and SH3 domains, PH domains may also mediate protein−protein interactions involved in cellular signaling events.

In the last year, considerable effort has been made to understand the biological role of PH domains with a focus on determining the three-dimensional structure of the PH domain and identifying the nature of its ligand(s). Recently, NMR structures of the PH domains from pleckstrin (Yoon et al., 1994) and β-spectrin (Macias et al., 1994) have been reported. Subsequently, the NMR (Downing et al., 1994; Fushman et al., 1995) and X-ray (Ferguson et al., 1994; Timm et al., 1994) structures of the PH domain of dynamin have been described. Although the protein sequence identity is poor between these PH domains, the cores of the structures are quite similar, featuring an antiparallel β-sheet consisting of seven strands and a C-terminal amphiphilic α-helix (Figure 1). All three PH domains have an electrostatically polarized surface and a cleft around a region of positive potential, suggesting that the positive cleft may be an important characteristic of PH domains and may play an essential role for their function. Indeed, an Arg28 → Cys point mutation in the PH domain of Bruton's tyrosine kinase (Btk) that is associated with X-linked agammaglobulinemia (Thomas et al., 1993; Rawlings et al., 1993) is located in this region of the PH domain.

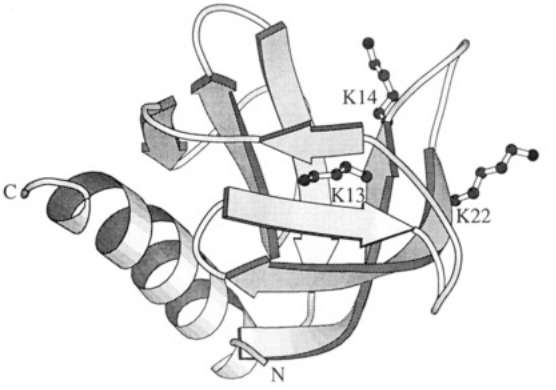

FIGURE 1: Molscript (Kraulis, 1991) representation of the N-terminal PH domain of pleckstrin. Side-chain atoms are shown for the three lysines (13, 14, and 22) which were changed to asparagines by site-directed mutagenesis.

Several studies have focused on a search for the natural ligand of the PH domains. The PH domains of the β-adrenergic receptor kinase (β-Ark) (Koch et al., 1993; Touhara et al., 1994) and other proteins (Touhara et al., 1994; Wang et al., 1994) have been shown to interact with $\beta\gamma$ subunits of G-proteins. The C-terminal α-helix and residues beyond the C-terminus of the PH domains appear to be

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[1] Abbreviations: PH, pleckstrin homology; PKC, protein kinase C; Btk, Bruton's tyrosine kinase; β-Ark, β-adrenergic receptor kinase; PIP_2, phosphatidylinositol 4,5-bisphosphate; IP_2, inositol 4,5-bisphosphate; IP_3, inositol 1,4,5-triphosphate; PI-4P, phosphatidylinositol 4-phosphate; GIP_2, 1-(α-glycerophosphoryl)-D-myoinositol 4,5-bisphosphate; CHAPS, 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate; DTT, dithiothreitol; PC, phosphatidylcholine; PS, phosphatidylserine; PG, phosphatidylglycerol; PI, phosphatidylinositol; PA, phosphatidic acid; dag-P, diacylglycerol phosphate; g-P, glycerol phosphate.

