Structural and Membrane Binding Analysis of the Phox Homology Domain of Bem1p

BASIS OF PHOSPHATIDYLINOSITOL 4-PHOSPHATE SPECIFICITY

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Robert V. Stahelin 1,5*, Dimitrios Karathanassis 1,1, Diana Murray**, Roger L. Williams 1, and Wonhwa Cho 1,2

From the 1Department of Chemistry, University of Illinois, Chicago, Illinois 60607-7061, the 8Department of Biochemistry and Molecular Biology, Indiana University School of Medicine, and the 6Department of Chemistry and Biochemistry and the Walther Center for Cancer Research, University of Notre Dame, South Bend, Indiana 46617, the 1Medical Research Council Laboratory of Molecular Biology, Cambridge CB2 2QH, United Kingdom, and the 8Department of Microbiology and Immunology, Weill Medical College of Cornell University, New York, New York 10021

Phox homology (PX) domains, which have been identified in a variety of proteins involved in cell signaling and membrane trafficking, have been shown to interact with phosphoinositides (PIs) with different affinities and specificities. To elucidate the structural origin of the diverse PI specificity of PX domains, we determined the crystal structure of the PX domain from Bem1p that has been reported to bind phosphatidylinositol 4-phosphate (PtdIns(4)P). We also measured the membrane binding properties of the PX domain and its mutants by surface plasmon resonance and monolayer techniques and calculated the electrostatic potentials for the PX domain in the absence and presence of bound PtdIns(4)P. The Bem1p PX domain contains a signature PI-binding site optimized for PtdIns(4)P binding and also harbors basic and hydrophobic residues on the membrane-binding surface. The membrane binding of the Bem1p PX domain is initiated by nonspecific electrostatic interactions between the cationic membrane-binding surface of the domain and anionic membrane surfaces, followed by the membrane penetration of hydrophobic residues. Unlike other PX domains, the Bem1p PX domain has high intrinsic membrane penetrating activity in the absence of PtdIns(4)P, suggesting that the partial membrane penetration may occur before specific PtdIns(4)P binding and last after the removal of PtdIns(4)P under certain conditions. This structural and functional study of the PtdIns(4)P-binding Bem1p PX domain provides new insights into the diverse PI specificities and membrane-binding mechanisms of PX domains.

Phosphoinositides (PIs), 3 phosphorylated derivatives of phosphatidylinositol (PtdIns), regulate diverse biological pro-

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1 Present address: Research Center for Biomaterials S. A., 15, 16562 Glyfada-Athens, Greece.
2 To whom correspondence should be addressed: Dept. of Chemistry (M/C 111), University of Illinois, 845 West Taylor St., Chicago, IL 60607-7061. Tel.: 312-996-4883; Fax: 312-996-2183; E-mail: wcho@uic.edu.
3 The abbreviations used are: PIs, phosphoinositides; PtdIns, phosphatidylinositol; PH, pleckstrin homology; FYVE, Fab1/YOTB/Vac1/EEA1; PX, Phox homology; ENTH, epsin N-terminal homology; SH3, Src homology 3; PLD, phospholipase D; CISK, cytokine-independent survival kinase; PI3K-C2α, phosphoinositide 3-kinase C2α; PS, phosphatidylserine; SPR, surface plasmon resonance; POPC, 1-palmitoyl-2-oleoyl-sn-glycerol-3-phosphocholine; POPS, 1-palmitoyl-2-oleoyl-sn-glycerol-3-phosphoserine; POPE, 1-palmitoyl-2-oleoyl-sn-glycerol-3-phosphoethanolamine; CHAPS, 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonic acid; MAD, multilayer anomalous dispersion; Bem1p-PX, Bem1p PX domain; OSBP, oxysterol-binding protein; Ppα, type II polyproline helix.
membrane during budding and mating, where it can serve as an adaptor for Cdc42p and other proteins (35, 36). The mechanism behind the plasma membrane translocation of Bem1p is still unknown. Interestingly, Bem1p has been shown to harbor a PX domain that binds PtdIns(4)P (37). PtdIns(4)P has been shown to be localized to both the plasma membrane and secretory machinery in yeast (27). The molecular details of a number of protein-protein interactions have been mapped out for Bem1p (38), but much less is known about its lipid binding properties, in particular the role of its PX domain in the membrane recruitment of Bem1p.

