The EMBO Journal vol.14 no.19 pp.4676–4685, 1995

Structure of the binding site for inositol phosphates in a PH domain

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Phosphatidylinositol bisphosphate has been found to bind specifically to pleckstrin homology (PH) domains that are commonly present in signalling proteins but also found in cytoskeleton. We have studied the complexes of the β-spectrin PH domain and soluble inositol phosphates using both circular dichroism and nuclear magnetic resonance spectroscopy, and X-ray crystallography. The specific binding site is located in the centre of a positively charged surface patch of the domain. The presence of 4,5-bisphosphate group on the inositol ring is critical for binding. In the crystal structure that has been determined at 2.0 Å resolution, inositol-1,4,5-trisphosphate is bound with salt bridges and hydrogen bonds through these phosphate groups whereas the 1-phosphate group is mostly solvent-exposed and the inositol ring has virtually no interactions with the protein. We propose a model in which PH domains are involved in reversible anchoring of proteins to membranes via their specific binding to phosphoinositides. They could also participate in a response to a second messenger such as inositol trisphosphate, organizing cross-roads in cellular signalling.

Keywords: binding sites/inositol phosphates/PH domain/pleckstrin/reversible anchoring

Introduction

Many proteins involved in signal transduction have a modular, multidomain structure. Some domains are shared by a number of proteins and employed in similar functions. Shared modules such as Src-homology domains SH2 and SH3 are used for specific protein–protein interactions (Cohen *et al.*, 1995; Pawson, 1995) whereas pleckstrin homology (PH) domain may specifically bind inositol lipids (Harlan *et al.*, 1994), key compounds in phospholipid signalling (Berridge, 1993; Divecha and Irvine, 1995).

PH domains are present not only in diverse signalling proteins such as protein kinases, guanine nucleotide releasing factors and GTPase-activating proteins of small G-proteins, but also in cytoskeletal proteins such as spectrin and syntrophin. They have been found in many oncogenic proteins (e.g. Vav and Dbl). Interestingly, a PH domain is present in many factors involved in budding of fission yeast and in dynamin, which also participates in intracellular vesicle budding (see Musacchio *et al.*, 1993; Gibson *et al.*, 1994; Ingley and Hemmings, 1994; Cohen *et al.*, 1995; Pawson, 1995; Saraste and Hyvönen, 1995).

The structure of PH domain (Downing *et al.*, 1994; Ferguson *et al.*, 1994; Macias *et al.*, 1994; Timm *et al.*, 1994; Yoon *et al.*, 1994; Fushman *et al.*, 1995) is formed by a seven-stranded antiparallel β-sheet with a strong bend that results in an orthogonal sandwich. The domain has a characteristic C-terminal α-helix which blocks one end of the twisted sheet. PH domains are widely spread in nature, occurring in both unicellular and multicellular eukaryotes. Their amino acid sequences are diverse. Only a single amino acid, a tryptophan that has a key structural role in the packing of the α-helix against the β-sheet, is invariant within the common 100–110 residues of the PH domain (Musacchio *et al.*, 1993; Gibson *et al.*, 1994). However, at several sequence positions charges are conserved and cluster in a way that leads to a strong electrostatic polarization of the protein (Ferguson *et al.*, 1994; Macias *et al.*, 1994). This may be a distinct and generally conserved feature of the PH domains.

The positively charged surface has been proposed to anchor PH domain and its host protein to membrane lipids (Ferguson *et al.*, 1994). Another mechanism by which some PH domains (in particular, the domain of β-adrenergic receptor kinase) have been proposed to anchor proteins to membranes is via interaction with the βγ subunits of trimeric G-proteins (Touhara *et al.*, 1994; Wang *et al.*, 1994) but this may also involve the flanking protein sequences. However, comparison of two homologous proteins, rhodopsin and β-adrenergic receptor kinase, indicates that the PH domain of the latter may have a role in membrane attachment. Rhodopsin kinase is modified by farnesylation at the C-terminus which provides the membrane-binding property, and has no PH domain. Conversely, the β-adrenergic receptor kinase is not isoprenylated but rather contains a C-terminal PH domain (Musacchio *et al.*, 1993; Wang *et al.*, 1994) which is required for its function. The substitution of the PH domain with an isoprenylation signal partially restores the function of β-adrenergic receptor kinase (Koch *et al.*, 1993).

Phosphoinositide-specific phospholipases C (PLC) make up a family of enzymes that hydrolyse the phosphoester bond between glycerol and inositol-1-phosphate. Cifuentes *et al.* (1993, 1994) and Yagisawa *et al.* (1994) have found that the N-terminal region of PLC-δ1 is important for the binding of this enzyme to membranes containing phosphatidylinositol-4,5-bisphosphate [PtdIns(4,5)P_2], as well as to D-*myo*-inositol-1,4,5-trisphosphate [Ins(1,4,5)P_3]. The N-terminal region is not involved in catalysis but its interaction with membranes enhances enzymatic activity (Cifuentes *et al.*, 1993), and this interaction can be inhibited with Ins(1,4,5)P_3 (Cifuentes *et al.*, 1994). A PH domain has been found in this region