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mediating this interaction. This portion of the PH domain is on the side opposite the positively charged cleft of this module (Figure 1). Due to the structural similarity between PH domains and proteins that bind lipophilic molecules such as retinol binding protein (Newcomer et al, 1984), fatty acid binding protein (Sacchettini et al., 1989), and bilin binding protein (Huber et al., 1987), we proposed that PH domains may bind to lipophilic molecules (Yoon et al., 1994). This hypothesis is consistent with the fact that many PH domain-containing proteins associate with phospholipid membranes, and disruption of the domain can interfere with this function (Koch et al., 1993; Davis et al., 1994; Rebecchi et al., 1992; Cifuentes et al., 1993). In order to test this hypothesis, the lipid binding characteristics of several PH domains were examined, and all five of the PH domains tested were shown to bind to the acidic phospholipid phosphatidylinositol 4,5-bisphosphate (PIP_2) (Harlan et al., 1994). Although earlier studies were not able to detect binding of the dynamin PH domain to PIP_2 (Ferguson et al., 1994), subsequent work has demonstrated that this PH domain does bind to PIP_2 (Fushman et al., 1995) with a binding affinity similar to that observed for the N-terminal PH domain of pleckstrin. We have also shown that the PIP_2 binding site is located at the positively charged cleft formed by loops between β-strands 1/2 and 3/4, with several residues implicated in the interaction by changes in the chemical shifts of the protein upon binding PIP_2 (Harlan et al., 1994).

Here we show, by site-directed mutagenesis and subsequent binding measurements, that specific lysine residues at the putative phospholipid-binding site are critical for interaction with PIP_2. In addition, we have examined the binding affinity of the N-terminal PH domain of pleckstrin to several PIP_2 analogs in order to examine the specificity of binding among these compounds and to identify the functional groups of PIP_2 that are important for protein−lipid complex formation.

MATERIALS AND METHODS

Materials. The N-terminal PH domain of pleckstrin used in this study was cloned from poly(A)$^+$ RNA using reverse transcriptase PCR as described previously (Harlan et al., 1994; Yoon et al., 1994). The amplified region was M^1−G^{105}, and the poly(A)$^+$ RNA was from the HL60 promyelocytic leukemia cell line. Inositol 4,5-bisphosphate (IP_2), inositol 1,4,5-triphosphate (IP_3), phosphatidylinositol 4-phosphate (PI-4P), and PIP_2 were purchased from Sigma Chemical Co. (St. Louis, MO). 1-(α-Glycerophosphoryl)-D-myoinositol 4,5-bisphosphate (GIP_2) was purchased from Calbiochem (San Diego, CA).

Alignment of PH Domain Sequences. PH domains of known structure were aligned on the basis of their conserved secondary structure. Other selected PH domain sequences were aligned using the multiple sequence portion of the program MacDNAsis (v 3.2, Hitachi Software, Inc.) and manually modified to conserve hydrophobic/aromatic residues within the putative secondary structural elements. Adjustments in the variable loop region were then made to align all residue types. All known PH domain sequences were examined for conservation of positively charged residues at positions near those corresponding to lysines 13, 14, and 22 of the N-terminal PH domain of pleckstrin.

Construction and Isolation of Mutant PH Domains. Four mutant proteins were prepared in which one (K13N, K14N, or K22N) or two (K13/22N) of the lysines shown to be in the putative lipid binding site (Harlan et al., 1994) were replaced by asparagines. For the mutagenesis, the following forward primers for the polymerase chain reaction were used: 5′-GCGCTCCATATGGAACCAAAGCGGATCA-GAGAGGGCTACCTTGTGAACAAGGGGAGC-3′-(K13N), 5′-GCGCTCCATATGGAACCAAAGCGGATCA-GAGAGGGCTACCTTGTGAAGAACGGGAGCGTG-3′-(K14N), and 5′-GCGCTCCATATGGAACCAAAGCGGA-TCAGAGAGGGCTACCTTGTGAACAACGG-GAGCGTGTTCAATACGTGGAACCCCATGTGG-3′-(K22N). 3′-TTCCGGTAATTTACGTAACTTCCTGAGCT-CTCGGCG-5′ was used as a reverse primer, and pEPLEC/NPHD1, which contains the N-terminal PH domain of pleckstrin, was used as a template. For the double mutant (K13/22N), the forward primer of K22N and the mutant cDNA coding for K13N were used. The yield of each cDNA fragment was treated with *Nde*I and *Xho*I and then subcloned into pET20b plasmid, and the mutant cDNA sequences were confirmed by dideoxy sequencing. The proteins were expressed, purified by affinity chromatography on a nickel−IDA column (Invitrogen) as described previously (Harlan et al., 1994; Yoon et al., 1994), and exchanged into a phosphate buffer [20 mM potassium phosphate, pH = 6.5, 100 mM NaCl, and 5 mM dithiothreitol (DTT)] containing 50% D_2O. Two additional double mutants (K13/14N and K14/22N) and a triple mutant (K13/14/22N) were cloned, but the proteins were expressed in insufficient quantities for our experiments.