The PX domain is a structural module composed of 100-140 amino acids that was first identified in the p40phox and p47phox subunits of NADPH oxidase (39) and has since been found in a variety of other proteins involved in membrane trafficking (e.g. Mvp1p, Vps5p, Bem1p, Grd19p, and the sorting nexin family of proteins) and cell signaling (e.g. phospholipase D (PLD), PI3-kinases, cytokine-independent survival kinase (CISK), and five SH3 domains (FISH)). Sequence comparisons of PX domains have shown that they contain several conserved regions, including a proline-rich stretch (PAXP) and a number of basic residues (13, 14). Subsequently, PX domains have been shown to interact with different PIs via conserved basic residues and to target the host proteins to specific subcellular locations (40-45). PX domains are similar to the PH domain in that they target the host proteins to specific subcellular locations (40-45).

For structure determination, DNA encoding the yeast Bem1p PX domain (residues 266-413) was cloned with a C-terminal His6 affinity tag in the pJL vector. The protein was expressed in Escherichia coli strain strain 834(DE3) and purified by Ni2+ affinity, heparin, and gel filtration chromatography. The protein in gel filtration buffer (20 mM Tris- HCl (pH 7.4 at 25 °C), 100 mM NaCl, and 5 mM dithiothreitol) was concentrated to 5 mg/ml. Crystals were obtained in sitting drops (3 l of reservoir solution) that were incubated at 14 °C over 24 h and grown to full size within 1 week.

Recent structural and modeling studies of a variety of PX domains have led to a better understanding of the mechanisms of stereospecific PI recognition and membrane binding by PX domains. Earlier structural studies focused on PX domains that interact with PtdIns(3)P. For example, the crystal structure of the p40phox-PtdIns(3)P complex illustrated how the domain achieves the stereospecific recognition of PtdIns(3)P (51). The structure revealed that basic residues Lys38 and Arg68 specifically form hydrogen bonds with the D1- and D3-phosphates of PtdIns(3)P, respectively. The crystal structure of the CISK PX domain showed that this domain also has all the basic residues necessary for binding the D3-phosphate of PtdIns(3)P (52). The crystal structures of the free and PtdIns(3)P-bound PX domains of the yeast Grd19p protein showed the lipid-induced local conformational changes in the membrane-binding loop (53). NMR studies of the Vam7p PX domain have also elucidated the origin of its PtdIns(3)P specificity and the membrane-docking mechanism (54, 55).

In addition to these studies on PtdIns(3)P-binding PX domains, structural studies on the p47phox (47) and PI3K-C2α (46) PX domains that specifically interact with PtdIns(3,4)P2 and PtdIns(4,5)P2, respectively, showed how these PX domains achieve different PI specificities. In particular, the crystal structure of the PX domain of p47phox revealed that this PX domain has a smaller secondary pocket that binds phosphatidic acid or phosphatidylinerine (PS) (47). A modeling study of the PLD1 PX domain also suggested that it has two binding pockets, a primary site specific for PtdIns(3,4,5)P3 and a second site that interacts nonspecifically with anionic phospholipids (49).

To date, no structural information is available for the PX domains with specificity for PtdIns(4)P. To gain a better understanding of differential PI recognition and membrane-binding mechanisms of PX domains, we determined the x-ray crystal structure of the Bem1p PX domain, which has been reported to bind PtdIns(4)P (37). We also measured the interaction of this domain and mutations with model membranes containing various PIs by surface plasmon resonance (SPR) and monolayer penetration analyses and calculated the electrostatic potential of the domain in the absence and presence of lipid ligand. The results provide new insight into how the Bem1p PX domain specifically recognizes PtdIns(4)P and how the domain may be targeted to the PtdIns(4)P-containing membranes.

