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Pleckstrin homology domains bind to phosphatidylinositol-4,5-bisphosphate

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THE pleckstrin homology (PH) domain is a new protein module of around 100 amino acids found in several proteins involved in signal transduction[1-5]. Although its specific function has yet to be elucidated, the carboxy-terminal regions of many PH domains bind to the $\beta\gamma$ subunits of G proteins[6,7]. On the basis of structural similarities between PH domains and lipid-binding proteins, we have proposed that PH domains may be binding to lipophilic molecules[8]. Indeed, many of the proteins that contain this domain associate with phospholipid membranes[6,9,10], and disruption of this domain can interfere with membrane association[6,11]. Here we report that PH domains bind to phosphatidylinosito1-4,5-bisphosphate and show that the lipid-binding site is located at the lip of the β-barrel. This suggests that PH domains may be important for membrane localization of proteins through interactions with phosphatidylinositol-4,5-bisphosphate.

The binding of PH domains to phospholipid vesicles was examined in a centrifugation assay. The amino-terminal PH domain of pleckstrin is unable to bind to many of the phospholipids examined (Fig. l*a*), but did bind to phosphatidylinosito1-4,5-bisphosphate (Ptdlns(4,5)P_2) in phosphatidylcholine vesicles (Fig. l*a*) in a concentration-dependent fashion (Fig. l*b*) as well as to Ptdlns(4,5)P_2 in other lipid backgrounds (data not shown). In addition, phosphatidylinositol-4-phosphate (Ptdlns(4)P) exhibits measurable binding to the PH domain, albeit with a lower apparent affinity (Fig. l*b*), and high levels of phosphatidylserine and phosphatidic acid (>25% vesicle components) display some affinity for the PH domains (data not shown). As a control, binding of other proteins to Ptdlns(4,5)P_2 was examined. Except for the PH domains, none of the proteins exhibits any binding affinity to vesicles containing Ptdlns(4,5)P_2 (Fig. 1*c*). In contrast, all the PH domains bind, including the C-terminal PH domain of pleckstrin as well as PH domains from T-cell-specific kinase (Tsk), rasGAP and the β-adrenergic receptor kinase (β-Ark) (Fig. l*c).*

To investigate the site of interaction of the PH domains with Ptdlns(4,5)P_2, the ^{1}H, ^{13}C and ^{15}N chemical shifts of the N-terminal PH domain of pleckstrin were followed as a function of added lipid (Fig. 2). On addition of Ptdlns(4,5)P_2, systematic chemical shift

changes were observed for signals corresponding to residues K13, K14, S16, V17, N19, T20, W21, K22, F35, Y36 and G46. All these residues are located in the N-terminal half of the PH domain at the lip of the β-barrel (Fig. 2). Lysines 13, 14 and 22, as well as T20, W21, F35, Y36 and G46, are conserved in many of the PH domains[2-5]. These results suggest that the positively charged lysines at the entry of the β-barrel may bind to the negatively charged phosphates of PtdIns(4,5)P_2. Further evidence of the importance of the lysines was obtained in binding studies of protein containing acetylated lysines. In these experiments the acetylated PH domain (Ac-PH) was unable to bind to the phospholipid vesicles containing PtdIns(4,5)P_2 (data not shown). Involvement of the PtdIns(4,5)P_2 phosphates in the binding interaction is supported by the changes in the ^{31}P NMR signals of PtdIns(4,5)P_2 on addition of the PH domain. As shown in Fig. 3, the phosphates at the 4,5 positions of the inositol ring exhibit the greatest changes in chemical shift during titration with PH domain. Addition of lysozyme, FK506-binding protein (FKBP), or the Ac-PH domain of pleckstrin, however, resulted in no chemical shift changes for any PtdIns(4,5)P_2 phosphates. Analysis of the changes in ^{31}P chemical shifts of PtdIns(4,5)P_2 on addition of PH domain gave an apparent dissociation constant of ~30 μM. Addition of the N-terminal PH domain of pleckstrin to both PtdIns(4)P and phosphatidylinositol-3-phosphate (PtdIns(3)P) also resulted in ^{31}P chemical shift changes, indicating that these lipids bind to PH domains (data not shown). These results suggest that the binding of PtdIns(4,5)P_2 to the PH domains is mediated primarily by ionic interactions. However, the apparent inability of $InsP_3$ to inhibit the binding of PtdIns(4,5)P_2 to PH domains at similar molar concentrations (Fig. 4) suggests that other interactions may help stabilize the complex. Although the lipophilic residues of the β-barrel of the PH domain could also contribute to the binding through interactions with the fatty acid chains of PtdIns(4,5)P_2 (ref. 8), no major chemical shift changes were detected for residues located deep in the hydrophobic core of the β-barrel.