Binding of PH Domains to PIP_2 and Analogs. The binding affinities of the PH domains to PIP_2 and its analogs were examined by ^{31}P NMR in the presence of 24.4 mM 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate (CHAPS). CHAPS was required to solubilize the PH domain−PIP_2 complex. The standard buffer for the titrations was 20 mM KPO_4 (pH 6.5), 100 mM NaCl, and 5 mM DTT in 50% D_2O. An alternate buffer system was used for binding studies in the presence of Ca^{2+} and Mg^{2+} in which 20 mM KPO_4 (pH 6.5) was replaced by 20 mM MES (pH 6.5). The initial concentration of PIP2 and all analogs was 0.2 mM. ^{31}P (242.94 MHz) NMR spectra of PIP_2 and all analogs were acquired at 298 K on a Bruker AMX600 NMR spectrometer using a 4.9-kHz sweep width, a 1-s total recycle delay, and 2048 scans. The spectra were acquired as a function of increasing concentration of wild-type (wt) or mutant PH domains. The ^{31}P chemical shifts were fit using the equation

$$\delta_{obs} = \delta_f + \chi_b \Delta_{b-f} \quad (1)$$

where δ_{obs} is the observed chemical shift, χ_b is the mole fraction of the bound state, δ_f is the chemical shift of the free state, and Δ_{b-f} is the difference in chemical shift between the free and bound states ($\delta_b - \delta_f$). Mole fractions were calculated from a dissociation constant, K_D, using the known initial concentrations of the protein and PIP_2. A least squares analysis was then performed by systematic variation of K_D, δ_f, and Δ_{b-f}. A minimum of five points was used in each analysis, and the error in the ^{31}P chemical shifts was estimated to be 0.01 ppm.

RESULTS

Alignment of PH Domain Sequences. Sequence alignment of the region spanning β-strands 1 and 2, the connecting loop

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Pleck/1     (7-30)       REGYLVKK--GSV------FNT--WKPMWVVLLE
Pleck/2     (247-270)    KQGCLLKQ--GHR------RKN--WKVRKFILRE
β-Ark       (561-585)    MHGYMSKM--GNP-----FLTQ--WQRRYFYLFP
Tsk         (7-35)       LEEQLIKK--SQQK-RRTSPSN--FKVRFFVLTK
Tec         (7-35)       LEEILIKR--SQQK-KKTSLLN--YKERLCVLPK
Btk         (6-34)       LESIFLKR--SQQK-KKTSPLN--FKKRLFLLTV
PKCμ        (424-447)    KEGWMVHY--TSK------DTL--RKRHYWRLDS
SIP3        (312-339)    KSGWLYMK--TQVG-KPTREI---WVRRWCFLKN
Bob1        (778-802)    CSGWMSKK--GTG-----AMGT--WKQRFFTLHG
RasGAP      (477-499)    KKGYLLKK--GKG------KR---WKNLYFILEG
RasGRF/1    (25-48)      RKGYLSKR--SSD------NPK--WQTKWFALLQ
Vav         (405-428)    IDGELKIT--SVE------RRS--KTDRYAFLLD
SOS         (482-506)    REDSLSKL--GSG------KRI--WSERKVFLFD
LBC         (314-426)    RDGSVFLK--NAA------GR---LKEVQAVLLT
3BP2A       (29-55)      KAGYLHKKGGTQLQLLK-------WPLRFVIIHK
PLC-δ       (20-41)      KGTMMRKV--RS-----KS-----WKKLRFFRLQ
β-Spec      (3000-3032)  MEGFLNRKHEWEAHNKKASSRS--WHNVYCVINN
AFAP-110/1  (155-177)       ICAFLLRK-------KRF-GQ--WIKLLCVIKE
Dynamin     (514-539)    RKGWLTIN--NIG----IMKGG--SKEYWFVLTA
β-Syn2/1    (22-43)      RSGLLEVL------VRD---R---WHKVLVNLSE