**EXPERIMENTAL PROCEDURES**

**Materials**—1-Palmitoyl-2-oleoyl-sn-glycero-3-phosphatic acid, 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine (POPC), 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphoserine (POPS), and 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphoethanolamine (POPE) were from Avanti Polar Lipids (Alabaster, AL). PtdIns(3)P, PtdIns(4)P, PtdIns(5)P, PtdIns(3,4)P2, PtdIns(3,5)P2, PtdIns(4,5)P2, and PtdIns(3,4,5)P3 were purchased from Cayman (Ann Arbor, MI). Phospholipid concentrations were determined by phosphorus analysis (56). The LiposoFast microextruder and 100-nm polycarbonate filters were from Avestin (Ottawa, Ontario, Canada). Fatty acid-free bovine serum albumin was from Bayer (Kankakee, IL). Restriction endonucleases and other enzymes for molecular biology were from New England Biolabs (Beverly, MA). CHAPS and octyl glucoside were from Sigma and Fisher, respectively. The Pioneer L1 sensor chip was from Biacore (Piscataway, NJ).

**Structure Determination**—For structure determination, DNA encoding the yeast Bem1p PX domain (residues 266–413) was amplified by PCR from yeast genomic DNA and subsequently cloned with a C-terminal His6 affinity tag in the pJL vector. The protein was expressed in the methionine-requiring auxotrophic Escherichia coli strain 834(DE3) and purified by Ni2+ affinity, heparin, and gel filtration chromatography. The protein in gel filtration buffer (20 mM Tris-HCl (pH 7.4 at 25 °C), 100 mM NaCl, and 5 mM dithiothreitol) was concentrated to 5 mg/ml. Crystals were obtained in sitting drops (3 l of reservoir solution) that were incubated at 14 °C over a reservoir consisting of 0.2 mM NaCl, 0.1 M sodium/potassium phosphate (pH 6.2), 10% polyethylene glycol 8000, and 2 mM dithiothreitol. Crystals were visible after 12 h and grew to full size within 1 week.

For diffraction data collection, crystals were cryoprotected by adding Paratone-N to the drop and removing excess mother liquor surrounding the crystal. Loops containing the crystal in...
Paratone-N with minimal mother liquor were flash-frozen in a nitrogen stream at 100 K. A three-wavelength multiwavelength anomalous dispersion (MAD) data collection was carried out. Table 1 summarizes the data collection statistics. Images were processed with the program MOSFLM (57) and refined with SCALA (58). Four selenium sites were located in the subphase (10 mm HEPES containing 0.16 m KCl (pH 7.4)) had been stabilized (after ~5 min), the protein solution was injected into the subphase through a small hole drilled at an angle through the wall of the trough, and the change in surface pressure (Δπ) was measured as a function of time. The maximal Δπ value at a given πo depended on the protein concentration, and thus, protein concentrations in the subphase were maintained high enough to ensure that the observed Δπ represented a maximal value. The critical surface pressure (πc) was determined by extrapolating the Δπ versus πo plot to the x axis.