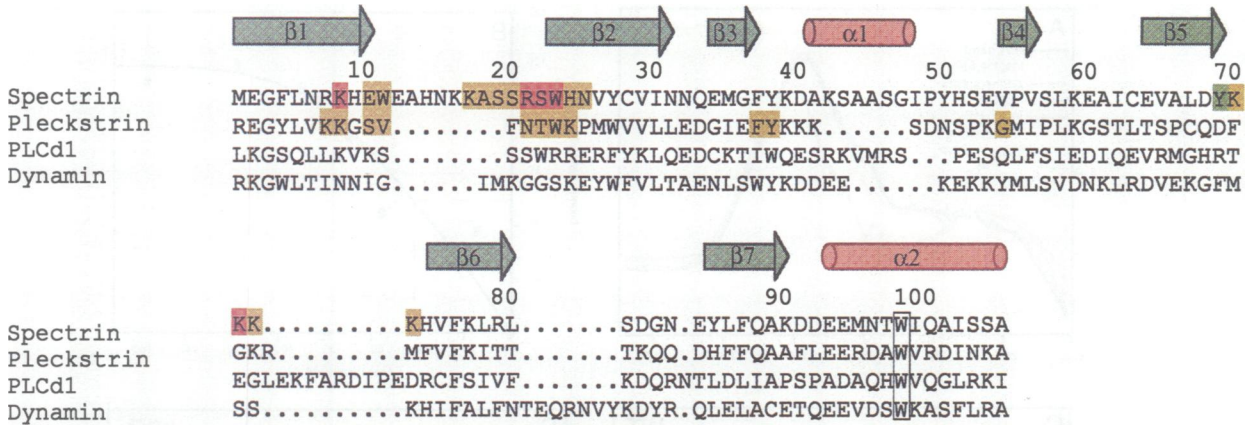

Fig. 1. An alignment of PH domain sequences. The following sequences are included with the EMBL database accession numbers and residue numbers in parentheses: mouse brain β-spectrin (M74773, residues 2198–2304), human pleckstrin (X07743, 11–95), rat phospholipase C-δ1 (M20637, 23–127) and rat dynamin-1 (X54531, 528–619). The residue numbers of the spectrin domain are given above the sequence. The β-strands and α-helices of the spectrin domain (Macias *et al.*, 1994) are shown by arrows and cylinders, respectively. All residues of the spectrin PH domain that shift upon binding of GPIns(4,5)P_2 and Ins(1,4,5)P_3 more than 0.1 p.p.m. in the NMR spectra are yellow, or red if they are involved in specific interactions with Ins(1,4,5)P_3 as determined from the crystal structure. Y69, marked with green, is not significantly shifted in the NMR spectra but is hydrogen bonded to Ins(1,4,5)P_3. The residues in the pleckstrin PH domain that are affected by the binding to PtdIns(4,5)P_2 (Harlan *et al.*, 1994) are also yellow.

(Musacchio *et al.*, 1993; Gibson *et al.*, 1994; Parker *et al.*, 1994), and in light of the present data, it is probable that this domain mediates association with the membrane via binding to PtdIns(4,5)P_2.

Fesik and colleagues have made the first explicit study on the interaction between phosphatidylinositol lipids and PH domains (Harlan *et al.*, 1994). In all cases they studied, PH domains were found to bind specifically to liposomes containing PtdIns(4,5)P_2. The estimated K_d for the binding of the N-terminal pleckstrin PH domain was 30 μM. Ins(1,4,5)P_3 inhibits the PtdIns(4,5)P_2 binding when it is added in a large excess. Using nuclear magnetic resonance (NMR), these authors also determined the changes of chemical shifts caused by the PtdIns(4,5)P_2 binding and mapped the binding site (Figure 1).

In this paper, we report a detailed characterization of the Ins(1,4,5)P_3 binding site of the PH domain from the β-chain of the cytoskeletal protein spectrin, using circular dichroic (CD) and NMR spectroscopy, and X-ray crystallography. The site is specific for inositol-4,5-bisphosphates and binds them in a 1:1 stoichiometry. It is surrounded by a positively charged surface patch which, together with the interaction with phosphatidylinositol-4,5-bisphosphate [PtdIns(4,5)P_2], is likely to facilitate anchoring of the PH domain to the membrane.

Results

Specificity of phosphoinositol binding

Figure 2 shows how binding of inositol phosphates to the spectrin PH domain perturbs circular dichroic spectrum of the protein in the near UV region. Three compounds, L-α-glycerophospho-D-*myo*-inositol-4,5-bisphosphate [GPIns(4,5)P_2] which corresponds to the water-soluble portion of PtdIns(4,5)P_2, Ins(1,4,5)P_3 and Ins(1,3,4,5)P_4 cause a spectral shift. Using this assay, we have tested several soluble inositol phosphates and found that only those with phosphate groups both in 4- and 5-positions bind to this domain. The inositol compounds lacking the bisphosphate such as Ins(1)P, Ins(1,4)P_2 or Ins(1,3,4)P_3 do not affect the CD spectrum (Figure 2A). The specificity for 4,5-bisphosphates has also been found in the previous binding data with PLC-δ1 (Cifuentes *et al.*, 1993, 1994; Yagisawa *et al.*, 1994).

Exact binding affinities could not be determined reliably due to high noise in these experiments, but titrations using the CD signal suggest that the wild-type spectrin domain binds GPIns(4,5)P_2 with a K_d in the 40 μM range (Figure 2B).

Mapping of the binding site by NMR spectroscopy

The binding of inositol phosphates to the spectrin PH domain was also investigated by NMR spectroscopy. The compounds used were GPIns(4,5)P_2, Ins(1,4,5)P_3, Ins(1)P and inositol. Strong perturbations of chemical shifts of the PH domain residues became evident when either of the two 4,5-bisphosphorylated ligands was added in 2-fold excess. The induced changes are confined to the positive side of the dipolar domain (Ferguson *et al.*, 1994; Harlan *et al.*, 1994; Macias *et al.*, 1994) where a number of residues are affected. In both complexes, the changes in chemical shifts involve the same amino acid residues, showing that the two compounds bind in a similar manner. In contrast, Ins(1)P or unsubstituted inositol do not cause any changes in the NMR spectrum.

The residues with the largest changes of chemical shifts (>0.3 p.p.m.) are K8, S22, W23 and K71 (Figure 1 and Table I). Some of the equivalent residues of the pleckstrin PH domain are also perturbed by the PtdIns(4,5)P_2 binding (Harlan *et al.*, 1994) as indicated in Figure 1. Among these, the K8 residue is particularly affected. Both $C_\varepsilon H$ protons of this highly ordered lysine dramatically change their spectral position by ~0.8 p.p.m. upon binding of the inositol bisphosphates (Table I).

In addition to the altered chemical shifts, some residues in this area show a change in line width. The signal of the imino proton of W23, for example, shows a very broad spectral line in the absence of ligands. This line becomes very sharp in the presence of either of the 4,5-bisphosphorylated ligands. Furthermore, the signals of