                         β1      variable loop      β2
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FIGURE 2: Alignment of PH domain sequences in the region of β-strands 1 and 2 and the variable loop between. Dashes indicate gaps, and numbers indicate the positions of the first and last amino acid shown in alignment. Boxes indicate positions corresponding to the lysines mutated in this study. The sequences are from the following PH domains: Pleck/1 and -2, human pleckstrin N- and C-terminus; β-Ark, human β-adrenergic receptor kinase; Tsk, human T-cell-specific kinase; Tec, mouse Tec tyrosine kinase; Btk, Bruton's tyrosine kinase; PKCμ, human atypical protein kinase C isozyme; SIP3, yeast protein, which interacts with SNF1 kinase; Bob1, yeast protein, which binds BEM1; RasGAP, human GTPase-activating protein; RasGRF/1, rat guanine nucleotide releasing factor N-terminus; Vav, mouse Vav protein; SOS, *Drosophila* Son of sevenless; LBC, human cdc24 homolog; 3BP2A, mouse SH3 binding protein; PLC-δ, bovine PI-specific phospholipase C; β-Spec, human muscle β-spectrin; AFAP-110/1, actin-associated pp60src substrate; Dynamin, human dynamin; β-Syn2/1, human dystrophin-associated protein N-terminus.

of PH domains of known structure, and a representative set of other PH domains is shown in Figure 2. Note the high degree of conservation of positive charge at or near positions corresponding to the lysines of the N-terminal PH domain of pleckstrin examined in this study. Examination of all known PH domain sequences revealed that 59%, 38%, and 54% have either lysines or arginines at positions homologous to lysine 13, 14, and 22, respectively, and 65%, 45%, and 80% have either lysines or arginines within one residue of these positions. Positive charge also appears highly conserved at positions corresponding to Arg28 of Btk (Met24 in the N-terminal PH domain of pleckstrin).

Binding of Wild-Type and Mutant PH Domains to PIP_2. The changes in the ^{31}P chemical shifts of PIP_2 upon the addition of the N-terminal PH domain of pleckstrin and three mutants (K13N, K14N, K22N) are shown in Figure 3A. A K_D of 51 ± 6.8 μM was measured for the binding of wt N-terminal PH domain to PIP_2 (Table 1), which is similar to a K_D of 46 ± 7.0 μM observed in a centrifugation assay using PIP_2-containing unilamellar phosphatidylcholine (PC) vesicles formed by extrusion (data not shown). The centrifugation assay contains no detergent, and the agreement between the two methods indicates that the presence of detergent in the ^{31}P measurements does not interfere with PH domain/PIP_2 complex formation. While changing buffer conditions from 20 mM PO_4 (pH 6.5) to 20 mM MES (pH 6.5) resulted in a modest (~3-fold) increase in binding affinity, the addition of either 2 μM Ca^{2+} or 500 μM Mg^{2+} had no significant effect on the binding of wt PH domain to