What is the functional relevance of the interaction between the PH domains and PtdIns(4,5)P_2? Most of the proteins that contain PH domains need to be associated with membranes for their function. But many of these proteins do not contain the classical membrane-anchoring groups such as a hydrophobic helix or sites for post-translational addition of lipid molecules. For example, the C-terminal region of β_G-spectrin, which contains a PH domain[4,9], has been shown to interact with brain membranes depleted of peripheral proteins[9], but does not contain a classical membrane-anchoring group. The Src family of tyrosine kinases, which are targeted to cellular membranes, contain a consensus sequence for myristylation in their N-terminal regions[12]. Three members of a subfamily of tyrosine kinases with extensive homology to the Src family (the Tec kinase from haematopoietic celis[13], Tsk from T cells[14], and Bruton's tyrosine kinase (Btk) from B and myeloid cells[15]) do not have an N-terminal myristylation site or any other candidate for membrane interaction. However, they do contain a PH domain at the N terminus[4]. Another kinase that contains a PH domain, β-Ark, must also be localized at the membrane surface in order to phosphorylate its receptor substrate effectively[6,16]. Although removal of the last 128 residues of β-Ark (essentially the entire PH domain) decreases the kinase activity to basal level, replacement of this domain with an isoprenylation site restores most of this activity[6]. A homologous protein, rhodopsin kinase, lacks the C-terminal PH domain of β-Ark, but contains an isoprenylation site instead[17]. Additional evidence for the functional correlation of PH domains and

membrane localization was found in studies of phospholipase C-δ1 (PLC-δ1). In this protein the N-terminal region containing the PH domain is necessary for binding to phospholipid vesicles containing Ptdlns(4,5)P_2, and this binding is necessary for processive catalytic activity[11]. All these observations suggest that PH domains may have a biologically significant role in the localization of proteins to phospholipid membranes.

Lefkowitz and co-workers have shown that several PH domains bind to the $\beta\gamma$ subunits of G proteins[6,7]. In these studies, the C-terminal region of the PH domain and residues extending beyond the C terminus of this module were required for binding[6,7]. Our results indicate that the N terminus of the PH domain is responsible for binding to Ptdlns(4,5)P_2, but they do not exclude the possibility that the PH domains also bind to $\beta\gamma$ subunits. Indeed, the binding of the N-terminal portion to Ptdlns(4,5)P_2 may localize the protein to the membrane, thus facilitating the interaction of the C-terminal portion with $\beta\gamma$ subunits.

Ptdlns(4,5)P_2 is a source of the important second messengers inositol trisphosphate and diacylglycerol, and in the past 10 years the importance of lipids as precursors of bioactive molecules and second messengers has become widely recognized[18]. Our results suggest that particular lipids may also be important for localizing proteins at the membrane surface. The PH domains may offer an alternative vehicle to hydrophobic helices or post-translational addition of lipid molecules for membrane localization. The interaction of PH domains with Ptdlns(4,5)P_2 provides a novel mechanism for membrane association and may be critical in controlling the biological functions of the many proteins that contain this module.