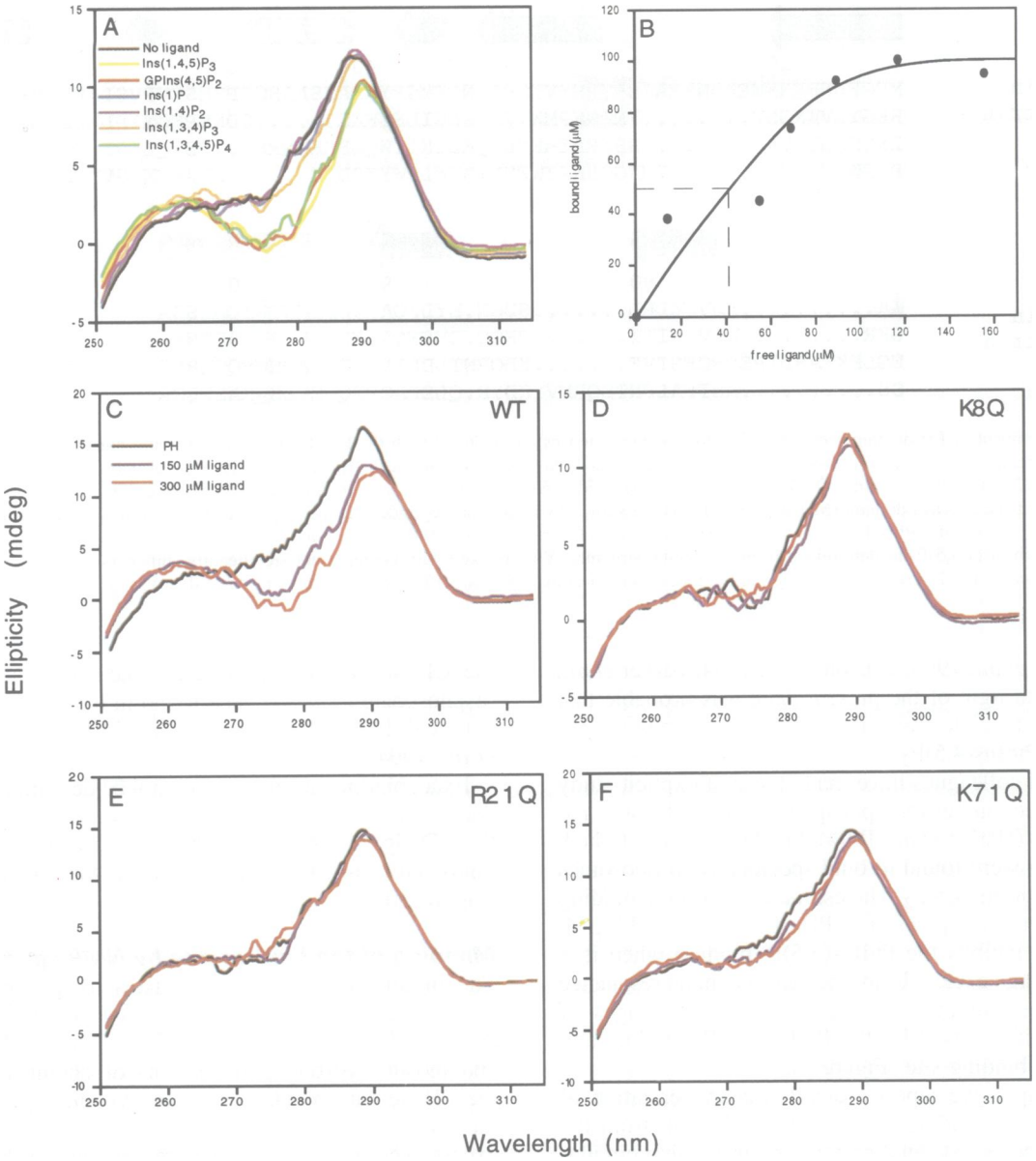

Fig. 2. (**A**) Near-UV CD spectra of the spectrin PH domain (100 μM) without a ligand and with 300 μM each of Ins(1)P, Ins(1,4)P_2, GPIns(4,5)P_2, Ins(1,3,4)P_3, Ins(1,4,5)P_3 and Ins(1,3,4,5)P_4. Colour coding is indicated in the figure. (**B**) The binding curve of GPIns(4,5)P_2 to the spectrin PH domain as monitored by changes in the CD spectrum at 290 nm. Protein concentrations were kept constant (100 μM). The data points are means of three experiments. (**C–F**) CD spectra of the wild-type PH domain and the K8Q, R21Q and K71Q mutants without the ligand (black lines), and with 150 μM (blue) and 300 μM (red) GPIns(4,5)P_2.

Table I. Changes in the NMR chemical shifts upon ligand binding

		None	GPIns(4,5)P_2		Ins(1,4,5)P_3		Ins(1)P
		δ[p.p.m.]	δ[p.p.m.]	Δδ[p.p.m.]	δ[p.p.m.]	Δδ[p.p.m.]	Δδ[p.p.m.]
K8	Hε1	2.64		–0.82		–0.83	0.00
	Hε2	2.61		–0.73		–0.75	+0.01
R21	HN	–	8.56		8.54		–
S22	HN	7.97		+0.32		+0.37	+0.01
	Hβ2	3.73		–0.07		–0.18	0.00
W23	HN	8.56		+0.32		+0.37	+0.01
	Hδ1	–	7.40		7.41		–
	Hε1	–	10.63		10.58		–
K71	HN	6.84		–0.50		–0.50	0.00
	Hδ1,2	1.26		–0.11		–0.10	0.00

The table shows the δ[p.p.m.] values of chemical shifts for protons in five residues of the PH domain as measured by 2-D NMR in the presence and absence of inositol phosphates. In three cases, the proton signals cannot be found in the absence of the ligand but appear when it is bound.

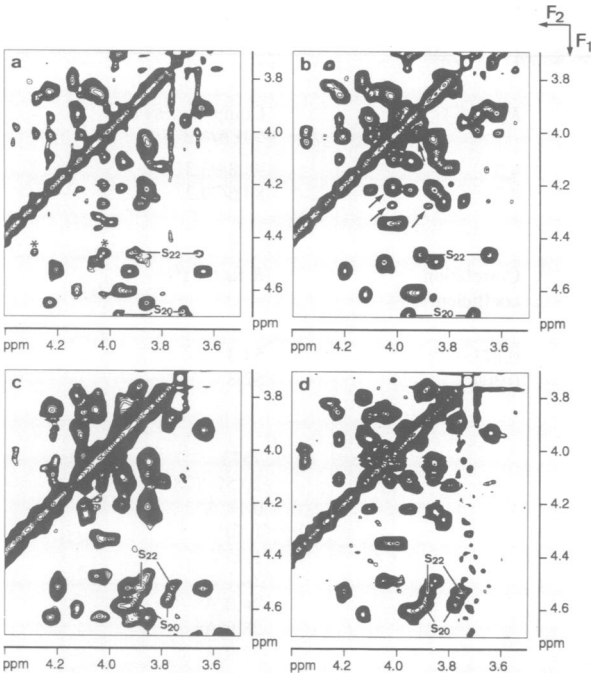

Fig. 3. (**a**) A region of the NOESY spectrum of the spectrin PH domain (1 mM protein) with GPIns(4,5)P_2 (2 mM) showing cross-peaks between the CaH of S22 at 4.47 p.p.m. and inositol resonances (*). (**b**) The same region of the TOWNY spectrum of the complex. The arrows indicate cross-peaks of the inositol coupling network. (**c** and **d**) The same regions of the NOESY and TOWNY spectra of the PH domain without the ligand.

R21 which could not be found in the absence of the ligands are readily observed in their presence (Table I). The bound ligand restricts the mobility of residues R21 and W23, and their signals return to normal line widths.