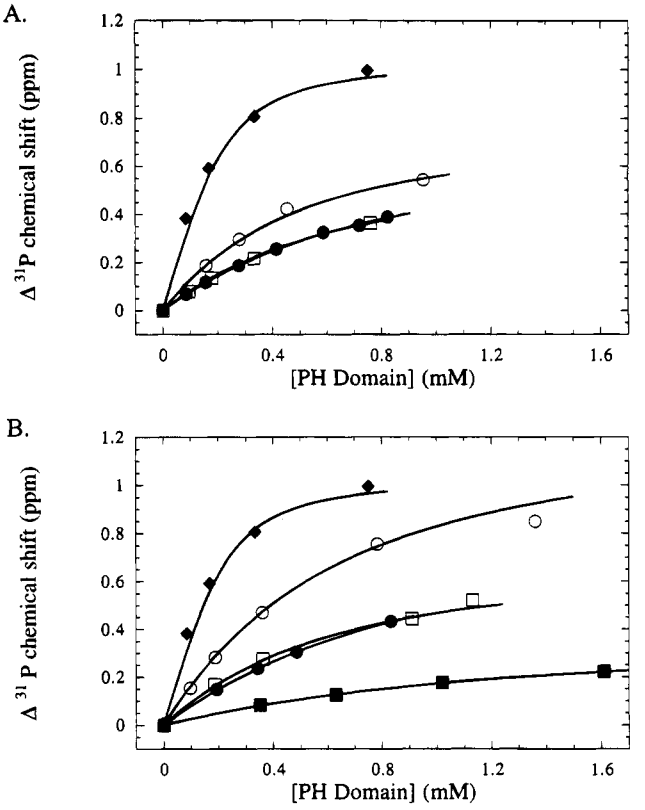
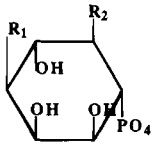


FIGURE 3: Change in ^{31}P chemical shift of the of the 4-phosphate of phosphoinositides upon the addition of PH domains. (A) Chemical shift changes of the 4-phosphate of PIP_2 upon the addition of wt PH domain (◆) and K13N (●), K14N (○), and K22N (□) mutants. Changes upon addition of the double mutant (K13/22N) or in the 5-phosphate of PIP_2 gave similar results (data not shown). (B) Chemical shift changes of the 4-phosphate of PIP_2 (◆), GIP_2 (○), IP_2 (●), IP_3 (□), and PI-4P (■) as a function of added wt PH domain. Changes in the 5-phosphate, where present, gave similar results (data not shown). The phosphates of PIP_2 and PI-4P were assigned previously (Harlan et al. 1994); those for all other analogs were assigned by comparison to PIP_2 and PI-4P.

PIP_2. As shown in Figure 3A and Table 1, each of the four mutant proteins, in which at least one lysine was replaced by an asparagine, exhibited a ~10-fold loss in binding affinity for PIP_2. This loss in affinity corresponds to a decrease in $\Delta G_{binding}$ of 1.4 kcal/mol. One-dimensional proton spectra were recorded for the mutant PH domains and compared to that of the native protein. Only minor chemical shift changes were detected, and no evidence for global conformational changes or unfolding was observed.

Binding of Wild-Type PH Domain to PIP_2 Analogs. The results of the titration of PIP_2 analogs with wt PH domain are shown in Figure 3B. All of these analogs display a 10−24-fold loss in binding affinity compared to the wt PH domain binding of PIP_2 (Table 1). The largest decrease (24-fold) was observed for PI-4P which lacks the 5-phosphate monoester. The other PIP_2 analogs, IP_2, IP_3, and GIP_2, exhibit 17-, 10-, and 10-fold decreases, respectively, relative to PIP_2 (Table 1). The 1-phosphates of PIP_2, PI-4P, and GIP_2 showed very little change in chemical shift upon the addition of PH domain, indicating little interaction. In contrast, the 1-phosphates of IP_3 showed a substantial change in chemical shift upon the addition of wt N-terminal PH domain of pleckstrin.