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FIG. 1 Protein binding to lipid vesicles as measured by a centrifugation assay. *a*, Binding of the N-terminal PH domain of pleckstrin to vesicles containing phosphatidylcholine (PC) and 5% of other lipids: phosphatidylserine (PS), phosphatidic acid (PA), phosphatidylethanolamine (PE), phosphatidylinositol (Ptdlns), sphingomyelin (Sph), Ptdlns(4)P and Ptdlns(4,5)P_2. *b*, Concentration dependence of the binding of the N-terminal PH domain of pleckstrin to PC vesicles containing various concentrations of PI (light grey), Ptdlns(4)P (hatched) and Ptdlns(4,5)P_2 (dark grey). *c*, Binding of proteins to PC: Ptdlns(4,5)P_2 (95:5) vesicles. These proteins include: cytochrome c, lysozyme, ribonuclease A (all sigma), the Shc SH2 domain containing a $(His)_6$ tag, the N- and C-terminal PH domains of pleckstrin (PHDN and PHDC, respectively), and PH domains of Tsk, rasGAP and β-Ark. The Shc SH2 domain and all PH domains were cloned and expressed in our laboratory. Binding was determined by total protein concentration in the supernatant; the apparent decrease in affinity of rasGAP for Ptdlns(4,5)P_2 compared with the other PH domains can be attributed to a protein contaminant by SDS-PAGE (data not shown).

METHODS. Centrifugation assays were carried out in 0.1 ml total volume using a Beckman TL-100 ultracentrifuge and a TLA-100 rotor. Protein (50 μl at 0.25 mg ml^{-1}) was added to centrifuge tubes. Lipid vesicles (50 μl at 25 mg per ml total lipid) of the desired composition were then added, and the mixture was incubated for 5 min before centrifugation at 100,000 g for 30 min. The vesicles pelleted with this treatment, removing any bound protein from the supernatant. The protein concentrations of the supernatants (50 μl) was then determined by the BCA protein assay (Pierce, Rockford, Illinois) and compared to that of protein not exposed to lipid. Phospholipid blanks were run to correct for any background due to phospholipid in the protein assay. Vesicles were formed by probe sonication of the lipid suspension for several minutes. Both protein and lipid suspensions were in 10 mM K-PO_4 pH 7.4, 100 mM NaCl, and 2.7 mM KCl (PBS). All PH domains in this study were cloned from poly$(A)^+$ RNA using reverse transcriptase-PCR. The amplified region and the source of poly$(A)^+$ RNA for each PH domain were as follows: the N-(M1-G105) and C-terminal (E240-K350) PH domains of pleckstrin were from poly$(A)^+$ RNA of HL60 promyelocytic leukemia cell line, TSK (M1-R111) was from Jurkat T-cell line poly$(A)^+$ RNA, and the Ras-GAP (A470-R579) and β-Ark (G556-A654) were from human placenta poly$(A)^+$ RNA. Complementary DNAs were then subcloned into pET20b plasmid and expressed in *Escherichia coli* (HMS174(DE3) or BL21(DE3)) cells, and all except the β-Ark PH domain contained an additional Leu-Glu-$(His)_6$ sequence at the C terminus. All proteins containing the Leu-Glu-$(His)_6$ sequence were purified by affinity chromatography on a nickel-IDA column (Invitrogen) and exchanged into PBS. The β-Ark PH domain was expressed within inclusion bodies which were washed with Tris buffer (50 mM Tris-HCl, 50 mM NaCl buffer, pH 7.0) three times. The second and third washes also contained 0.2% dodecylmaltoside ($C_{12}M$), and 0.32% $C_{12}M$ plus 1 M NaCl respectively. The washed inclusion bodies were solubilized in PBS containing 6 M urea, 20 mM dithiothreitol (DTT) and subjected to chromatography on Superdex 75. The purified β-Ark PH domain

was then refolded by dialysis against decreasing amounts of urea in PBS with 20 mM DTT. The refolded protein was exchanged into PBS without DTT before assay.