We also found two NOEs between the inositol compounds and the $C_\alpha H$ of residue S22 (Figure 3). It was possible to assign them because this $C_\alpha H$ signal at 4.47 p.p.m. shows no overlap with other proton signals in the spectra of the complexes. The relevant regions of the TOWNY (TOCSY without NOESY, see Kadkhodaei *et al.*, 1993) and NOESY (Jeener *et al.*, 1979) spectra with and without GPIns(4,5)P_2 are shown in Figure 3. The additional signals (* in Figure 3a) have considerable intensity and do not occur in the TOWNY spectrum (Figure 3b) or in the NOESY spectrum in the absence of the ligand (Figure 3c). The arrows in Figure 3b indicate the cross-peaks of the inositol coupling network. These are absent from the TOWNY spectrum without the ligand (Figure 3d).

Figure 1 highlights the residues that are strongly (>0.3 p.p.m., red) or weakly (>0.1 p.p.m., boxed) affected upon ligand binding to the spectrin PH domain. Some of the equivalent residues of the pleckstrin PH domain are also perturbed by the PtdIns(4,5)P_2 binding (Harlan *et al.*, 1994; yellow in Figure 1). The NMR data and the sequence conservation suggest that the binding sites are essentially the same in the spectrin and pleckstrin domains.

Three-dimensional structure of the binding site

The crystal structure of the PH domain of β-spectrin in a complex with Ins(1,4,5)P_3 was determined at 2.0 Å resolution (Table II). This structure was solved using molecular replacement methods with the previous NMR structure of the same protein (Macias *et al.*, 1994) as a template. The refined crystal structure deviates from the used NMR model template by ~1.6 Å if the main chain atoms are used for superposition. The strongest deviations are found in the loops close to the Ins(1,4,5,)P_3 binding site. A detailed comparison of these structures will be published separately.

Ins(1,4,5)P_3 could be located unambiguously in the electron density map (Figure 4A). It is bound between two loops that connect β-strands 1–2 and 5–6 (Figure 4B). Most of the interactions are salt bridges between phosphate groups and positively charged amino acid residues (Figure 5). The 4- and 5-phosphate groups are anchored by salt bridges with K8 and R21 and by hydrogen bonds with W23 and Y69. The side chain of K71 is in the close vicinity of the 4-phosphate group. The terminal amino group of K71 is, however, not in a position that would clearly define a salt bridge. The key residue K8 is entirely buried upon Ins(1,4,5)P_3 binding. The 1-phosphate group, in contrast, is only linked through one hydrogen bond to S22 and is mostly solvent-exposed. The are no specific bonds between the remaining hydroxyl groups of the inositol ring and PH residues except one hydrogen bond between the 3-hydroxyl group and a bound water molecule that is in turn hydrogen-bonded to the main chain NH group of W23.

The crystal structure of the binding site closely agrees with the solution binding data (Table I and Figure 1). The key residues are shown in Figure 5A. To verify the roles of residues K8, R21 and K71 in binding, they were individually substituted with glutamines, and the effects of these mutations on the interaction with GPIns(4,5)P_2 were measured using near-UV CD spectroscopy. Figure 2D and E show that the K8Q and R21Q mutations abolish the binding, as expected. The K71Q mutation does not abolish the binding completely, but clearly lowers the affinity (Figure 2F). As a negative control, mutagenesis of the K17 residue which is not involved in the binding site does not abolish ligand binding (not shown).

Structural basis of specificity

We have analysed the binding site by modelling several inositol phosphates onto the bound Ins(1,4,5)P_3 ligand. The orientation of Ins(1,4,5)P_3 demonstrates the importance of the 4- and 5-phosphate groups as major binding determinants. The structure implies that the L-isoform of Ins(1,4,5)P_3 cannot fit to the binding site, consistent with the finding that it does not inhibit binding of PtdIns(4,5)P_2 to PLC-δ1 (Cifuentes *et al.*, 1994). The presence of a 3-phosphate group in the inositol ring could be accommodated without any steric clashes. There are, however, no side chains in a potential 3-phosphate group binding site of the β-spectrin PH that could generate specific interactions and increase binding affinity. This is reflected in the decreased affinity of Ins(1,3,4)P_3 for the domain (Figure 2A).

In a recent paper, Heinz *et al.* (1995) describe how inositol binds to a bacterial phosphatidylinositol-specific phospholipase C (inositol is a weak inhibitor of this enzyme). The crystal structure of the complex shows a large number of interactions via hydrogen bonds and van der Waals contacts. This is different from the interactions

Table II. Summary of crystallographic analysis

Crystallographic data sets				
	Resolution (Å)	Reflections measured/unique	R_{sym} (%)	Completeness (%) ≥0 σ/≥3 σ
Native I	2.8	19 481/3261	4.3	98.8/93.3
Native II	2.0	41 713/7830	4.7	89.6/79.2
Molecular replacement				
	Rank	Peak height (σ)	Correlation coefficient	*R*-factor (%)
Rotation	7	4.0		
Translation	1		0.271	54.3
Fitting			0.309	52.8
Structure refinement				
Data used for X-PLOR refinement and map calculations:				7573
R-factor (R-free) of final model:				20.5% (28.6%)
R.m.s. deviation from ideal bond length:				0.11 Å
R.m.s. deviation from ideal angles:				1.50°
R.m.s. deviations from ideal planar groups:				1.35°
B(average) of 526 protein main chain atoms:				20.1 Å^2
B(average) of 338 protein side chain atoms:				24.0 Å^2
B(average) of 77 water oxygen atoms:				28.6 Å^2
B(average) of 24 Ins(1,4,5)P_3 atoms:				27.9 Å^2

$R_{sym} = \Sigma_{i,h}|I(i,h)-<I(h)>|/\Sigma_{i,n}|I_{(i,h)}|$ where $<I(h)>$ is the mean of the i observations of reflection h. Correlation coefficient and *R*-factor are defined by the program AMORE (Navaza, 1987). All data above 1 σ of the native II dataset between 8.0 and 2.0 Å resolution were used for structure refinement. 10% of the data were used for the calculation of R_{free}.

seen in the complex of PH domain and Ins(1,4,5)P_3 in which the inositol ring plays virtually no role (Figure 5A).

Discussion

Anchoring to the membrane

The Ins(1,4,5)P_3 binding sites of the pleckstrin and β-spectrin PH domains are in the centre of a large, positively charged surface, illustrated in Figure 6. This surface patch is rather flat and has a triangular shape. In the spectrin domain, it can be subdivided into three clusters of residues with positively charged side chains: (i) K16 and K17; (ii) H24, H51 and K39; and (iii) K70, K71, K72 and K73.

We propose a model where binding to membrane-bound PtdIns(4,5)P_2 is facilitated through an ensemble of bonds between positive residues of this surface patch on PH and negatively charged head groups of the lipid bilayer (Figure 7). In the orientation of the bound Ins(1,4,5)P_3 ligand (Figure 5), the 1-phosphate group is solvent exposed, which would enable PtdIns(4,5)P_2 to reach the binding site from membrane surface. The PtdIns(4,5)P_2-complexed PH domain would be attached to the membrane surface through salt bridges and hydrogen bonds. In the case of PLC-δ1, the binding to PtdIns(4,5)P_2-containing vesicles is enhanced by phosphatidylserine (Cifuentes *et al.*, 1994). Supposing that this binding is mediated by the PH domain, it implies that the interaction between positive charges on the protein and negative charges on the membrane contributes to the stability of the attachment.