Table 1: Binding Affinity of Wild-Type and Mutant PH Domains to Phosphoinositides as Measured by ^{31}P NMR[a]



compound	buffer	PH domain	R1	R2	K_D (μM)
PIP_2	PBS[b]	wt	dag-P	PO_4	51 ± 6.8
PIP_2	PBS	K13N	dag-P	PO_4	650 ± 1.2
PIP_2	PBS	K14N	dag-P	PO_4	370 ± 59
PIP_2	PBS	K22N	dag-P	PO_4	550 ± 140
PIP_2	PBS	K13/22N	dag-P	PO_4	615 + 131
PIP_2	MES[c]	wt	dag-P	PO_4	13.4 + 1.5
PIP_2	MES + Ca[d]	wt	dag-P	PO_4	13.5 + 1.3
PIP_2	MES + Mg[e]	wt	dag-P	PO_4	20.2 + 1.9
IP_2	PBS	wt	OH	PO_4	870 ± 150
IP_3	PBS	wt	PO_4	PO_4	500 ± 95
GIP_2	PBS	wt	g-P	PO_4	520 ± 46
PI-4P	PBS	wt	dag-P	OH	1200 ± 230

[a] Changes in the ^{31}P chemical shifts of the 4-phosphate of PIP_2 and all analogs upon addition of wt or mutant PH domains were fit using eq 1, as described in Materials and Methods. [b] PBS: 20 mM KPO_4 (pH 6.5), 100 mM NaCl, and 5 mM DTT. [c] MES: 20 mM MES (pH 6.5), 100 mM NaCl, and 5 mM DTT. [d] MES + Ca: MES buffer + 2 μM Ca^{2+}. [e] MES + Mg: MES buffer + 500 μM Mg^{2+}.

DISCUSSION

The results presented in this study indicate that (1) the binding of the N-terminal PH domain of pleckstrin to PIP_2 is mediated through charge−charge interactions involving three lysines in the positive cleft of the PH domain and the 4,5-monoesters of PIP_2, (2) the affinity of this PH domain for PIP_2 is sufficient to provide a tendency for PH domain-containing proteins to localize to the membrane (see below), (3) this binding is specific for PIP_2, and (4) the conservation of positively charged residues at positions corresponding to these lysines may imply a generalization of PIP_2 binding to other PH domains.

Each of the lysines shown in Figure 1 plays a critical role in the interaction of the PH domain with PIP_2. Interestingly, the $\Delta G_{binding}$ observed upon mutation of any one of these lysines (~1.4 kcal/mol) is similar to that observed in studies of the binding of basic peptides [$(Lys)_n$, n = 3, 4, or 5] to lipid vesicles containing phosphatidylserine (PS) or phosphatidylglycerol (PG) (Kim et al., 1991). In the peptide studies, each additional lysine above n = 2 interacted with a single acidic headgroup and contributed an additional 1.4 kcal/mol to the binding interaction (Kim et al., 1991). The lysines of the PH domain are interacting with the 4- and 5-monoesters of PIP_2. The 24-fold decrease in binding of PH domain to PI-4P relative to PIP_2 attests to the importance of the phosphate monoesters in this charge−charge interaction.

In the MES buffer, the binding affinity of the N-terminal PH domain of pleckstrin for PIP_2 is 15 μM, which is similar to that observed for dynamin (D. Cowburn, personal communication). This affinity is ~3-fold greater than that observed in the presence of 20 mM phosphate (see Table 1). On the basis of these data, organic phosphate inhibits the binding of the PH domain to PIP2 with an apparent K_I of ~8 mM. Surprisingly, the effect of the double mutation (K13/22N) was not additive (Table 1). Although it is possible that unfolding or large conformational changes in the single- and double-mutant proteins could explain this result, no significant changes in the 1D NMR spectra were observed in any of these proteins compared to the native protein. Furthermore, acetylation of all surface lysines completely abolished binding (Harlan et al., 1994), supporting the essential role of lysines in the interaction with PIP_2. One possible explanation for the nonadditive effect of the double mutant is that the interaction of the individual lysines of the PH domain with PIP_2 may be cooperative. In this case, the absence of a single lysine may disrupt the cooperativity, and the removal of additional lysines will have a significantly smaller overall effect. Proximal charged residues, while not contributing to the cooperativity, may compensate for the loss of additional lysines.