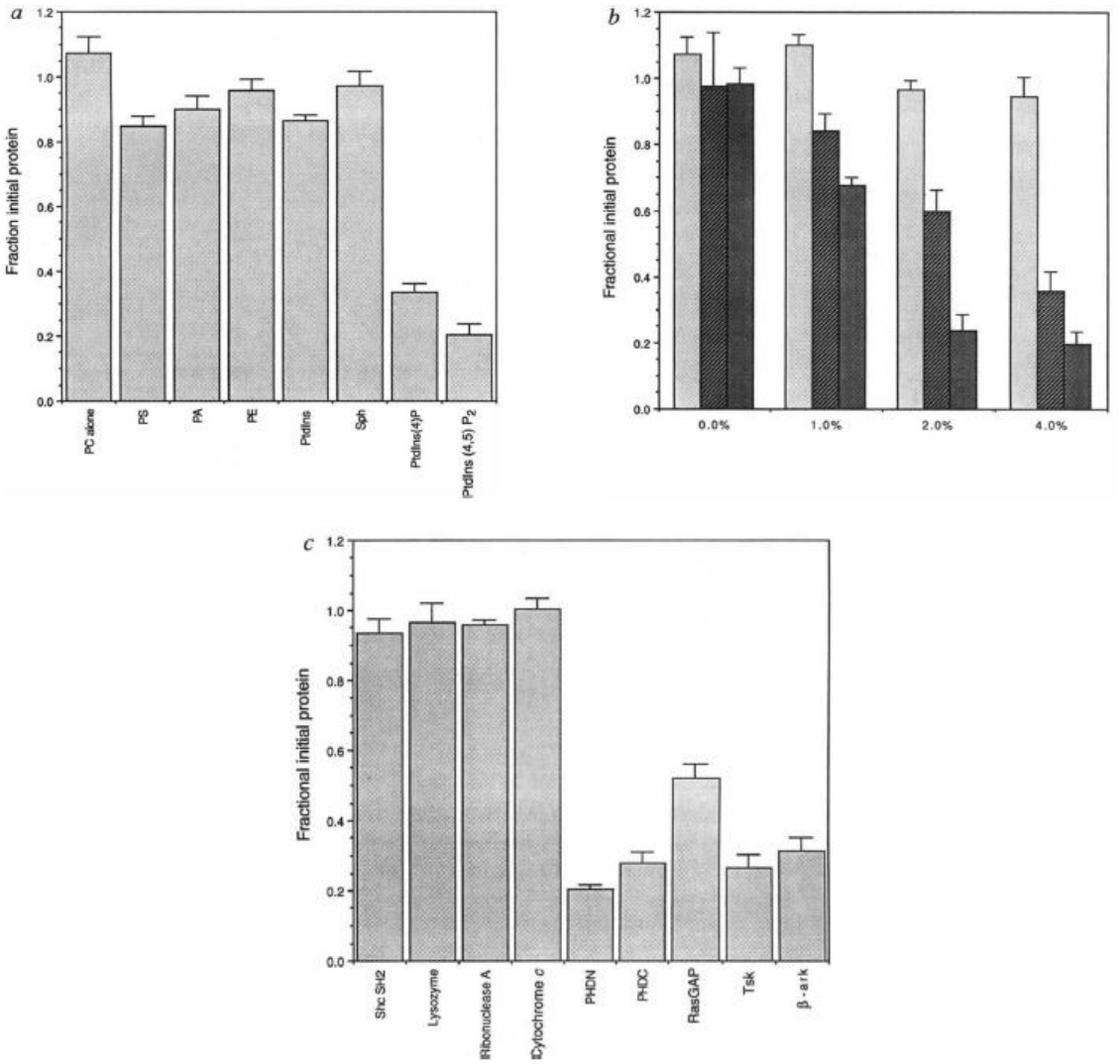
FIG. 2 Ribbon plot[19] of the N-terminal PH domain of pleckstrin. Residues whose backbone resonances (N, HN, Cα or Hα) showed significant chemical shift changes on addition of Ptdlns(4,5)P_2 are shown in red. Also shown in red are side chain heavy atoms for which the heteroatom and attached proton(s) showed significant chemical shift changes (side chain heavy atoms shown in blue either did not shift or could not be followed as a result of signal overlap). Chemical shift changes were considered significant if (1) they were systematic as a function of Ptdlns(4,5)P_2 concentration, and (2) the magnitude of the change was greater than twice the digital resolution of the spectra (0.005, 0.04 and 0.05 p.p.m. for ^{1}H, ^{15}N and ^{13}C, respectively).