The multiple alignment of 92 PH domains identified so far (Musacchio *et al.*, 1993; Gibson *et al.*, 1994; T.Gibson, personal communication) reveals an outstanding sequence diversity. There is neither a conserved surface patch nor a cavity in the known PH structures that could help to identify areas crucial for a common function. Some of the residues involved in the Ins(1,4,5)P_3 binding site and most of the residues that contribute to the positively charged surface patch in the β-spectrin PH cannot be aligned unambiguously with the remaining PH sequences due to divergent loops with variable lengths. The only exceptions seem to be positions 8 and 23, K8 and W23 in the β-spectrin PH. The first position is a positively charged residue only in about half of the aligned sequences. The latter is an aromatic residue, mostly tryptophan in ~75% of all sequences. Both residues are conserved in the pleckstrin and PLC-δ1 PH domains (Figure 1). The binding site in the PLC-δ1 PH domain shares the key K8 residue with the spectrin and pleckstrin domains, and the RSW motif (residues 21–23, NTW in the pleckstrin domain) is replaced with a SWR sequence (Figure 1). In contrast, these key residues are absent from the dynamin PH domain (Figure 1) for which the PtdIns(4,5)P_2 binding data are controversial (Ferguson *et al.*, 1994; Fushman *et al.*, 1995).

Biological implications

Further support for the role of the spectrin PH domain in membrane anchoring comes from experimental results showing that the C-terminus of β-spectrin binds to alkali-washed brain membranes. This binding is restricted to the general form of β-spectrin which contains the PH domain. The corresponding fragment of the human erythrocyte spectrin without this domain does not bind (Davis and Bennett, 1994).

We have estimated that the dissociation constant for the spectrin PH domain complex is ~40 μM using 100 μM protein in CD measurements. A similar value (30 μM) has been published by Harlan *et al.* (1994) for the binding of PtdIns(4,5)P_2 to the PH domain of pleckstrin. The K_d values in this range are probably too high to account for binding to soluble inositol phosphates such as Ins(1,4,5)P_3. In contrast, the local concentrations of PtdIns compounds can be rather high in cellular membranes. For instance, it

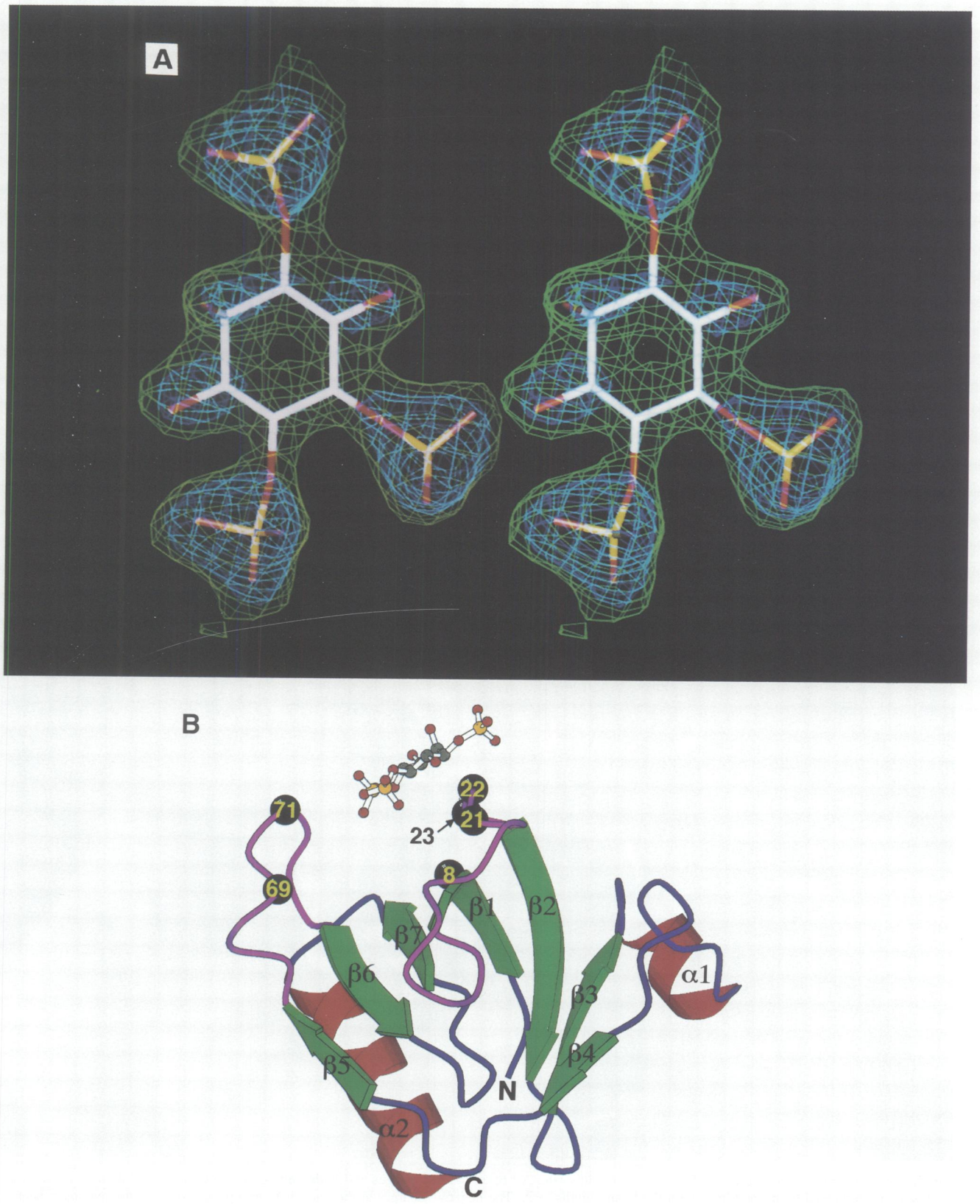

Fig. 4. (**A**) Ins(1,4,5)P_3 in a $2F_o$–F_c electron density map with coefficients derived by the program SIGMAA (Read, 1986). Contour levels: 1 σ, green; 2 σ, blue. The refined structure without Ins(1,4,5)P_3 and four water molecules in the close vicinity of Ins(1,4,5)P_3 were submitted to positional refinement by X-PLOR (Brünger, 1992) refinement prior map calculation. (**B**) A schematic drawing of the structure of the β-spectrin PH domain:Ins(1,4,5)P_3 complex. The PH domain is shown by a ribbon with colour codes: β-strands, green; α-helices, red; Ins(1,4,5)P_3 binding site forming loops 1–2 and 5–6, pink; remaining loops, blue. The amino acids that are involved in specific interactions with Ins(1,4,5)P_3 are highlighted by black spheres with the residue numbers. The secondary structure elements and the termini are labelled. Ins(1,4,5)P_3 is shown by ball-and-stick with colour codes: carbon, grey; oxygen, red; phosphorus, yellow. The drawing was made with MOLSCRIPT (Kraulis, 1991).