The wt N-terminal PH domain of pleckstrin binds to PIP_2 with an affinity of 15−50 μM. Is this relatively weak association physiologically relevant for membrane association? Numerous studies of peptides with membrane anchoring groups suggest that it is. Apparent dissociation constants of 5−39 μM have been observed for farnesylated peptides containing an alkylated (*O*-methyl) cysteine C-terminus (Silvius & l'Heuraux, 1994). Studies of myristoylated peptides have shown apparent dissociation constants on the order of 100 μM (Silvius & l'Heuraux, 1994; Peitzsch & McLaughlin, 1993; Sankaram, 1994). Thus, the binding of PH domain to PIP_2 is on the same order as the binding of these peptides to lipid vesicles, and it could therefore serve the same function as myristoylation or farnesylation. Several lines of evidence support this intriguing possibility. Three members of a subfamily of tyrosine kinases with high homology to the Src family have no N-terminal myristoylation site. However, they do have an N-terminal PH domain (Rawlings et al., 1993; Mano et al., 1993; Gibson et al. 1993). It is possible that the PH domain has replaced the function of the myristoylation in this subfamily of tyrosine kinases. Removal of the PH domain in β-Ark resulted in a decreased ability of this kinase to phosphorylate the β-adrenergic receptor (Koch et al., 1993). Replacement of these residues with an isoprenylation site restored most of this activity (Koch et al., 1993). Finally, removal of the C-terminal farnesylation site of Ras results in a protein unable to support Ras-dependent cell growth. Replacement of this site with a polybasic extension restores this ability, indicating that charge−charge interactions with the membrane can replace the function of farnesylation in Ras (Michell et al., 1994).

The binding affinity of PH domain for PIP_2 and of myristoylated or farnesylated peptides for lipid vesicles may not be strong enough to provide a firm anchor to the membrane (Silvius & l'Heuraux, 1994; Peitzsch & McLaughlin, 1993). However, additional interactions may stabilize this association, as has been observed for basic peptides related to the N-terminus of Src (Busier et al., 1994). Pleckstrin contains two PH domains (Tyers et al., 1988), both of which can interact with PIP_2 (Harlan et al., 1994) and therefore enhance membrane association. Several other proteins also contain two or more PH domains (Gibson et al., 1994). Many PH domains have been shown to interact with $\beta\gamma$ subunits of G-proteins (Koch et al., 1993; Touhara et al., 1994; Tsukada et al., 1994), and the PH domains of Bruton tyrosine kinase (Btk) and T-cell-specific kinase (Tsk) have recently been shown to interact with both Ca^{2+}-dependent and Ca^{2+}-independent isoforms of PKC (Yao et al., 1994). Such interactions could act synergistically with

PIP_2/PH domain binding and substantially increase the affinity of PH domain-containing proteins for the membrane.