METHODS. All NMR spectra were acquired at 298 K on a Bruker AMX 600 NMR spectrometer. All samples were in exchanged into phosphate buffer (20 mM, pH = 6.5) containing 100 mM NaCl and 5 mM DTT. The ^{1}H, ^{13}C and ^{15}N assignments were taken from Yoon *et a1*.[8]. The large size of the protein-vesicle complex precluded direct observation of the protein resonances, and the addition of Ptdlns(4,5)P_2 alone caused precipitation of the complex. Therefore, titrations were performed in 24.4 mM 3[(3-cholamidopropyl)dimethylammonio]-1-propanesulphonate (CHAPS), where the protein, Ptdlns(4,5)P_2 and the complex were soluble. The detergent alone had no apparent effect on the chemical shifts of the protein. Titrations were performed on samples of 0.5-1.0 mM protein, and Ptdlns(4,5)P_2 was added sequentially out to a maximum of 2.0 molar equivalents.

FIG. 3 ^{31}P NMR spectra of Ptdlns(4,5)P_2 (0.2 mM in 24.4 mM CHAPS buffer as described in Fig. 2) as a function of increasing concentration of the N-terminal PH domain of pleckstrin (final mM protein concentration given to the right of each spectrum). Numbers above the peaks correspond to the phosphates at positions 1, 4, and 5 of the inositol ring of Ptdlns(4,5)P_2 (phosphate assignments were obtained by comparing spectra of Ptdlns(4,5)P_2, (Ptdlns(4)P and PC). Phosphate present in the buffer (large central peak) was referenced to 0.0 p.p.m. The inset shows a fit to the chemical shift data using the following equation: $\delta_{obs} = \delta_f + \chi_b \Delta_{b-f}$, where δ_{obs} is the observed chemical shift, χ_b is the mole fraction in 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 Ptdlns(4,5)P_2. A least-squares analysis was then performed by systematic variation of K_d, δ_f and Δ_{b-f}. Fitted parameters and estimated errors (in parentheses) for resonances corresponding to positions 4 and 5 were the following: Position 4: K_d =32 (11) μM; δ_f= 2.22 (0.02) p.p.m.; Δ_{b-f} =0.99 (0.05) p.p.m. Position 5: K_d = 23 (8) μM; δ_f=1.10 (0.02) p.p.m.; Δ_{b-f} = 1.29 (0.05) p.p.m. Fits of data for position 1 were insensitive to K_d because of the small chemical shift changes. Holding

K_d constant at 27 μM yielded $\delta_f = -1.91$ (0.02) p.p.m. and $\Delta_{b-f} = -0.16$ (0.05) p.p.m. for position 1.

FIG. 4 Inhibition of binding of the N-terminal PH domain of pleckstrin to 98:2 (PC: Ptdlns(4,5)P_2) vesicles. The N-terminal PH domain was incubated with the additives for 20 min at room temperature before the addition of the lipid vesicles. Binding assays were performed as described in Fig. 1. Stippled box, $InsP_3$; hatched box, inositol.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1.0
0.8
0.6
0.4
0.2
0.0
PC alone
PS
PA
PE
PtdIns
Sph
PtdIns(4)P
PtdIns (4,5) P2
b
Fractional initial protein
0.0%
1.0%
2.0%
4.0%
c
Fractional initial protein
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Lysozyme
Ribonuclease A
Cytochrome c
PHDN
PHDC
RasGAP
Tsk
β - ark