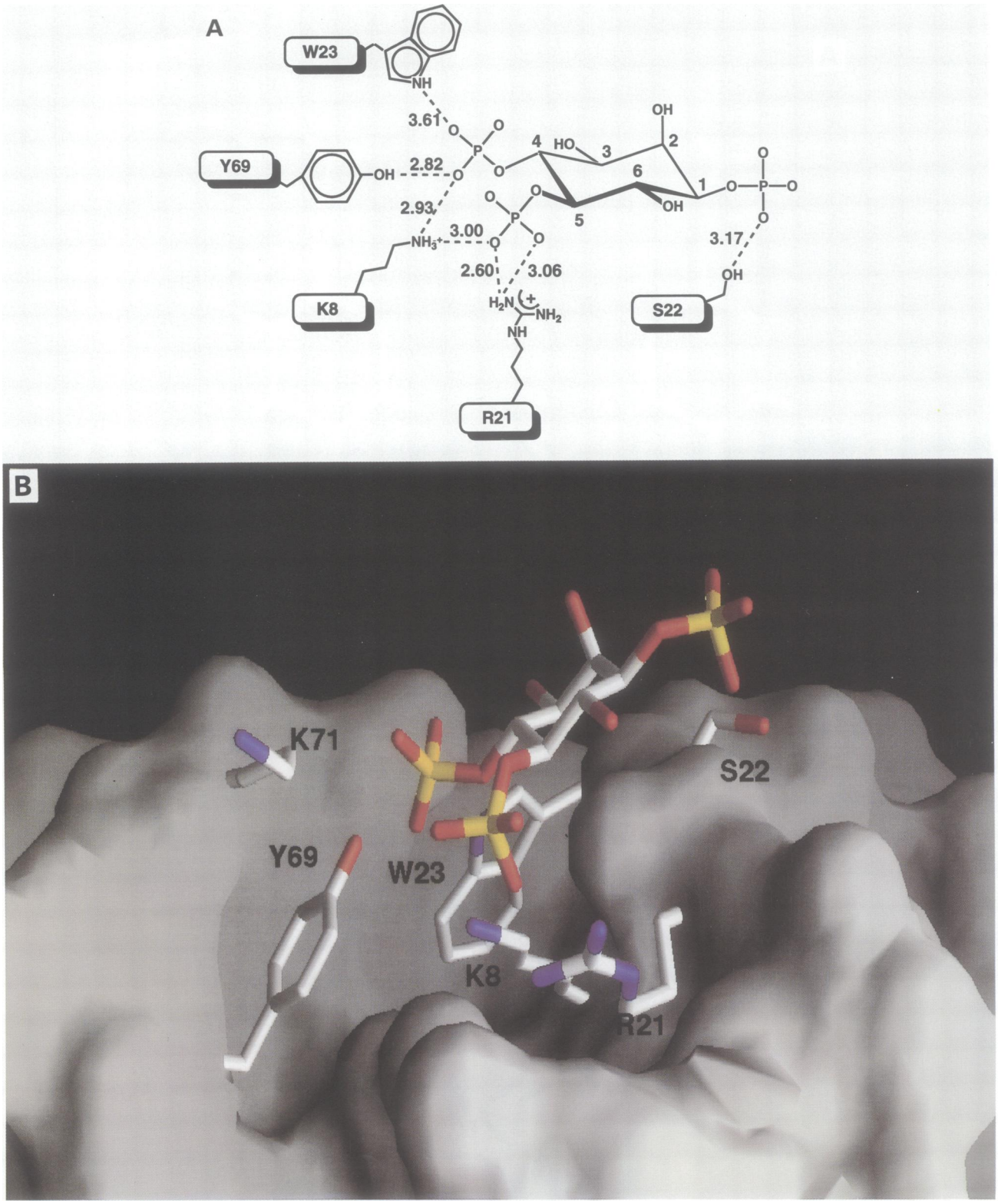

Fig. 5. (**A**) A schematic drawing of the Ins(1,4,5)P_3 binding site. All observed salt bridges and hydrogen bonds between the PH domain residues and the ligand are indicated by dashed lines with the bond lengths in Å. The negative charges at the phosphate groups and some hydrogens have been omitted from the drawing. (**B**) A three-dimensional representation of the Ins(1,4,5)P_3 binding site. Those residues that are specifically involved in the Ins(1,4,5)P_3 binding and the bound ligand are shown by sticks. Colour codes and the view are as in Figure 4B. This drawing was made with GRASP (Nicholls *et al.*, 1991).

has been reported that the PtdIns(4,5)P_2 concentrations in cultured cells are 30–160 mM when averaged throughout the cell volume (Bunce *et al.*, 1993), and the local concentration of the phospholipid could be considerably higher.