The known structures of PH domains all exhibit a conserved electrostatic polarization (Yoon et al., 1994; Macias et al., 1994; Downing et al., 1994; Fushman et al., 1995: Ferguson et al., 1994; Timm et al., 1994) which could serve to orient the PH domain-containing proteins with respect to the membrane and facilitate other interactions. This structural conservation is interesting given the low degree of sequence homology between the various PH domains. In fact the only conserved amino acid in all of the known PH domain sequences is a C-terminal tryptophan. As was previously pointed out (Yoon et al., 1994), this tryptophan, which is in the C-terminal α-helix, is located within the hydrophobic core of the domain and is probably important for the structural integrity of the module. However, closer inspection of the sequences in the vicinity of the postulated PIP_2 binding site (Harlan et al., 1994) reveal a conservation of positively charged residues (Figure 2). Indeed, 65%, 45%, and 80% of the known PH domain sequences have either lysines or arginines at or within one residue of positions homologous to lysine 13, 14, and 22, respectively, and all of the published consensus sequences (Mayer et al., 1993; Haslam et al., 1993; Musacchio et al., 1993; Shaw, 1993; Gibson et al., 1994) have positively charged residues at these positions. The binding results of the mutant N-terminal PH domains of pleckstrin highlight the importance of these residues in the interaction with PIP_2. This interaction is not unique to the N-terminal PH domain of pleckstrin, as four other PH domains have been shown to bind to PIP_2 in a centrifugation assay (Harlan et al., 1994). The PH domain of dynamin, while initially thought to not interact with PIP_2 (Ferguson, et al., 1994), has been shown to exhibit a binding affinity for PIP_2 similar to that of the N-terminal domain of pleckstrin (Fushman et al., 1995; D. Cowburn, personal communication). Given the high degree of conservation in the positioning of these charged residues (Figure 2) and their importance in the PH domain/PIP_2 interaction, the binding to PIP_2 may well be a general function of many PH domains and may be one mechanism whereby PH domains can accomplish membrane localization.

The membrane localization postulated here is most likely specific to PIP_2/PH domain binding. We have previously shown that the N-terminal PH domain of pleckstrin did not bind to vesicles containing acidic lipids such as PS, phosphatidylinositol (PI), or phosphatidic acid (PA), except at relatively high molar ratios (Harlan et al., 1994). We have also observed dose-dependent binding of this PH domain to PIP_2 in PC vesicles containing 17% (mol) PS or composed entirely of sphingomyelin and cholesterol (9/1, mol/mol).[2] In the ^{31}P NMR titrations, the 1-phosphate diesters of PIP_2, PI-4P, and GIP_2 did not shift significantly upon the addition of PH domain, indicating little interaction. This evidence implicates the importance of features unique to PIP_2, the 4- and 5-monoesters, in the binding of PH domains to lipid membranes. Given the 24-fold decrease in binding affinity for wt PH domain relative to PIP_2, PI-4P, which has a single phosphate monoester, is not likely to be involved in membrane localization of the N-terminal domain of pleckstrin.

[2] J. E. Harlan, unpublished observations.

The PIP_2 analogs, IP_2, IP_3, and GIP_2, all exhibit a ~10-fold decrease in binding affinity relative to PIP_2 (Table 1). These results imply that other portions of PIP_2, including the acyl chains, contribute to PH domain binding. However, no evidence for direct interaction of the acyl chains with the PH domain was observed in our previous study (Harlan et al., 1994). It may be possible that the acyl chains serve to orient the inositol ring, either in lipid membranes or mixed detergent−lipid micelles. From entropic arguments, binding of the oriented PIP_2 would be more favorable than binding of the unoriented analogs.

In summary, we have shown that the binding of PH domains to PIP_2, as measured by ^{31}P NMR, is on the order of that seen for myristoylated or farnesylated peptides and could thus serve a similar function. The membrane localization posulated here would be specific for PH domain/PIP_2 interactions, given the relative affinity of PIP_2 and PI-4P and the lack of interaction seen with other acidic lipids (Harlan et al., 1994). This interaction appears to be mediated by charge−charge interactions as seen by the dramatic loss of affinity observed by mutation of any of the three lysines implicated in binding, or the removal of one of the two phosphate monoesters of PIP_2 involved in this interaction. The 10-fold loss of affinity observed upon mutation of lysine 13, 14, or 22 of the N-terminal PH domain or homologous residues in other PH domains could be enough to interfere with the membrane association. Site-directed mutagenesis of residues homolgous to these lysines in the other PH domain-containing proteins may help define the functional significance of the PH domain/PIP_2 interaction and the role of this domain in signal transduction.

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