SPR Measurements—All SPR measurements were performed at 23 °C in 10 mm HEPES (pH 7.4) containing 0.16 m KCl as described previously (68, 70, 71). Following washing of the sensor chip surfaces, POPC/POPE/PI (77:20:3) and POPC/POPE (80:20) vesicles were injected at 5 ml/min onto the active surface and the control surface, respectively, to give the same resonance unit (RU) values. The level of lipid coating for both surfaces was kept at the minimum that is necessary for preventing nonspecific adsorption to the sensor chips. This low surface coverage minimized the mass transport effect and kept the total protein concentration (Pp) above the total concentration of protein-binding sites on vesicles (72). Under our experimental conditions, no binding to the control surface was detected beyond the refractive index change for all proteins. Each lipid layer was washed three times with 10 ml of 50 mM NaOH at 100 ml/min. Typically, no decrease in lipid signal was seen after the first injection. Equilibrium SPR measurements were done at the flow rate of 2 ml/min to allow sufficient time for the R values of the association phase to reach near-equilibrium values (R_{eq}) (46). After sensorgrams were obtained for five or more different concentrations of each protein within a 10-fold range of Kp each of the sensorgrams was corrected for refractive index change by subtracting the control surface response from it. Assuming a Langmuir-type binding between the protein (P) and protein-binding sites (M) on vesicles (i.e. P + M ⇌ PM) (72), R_{eq} values were then plotted versus P_{0}, and the Kp value was determined by a nonlinear least-squares analysis of the binding isotherm using the following equation: R_{eq} = R_{max}/(1 + K_{p} P_{0}) (72). Each data set was repeated three or more times to calculate the means ± S.D.
Structure of the Bem1p PX Domain

Molecular Modeling and Electrostatic Potential Calculations—The electrostatic properties of the Bem1p PX domain with and without bound lipid were calculated with a modified version of the program Delphi and visualized in the program GRASP (73) as described previously (74). The electrostatic calculations performed used partial charges taken from the CHARMM27 force field (75) and spatial coordinates from the structure of the Bem1p PX domain. Inositol 1,4-bisphosphate was docked onto Bem1p using superposition of the p40phox PX domain (Protein Data Bank code 1H6H) (51) with Bem1p and copying the coordinates of Bem1p from the superposition and those of the ligand from 1H6H. Steric clashes were fixed by side chain minimization calculation with MODELLER (76).

RESULTS

Description of the Overall Structure—Recent structural studies have elucidated the basis of the PI specificity of several PX domains, including the PtdIns(3)P-binding p40phox (51), Grd19p (53), and Vam7p (54, 55) PX domains; the PtdIns(3,4)P2-coordinating p47phox PX domain (47); the PtdIns(3,4,5)P3-binding CISK PX domain (77); and the PtdIns(4,5)P2-binding PI3K-C2a PX domain (46). To understand the mechanism by which small and structurally similar PX domains achieve such diverse PI specificity, we determined by x-ray diffraction analysis the crystal structure of the Bem1p PX domain (PDB ID: 2v6v) that has unique specificity for PtdIns(4)P (37).

The Bem1p PX domain crystallized in space group P212121 with two molecules in the asymmetric unit. The crystals diffracted to 1.5-Å resolution, and the structure was determined using MAD phasing for a selenomethionine-substituted protein. The two molecules in the asymmetric unit are nearly identical. The Bem1p PX domain features the common PX domain fold consisting of a three-stranded meander topology β-sheet packed against a helical subdomain, which contains three α-helices, a type II polyproline helix (PPII), and a 3₁₀ helix (Fig. 1A). The largest differences in the fold with respect to other PX domains are in the N- and C-terminal extensions and in the α₁-PPII loop (Fig. 1B). The Bem1p PX domain has a fourth short β-sheet (B4) at its C terminus. The α₁-PPII loop is a region that is variable among the PX domains.

The most striking difference between a PtdIns(3)P-binding PX domain and the Bem1p PX domain is that a basic residue critical for the D3-phosphate interaction in PtdIns(3)P binders (e.g. Arg₅₈ in p40phox-PX) is replaced by Tyr₃¹⁷ in Bem1p. The structure of the Bem1p PX domain reveals that Tyr₃¹⁷ is actually pointing in the opposite direction of the pocket itself (Fig. 2, A and C), leaving much of the space occupied by the side chain of Arg₅₈ in p40phox empty (Fig. 2D). Only the rotamer of Tyr₃¹⁷ pointing away could provide sufficient volume to the pocket for PI binding. Another important determinant of PtdIns(3)P binding is Lys₉² in p40phox-PX, which interacts with the D1-phosphate of PtdIns(3)P, along with Arg₆⁰, and helps orient the lipid in the correct position relative to the pocket (Fig. 2D). This interaction is absent in Bem1p, as the position corresponding to Lys₉² in p40phox-PX is occupied by the buried Pro₃⁵⁷ (Fig. 2, A and C).