In the cases of PLC-δ1 and PLC-γ, which both contain

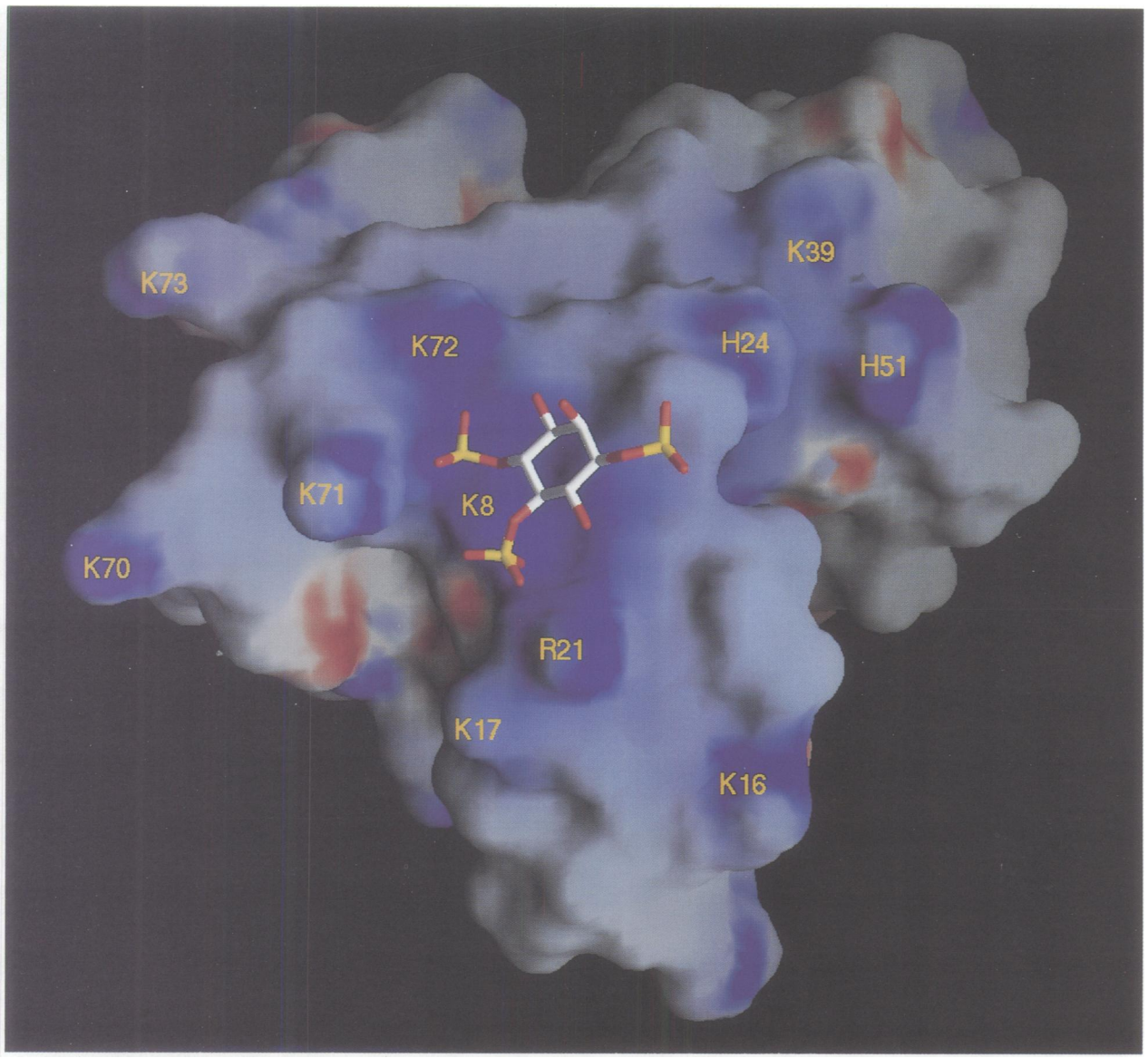

Fig. 6. Top view to the surface of β-spectrin PH domain around the Ins(1,4,5)P_3 binding site. The PH domain: Ins(1,4,5)P_3 complex is rotated by ~90° around a horizontal axis with respect to the orientation given in Figure 4B. The electrostatic surface potential is represented by a colour range from blue (positive) to red (negative). All charged residues that are exposed to the surface patch surrounding the binding site are labelled at their approximate positions. Ins(1,4,5)P_3 is shown by sticks with colour codes as in Figure 4. The figure was prepared with GRASP (Nicholls, 1993).

a PH domain (Musacchio *et al.*, 1993; Parker *et al.*, 1994), PH domains may be involved in localization of the catalytic activity close to the substrate (Rebecchi *et al.*, 1992; Cifuentes *et al.*, 1993). These phospholipases are released from interfacial position on the membrane when the concentration of the soluble inositol phosphates increases due to enzymatic hydrolysis (Cifuentes *et al.*, 1993; Yagisawa *et al.*, 1994). In an analogous manner, the interaction of PH domains with membranes containing phosphoinositides could be controlled by the concentrations of these phospholipids and soluble phosphoinositols. A high PLC activity, for instance, might lead to a release of signalling proteins with PH domains from their target membranes.

Another function of PH domains might be the interaction with second messengers such as Ins(1,4,5)P_3. They could have a receptor function, transmitting signals to other domains in the host protein. There is a strict neighbouring arrangement (Musacchio *et al.*, 1993) of Cdc24 and PH domains in a family of proteins that, among other things, possess guanine nucleotide exchange factor activity for Rho and Rac, small G-proteins that are involved in cytoskeletal dynamics (Boguski and McCormick, 1994). This grouping is in contrast to the generally promiscuous positioning of the PH domain in its host proteins. The PH domain could regulate the nucleotide exchange activity of the adjacent Cdc24 domain in a way that could be controlled by the binding of an inositol phosphate compound. This could link G-protein-coupled control on cytoskeletal dynamics to phospholipid signalling.

A number of point mutations have been identified within the PH domain of the Bruton's tyrosine kinase (Btk) that is involved in the maturation of B cells. These mutations cause an immunodeficiency disease known as X-linked agammaglobulinaemia (Rawlings *et al.*, 1993; Thomas *et al.*, 1993). However, the proposed arrangement of these

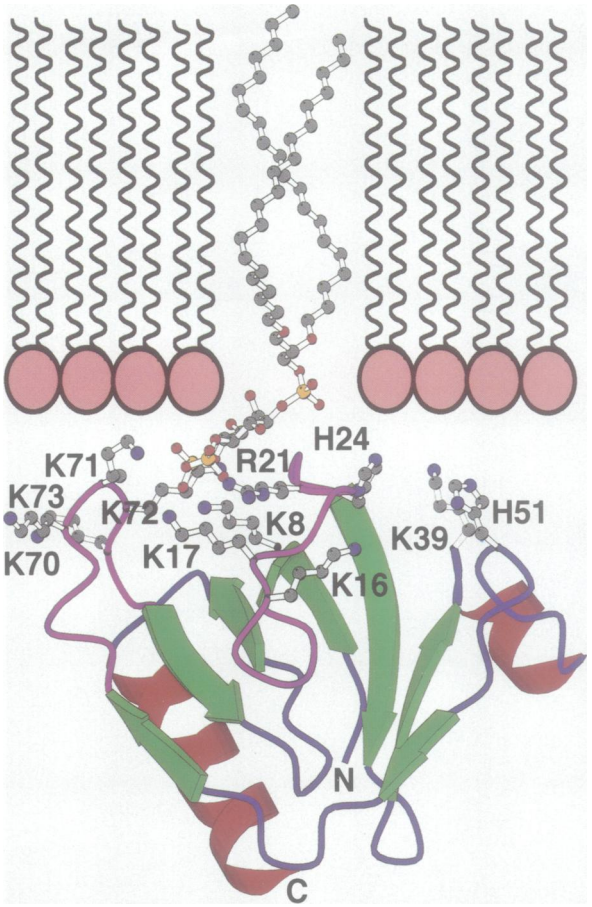

Fig. 7. A model of the membrane-bound PtdIns(4,5)P_2:spectrin PH domain complex. The ribbon drawing is in the same orientation and has the same colour codes as Figure 4B. The side chains of the positively charged residues surrounding the Ins(1,4,5)P_3 binding site (Figure 6) are shown by ball-and-stick. The Ins(1,4,5)P_3 moiety of PtdIns(4,5)P_2 is based on the coordinates of the crystal structure whereas the rest of this phospholipid is a model. Phospholipid head groups are oriented towards the positively charged surface of the PH domain. The figure was prepared with MOLSCRIPT (Kraulis, 1991).

mutations in the modelled Btk PH domain (Vihinen *et al.*, 1995) would not map with the Ins(1,4,5)P_3 binding site in the spectrin PH domain. Binding to inositol phosphates may not be the property of all PH domains, but they may have other functions which remain to be discovered.