FIG. 1

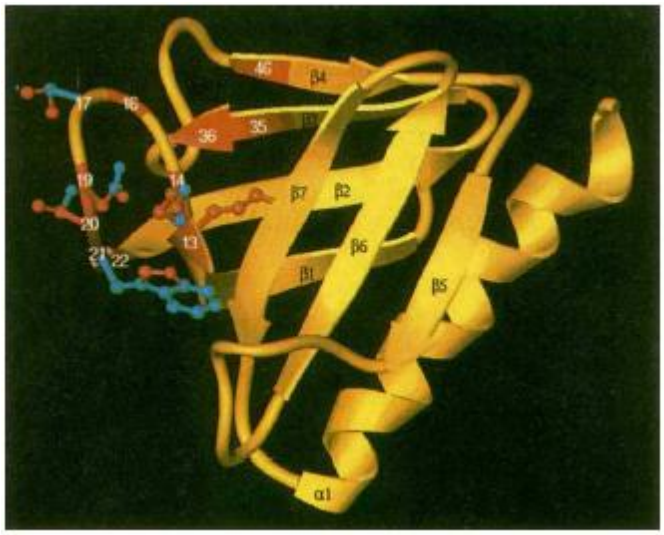

FIG. 2

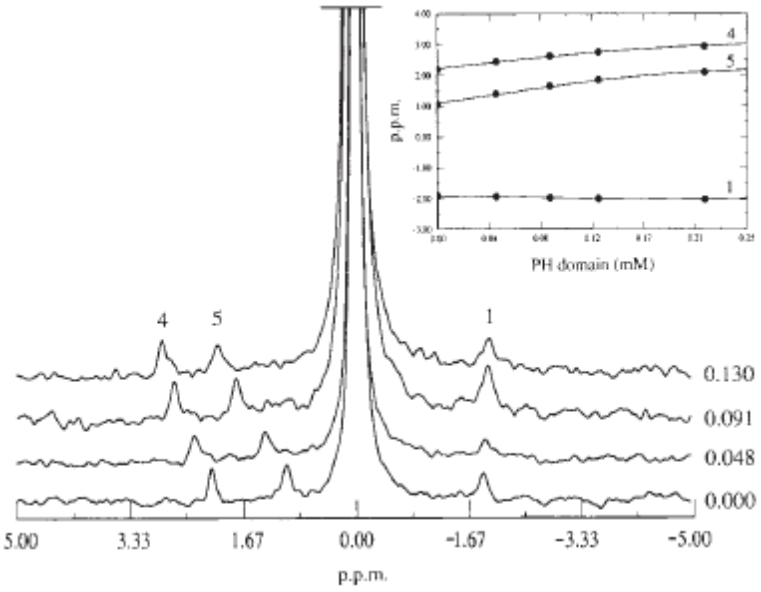
4
5
1
p.p.m.
PH domain (mM)
4
5
1
0.130
0.091
0.048
0.000
5.00
3.33
1.67
0.00
-1.67
-3.33
-5.00
p.p.m.

FIG. 3

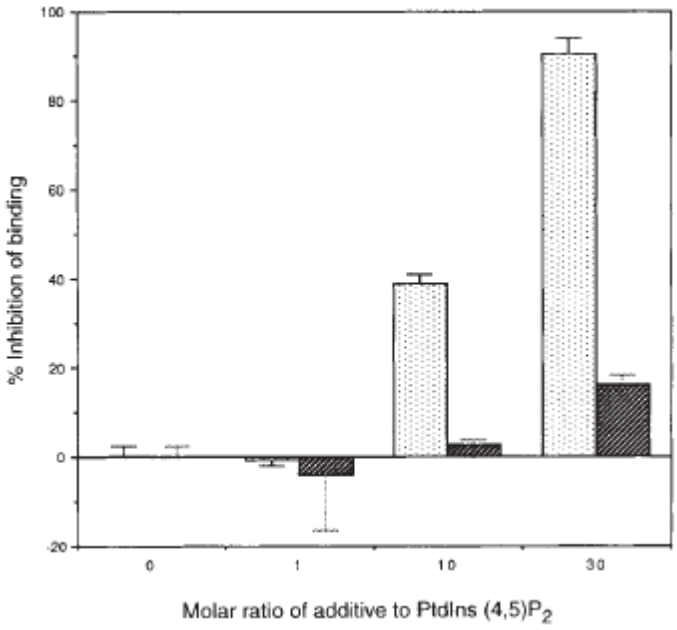
100
80
60
40
20
0
-20
% Inhibition of binding
0
1
10
30
Molar ratio of additive to PtdIns (4,5)P2

FIG. 4