The Bem1p-PX Tyr₃¹⁸ side chain and its analogs in the other PX domains mark the floor of the lipid-binding pocket (Fig. 2C), under which a hydrophobic core is conserved throughout the PX domains. Besides Tyr₃¹⁸, this core is made up of the conserved residues Phe₃⁰⁹, Phe₃²₁, and Leu₃⁷₃. Bem1p-PX Tyr₃¹⁸ clearly superimposes with p40phox-PX Tyr₅⁹ (Fig. 2D), but in contrast to p40phox-PX, this residue cannot contribute to inositol ring stacking interactions, as it is sheltered by the Pro₃⁵⁷ side chain (Fig. 2C). This is reminiscent of p47phox-PX, in which, again, a completely buried Pro₃⁷₅ prohibits access to the aromatic ring.

In addition to other conserved lipid-binding determinants, the loop spanning PPII and helix α₂, which is the region with least sequence similarity among PX domains, seems to be instrumental in the lipid binding selectivity of PX domains. In the Bem1p PX domain, two features of this variable loop prevent PIs from binding in an orientation similar to that seen for PtdIns(3)P-binding p40phox and Vam7p PX domains. The position equivalent to Tyr₉⁴ in p40phox-PX, which is responsible for hydrophobic contacts with the diacylglycerol moiety of...
PtdIns(3)P (Fig. 2D), is occupied in Bem1p by an exposed Pro359 (Fig. 2C), which precludes the possibility of interactions with the diacylglycerol moiety in a p40<sub>phox</sub>-like manner. Additionally, the backbone of the loop leading into helix α2 forms an accentuated curve between residues 360 and 362, which forces Val361 to sway inwards into the pocket (Fig. 2A–C). Compared with the extended conformation of the analogous region in p40<sub>phox</sub>-PX, the specific turn in Bem1p is much tighter, and the Val361 side chain is sufficiently bulky to make the site too cramped for lipids to fit. To model plausible binding modes, it is essential to consider both orientations of the inositol ring in which the axial 2-OH points down toward the floor of the binding pocket (as it does for p40<sub>phox</sub>-PX) and orientations with the inositol ring flipped 180° around the C1-C4 axis. In our proposed orientation of PtdIns(4)P (Fig. 2), Val361 would prevent access to doubly phosphorylated PIs, i.e. PtdIns(3,4)P<sub>2</sub> with the D2-OH pointing down or PtdIns(4,5)P<sub>2</sub> with the D2-OH pointing up or down, because either possibility would position a phosphate adjacent to Tyr<sup>318</sup> that cannot effectively neutralize negative charges. The possibility of PtdIns(5)P binding the pocket in a similar orientation to the one suggested for PtdIns(4)P is unlikely, as it would disrupt hydrogen bonds with the inositol ring and the D1-phosphate.