In conclusion, our structural analysis leads to a model that explains how a PH domain complexed with PtdIns(4,5)P_2 could associate with the membranes. This paper presents a high-resolution structure of a complex between a protein and a phospholipid head group. Protein–lipid interactions are important for many processes in intracellular sorting and signalling. The structural basis for the proposed specific binding of inositol lipids as well as soluble phosphoinositols to a ubiquitous protein domain may help to understand these interactions in molecular detail.

Materials and methods

Purification

The spectrin PH domain was expressed in *Escherichia coli* as described previously (Macias *et al.*, 1994). Purification from the soluble fraction of cell lysate was achieved using S-Sepharose cation exchange, Superdex 75 gel filtration and Q-Sepharose anion exchange columns. Protein concentrations were determined spetrophotometrically in 6 M guanidinium chloride at 280 nm using the calculated molar absorptivity 23 590 M^{-1} cm^{-1}. Mutagenesis of the domain was carried out using polymerase chain reaction; all DNA constructs were verified by dideoxy sequencing. Mutants were cloned under the T7 promoter in the pBAT expression vector (J.Peränen, unpublished). Expression and purification were performed as with the wild-type protein.

Spectroscopy

Inositol phosphates were purchased from Sigma (St Louis, USA), LC Laboratories Europe (Läufelingen, Switzerland) or Calbiochem (San Diego, USA). They were dissolved at 20 mM concentration in MilliQ pure water and kept at –80°C until use. CD spectroscopy was performed with a Jasco J-710 spectropolarimeter in 10 mM sodium phosphate, 100 mM NaCl, pH 6.5, in a 1 cm cuvette at 25°C. Protein concentration was always 100 mM. Spectra were smoothed with a 2.5 nm window for clarity.

The NMR measurements were carried out using a 1 mM sample of the mouse β-spectrin PH domain in 90% H_2O/10% D_2O, 10 mM sodium acetate, pH 6.5, and 2-fold excess of the ligands GPIns(4,5)P_2, Ins(1,4,5)P_3, Ins(1)P and inositol with a Bruker AMX-600 spectrometer at 30°C. NOESY (Jeener *et al.*, 1979) and TOWNY (Kadkhodaei *et al.*, 1993) spectra were recorded using the WATERGATE pulse sequence for water suppression (Piotto *et al.*, 1992). Mixing times were 40 ms for the TOWNY and 80 ms for the NOESY experiments.

Crystallographic analysis

For crystallization, the β-spectrin PH domain (20 mg/ml in 10 mM sodium phosphate, pH 6.5) was mixed with 3-fold excess of Ins(1,4,5)P_3. The resulting solution was mixed with the reservoir solution [10–15% (w/v) PEG-6000 and 30% (v/v) ethanol in 100 mM sodium acetate, pH 4.8] in a 1:1 ratio. Crystals were obtained by the hanging-drop method after 2 days at room temperature. The crystals were grown as needles up to 3 mm long. The maximum thickness of these needles was 0.2 mm in both dimensions. The crystallographic parameters of these crystals are: space group $P42_12$, a = 68.9 Å, c = 50.8 Å, one molecule per asymmetric unit.

Two crystallographic data sets were collected in-house from Cu-K_α radiation. The first data set to 2.8 Å resolution was recorded on a Mar Imaging plate detector with a radius of 90 mm, mounted on a Siemens/MacScience generator MX18 with a 300 mm focus operating at 40 kV and 90 mA. The second data set was recorded on a Mar Imaging plate detector with a radius of 150 mm, using an Enraf–Nonius generator FR571 with a 300 mm focus operating at 40 kV and 70 mA. Both data sets were reduced and processed with the XDS program package (Kabsch, 1988).

The structure of the β-spectrin PH domain–Ins(1,4,5)P_3 complex was solved by molecular replacement with a refined NMR model of the spectrin PH-domain (Macias *et al.*, 1994; M.Nilges, unpublished results) using the AMORE program package (Navaza, 1987). All available crystallographic data between 8.0 and 3.0 Å were used in each step of AMORE. The radius of integration was 19 Å. The NMR model contained pseudo B-factors for each scattering atom that were derived from spatial atomic distances averaged over 15 refined NMR structures. Details on the derivation of these pseudo B-factors will be published elsewhere. The used translation solution was validated by successful rigid-body refinement and graphical inspection of the packing of the molecules within the unit cell.

The structure was refined as follows. The SIGMAA-weighted (Read, 1986) $2F_o$–F_c map with phases from the correctly positioned NMR model was immediately interpretable for about two-thirds of the sequence. The electron density was iteratively improved by employing a cyclic procedure that included model building using the crystallographic graphics program O (Jones *et al.*, 1991), molecular dynamics refinement with XPLOR (Brünger, 1992) and improvement of electron densities with the program ARP (Lamzin and Wilson, 1993). In addition, several programs of the CCP4 suite (1994) were used. The refinement procedure was repeated ten times.

In the course of the refinement, we were able to reinterpret all segments of the β-spectrin PH domain X-ray structure which were considerably different from the NMR structure. After the fifth refinement cycle non-protein atoms were included into subsequent models. The three highest peaks in the difference electron density map were interpreted as phosphate groups of the Ins(1,4,5)P_3 molecule. All three peaks showed a tetrahedral shape typical for phosphate groups. The sugar moiety of Ins(1,4,5)P_3 became interpretable in later stages of refinement. The final

position of Ins(1,4,5)P_3 was validated by monitoring decrease of R_{free} and calculation of OMIT maps of molecular dynamics refined models without the Ins(1,4,5)P_3. The orientation of Ins(1,4,5)P_3 could be identified unambiguously from the arrangement of the three phosphate positions and the axial orientation of the 2-hydroxyl group of the inositol ring. The statistics of the final model are given in Table II.

Acknowledgements

We are grateful to Drs Sara Courtneidge, Toby Gibson, Werner Kühlbrandt and Andrea Musacchio for critical reading of the manuscript, and to Dr Ana Rosa Viguera for her help with CD spectroscopy.

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Received on May 30, 1995; revised on June 29, 1995