Two residues in Bem1p that remain in positions closely related to their equivalents in p40<sub>phox</sub>-PX are Arg<sup>369</sup> in helix α2, which superimposes with Arg<sup>105</sup> of p40<sub>phox</sub>-PX, and Gln<sup>319</sup> which replaces Arg<sup>60</sup> of p40<sub>phox</sub>-PX with its shorter side chain tilted more toward helix α1 (Fig. 2). Arg<sup>105</sup> in p40<sub>phox</sub>-PX is responsible for interaction with the D4-OH in PtdIns(3)P. In our model of PtdIns(4)P bound to Bem1p (Fig. 2C), its Bem1p equivalent, Arg<sup>369</sup>, is mediating interaction with the D4-phosphate of PI. With PtdIns(4)P placed in this orientation, the D4-phosphate would form hydrogen bonds with Arg<sup>369</sup> and the backbone amide of Val358 at the start of the loop following PPII. With the inositol ring positioned at a “slant” relative to the pocket, our model of PtdIns(4)P steers clear of Val361 while permitting the N-H of the Lys297 side chain to assume a role of stabilizing the D1-phosphate, as seen with Lys<sup>92</sup> in p40<sub>phox</sub>-PX. Lys297 is not conserved among the PX domains. Instead, this position is usually occupied by a valine. The Vam7p PX domain has Lys25 in this position, but the side chain of this lysine points away from the lipid-binding pocket. In one of the two Bem1p molecules in the asymmetric unit, part of the side chain of Lys297 is disordered, suggesting flexibility.

In general, the membrane-binding surfaces of PI- or other lipid-binding proteins contain basic and hydrophobic residues, which are involved in initial membrane adsorption and membrane penetration, respectively (7). In the case of PX domains, clustered basic residues are often found in the α1-PP<sub>II</sub> loop (7). Also, hydrophobic residues are present in the PP<sub>II</sub>-α2 loop of most PX domains (7) and also in the α1-PP<sub>II</sub> loop of some PX
domains, such as p47\textsuperscript{phox}-PX (47, 78). The crystal structure of Bem1p-PX shows the presence of two basic residues, Lys\textsuperscript{338} and Arg\textsuperscript{349}, in the α'-PPII loop (Fig. 2B), which may either form a secondary lipid pocket, as seen with p47\textsuperscript{phox}-PX (47) and PLD1-PX (49), or interact nonspecifically with anionic phospholipids. Also, both the PP II-α2 and α1-PP II loops of Bem1p-PX contain exposed hydrophobic residues (i.e. Tyr\textsuperscript{346} in the PP II-α2 loop and Trp\textsuperscript{346} in the α1-PP II loop) (Fig. 2B), suggesting that both loops are involved in membrane penetration.

**Membrane Binding of Bem1p-PX**—To determine the functional roles of the putative phospholipid-binding residues in Bem1p-PX, we measured the vesicle binding of wild-type Bem1p-PX and a series of site-specific mutants by SPR and monolayer penetration analyses. We first measured the PI specificity of the Bem1p PX domain by SPR analysis. In these experiments, an active surface was coated with POPC/POPE/PI (77:20:3), whereas a control surface was coated with POPC/POPE (80:20). Initial screening was performed with the injection of 1 \( \mu M \) Bem1p-PX into the sensor coated with various PI-containing vesicles (Fig. 3A). Clearly, the Bem1p PX domain has specificity for PtdIns(4)P, as it exhibited no detectable binding to other PI-containing vesicles. Fig. 3B shows representative sensorsgrams for Bem1p-PX-POPC/POPE/PtdIns(4)P (77:20:3) vesicle binding, and Fig. 3C illustrates the binding isotherm from the sensorsgrams. The \( K_d \) values determined for Bem1p-PX and its mutants are listed in Table 2.

Although the Bem1p PX domain was highly specific for PtdIns(4)P, it had modest affinity (\( K_d = 1.2 \mu M \)) for POPC/POPE/PtdIns(4)P (77:20:3) vesicles. Other PX domains, including p40\textsuperscript{phox}-PX, p47\textsuperscript{phox}-PX, and PI3K-C2α-PX, were shown to have >10-fold higher affinities for POPC/POPE/PI (77:20:3) vesicles containing their cognate PI molecules under similar conditions (46, 78). Because the Bem1p PX domain has a cationic patch on its putative membrane-binding surface, it was expected to have higher affinity for vesicles with higher anionic lipid contents. Indeed, the affinity of Bem1p-PX gradually increased as the POPS concentration in POPC/POPE/POPS/PtdIns(4)P (77:20:3:20) vesicles increased (data not shown); the Bem1p PX domain bound POPC/POPE/POPS/PtdIns(4)P (57:20:20:3) vesicles ~8-fold more tightly than POPC/POPE/PtdIns(4)P (77:20:3) vesicles (see Table 2, third column). The addition of 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphatidic acid up to 20 mol % had the same effect (data not shown), indicating that anionic phospholipids enhance binding through nonspecific electrostatic interactions. To determine whether the membrane affinity of Bem1p-PX is comparable with that of other known PtdIns(4)P-binding domains, we also measured the membrane binding of the PH domains of OSBP (79) and FAPP1 (80) that were reported to interact with PtdIns(4)P and PtdIns(4,5)P\(_2\). As indicated in Table 2, both PH domains had modest selectivity for PtdIns(4)P over PtdIns(4,5)P\(_2\) (see the second and fourth columns). As far as the affinity for PtdIns(4)P-containing vesicles is concerned, these PH domains had only 2–4-fold higher affinity for POPC/POPE/PtdIns(4)P (57:20:20:3) vesicles compared with Bem1p-PX (Table 2). Collectively, these results establish that the Bem1p PX domain is the genuine PtdIns(4)P-specific domain, with overall membrane affinity comparable with other reported PtdIns(4)P-binding domains.

**FIGURE 3. Equilibrium SPR binding analysis of the Bem1p PX domain.** A, the Bem1p PX domain (1 \( \mu M \)) was injected over a POPC/POPE/PI (77:20:3) surface to gauge the affinity and specificity for different PIs, including PtdIns(3)P, PtdIns(4)P, PtdIns(5)P, PtdIns(3,4)P\(_2\), PtdIns(3,5)P\(_2\), and PtdIns(4,5)P\(_2\). The SPR response curves for respective PIs are shown after background correction for binding to the control surface coated with POPC/POPE (80:20). Binding to the control surface was minimal, and little evidence of nonspecific binding was evident at 1 \( \mu M \) protein. B, the Bem1p PX domain was injected at 2 \( \mu M \) min at varying concentrations (0.1, 0.4, 1, 4, and 8 \( \mu M \) from bottom to top) over the POPC/POPE/PtdIns(4)P (77:20:3) surface, and \( R_{\text{max}} \) values were measured. C, a binding isotherm was generated from the \( R_{\text{eq}} \) (n = 3) versus concentration of Bem1p-PX plot. The solid line represents a theoretical curve constructed from \( R_{\text{max}} \) (51 ± 0.5) and \( K_d \) (1.2 ± 0.1 \( \mu M \)) values determined by nonlinear least-squares analysis of the isotherm using \( R_{\text{eq}} = R_{\text{max}}/(1 + K_d/3) \). 10 mM HEPES (pH 7.4) containing 0.16 M KCl was used for all measurements. RU, resonance units.

**TABLE 2. Membrane binding properties of the Bem1p PX domain and mutants**

| Protein     | \( K_d \) (\( \mu M \)) | \( K_d \) (\( \mu M \)) | \( K_d \) (\( \mu M \)) |
|-------------|-------------------------|-------------------------|-------------------------|
| Bem1p-PX    | 1.2 ± 0.1               | 0.15 ± 0.02             | ND*                     |
| K297A       | 6.0 ± 0.4               | 0.49 ± 0.06             | NM                      |
| K338A       | 1.5 ± 0.3               | 0.43 ± 0.04             | NM                      |
| W346A       | 4.6 ± 0.4               | NM                      | NM                      |
| R349A       | 2.0 ± 0.3               | 0.54 ± 0.04             | NM                      |
| K338A/R349A | 3.5 ± 0.6               | 1.7 ± 0.6               | NM                      |
| Y360A       | 9.4 ± 0.5               | NM                      | NM                      |
| R369A       | ND                      | >15                     | NM                      |
| OSBP-PH     | 0.1 ± 0.02              | 0.04 ± 0.008            | 0.18 ± 0.02             |
| FAPP1-PH    | 0.23 ± 0.03             | 0.080 ± 0.002           | 0.4 ± 0.03              |

* ND, not detectable; NM, not measured.
We then measured the membrane binding of Bem1p-PX mutants to vesicles with different compositions (Table 2). In agreement with our structural analysis, mutation of a conserved Arg residue (i.e. R369A) abolished binding to POPC/POPE/PtdIns(4)P (77:20:3) vesicles, corroborating the notion that Arg369 is essential for binding to the D4-phosphate. Mutation of another cationic residue in the PtdIns(4)P-binding pocket of Bem1p-PX (K297A) had a smaller but significant effect (i.e. 5-fold decrease) on binding to the same vesicles, supporting the notion that this residue is also involved in PtdIns(4)P binding, presumably through coordinating the D1-phosphate.

We also determined the roles of two cationic residues in the α1-PPII loop of the Bem1p PX domain (Table 2). In contrast to mutation of Arg369 in the PI-binding pocket, mutation of Lys338 or Arg349 had little effect on the binding of Bem1p-PX to POPC/POPE/PtdIns(4)P (77:20:3) vesicles, indicating that PtdIns(4)P does not interact with this site. However, K338A and R349A showed 3- and 4-fold reduced affinity, respectively, for more anionic POPC/POPE/POPS/PtdIns(4)P (57:20:20:3) vesicles. Also, the double mutant K338A/R349A had 11-fold lower affinity compared with the wild type for POPC/POPE/POPS/PtdIns(4)P (57:20:20:3) vesicles. Furthermore, this mutant exhibited only a 2-fold difference in affinity between POPC/POPE/PtdIns(4)P (77:20:3) and POPC/POPE/POPS/PtdIns(4)P (57:20:20:3) vesicles, indicating that Lys338 and Arg349 play a significant role in nonspecific electrostatic interaction with anionic phospholipids.

Finally, we measured the effects of mutating hydrophobic residues in the PPα2 and α1-PPII loops (Trp346) on the membrane binding of Bem1p-PX to see if they are involved in membrane penetration. Y360A exhibited 8-fold lower membrane affinity compared with the wild type for POPC/POPE/PtdIns(4)P (77:20:3) vesicles, whereas W346A showed 4-fold lower affinity compared with the wild type for the same vesicles. Thus, hydrophobic residues adjacent to the PI-binding pocket (i.e. PPα2 loop) and in the α1-PPII loop play a significant role in membrane binding and may be involved in membrane penetration.

**Membrane Penetration of Bem1p-PX**—Recent studies have shown that PIs can specifically induce the membrane penetration of the FYVE (68), PX (46, 49, 54, 78), and ENTH (81) domains. To determine whether or not PtdIns(4)P can also elicit the membrane penetration of Bem1p-PX, we first measured the penetration of the PX domain into monolayers with different lipid compositions (Fig. 4A). Interestingly, the Bem1p PX domain was able to penetrate the POPC/POPE (80:20) monolayer with surface pressure up to 30 dynes/cm. PI-independent membrane penetration has been reported for a few domains, including the PH domain of phospholipase Cδ1 (82) and p47pox-PX (78). However, this type of strong PI-independent monolayer penetrating activity has not been seen with any PI-binding domains that typically cannot penetrate the monolayer with surface pressure above 25 dynes/cm in the absence of their cognate PI molecules (46, 49, 78). Because the surface pressure of cell membranes has been estimated to be 31–35 dynes/cm (83–85), this also implies that Bem1p-PX may be able to partially penetrate cell membranes even in the absence of PtdIns(4)P under certain conditions.

Although Bem1p-PX had high intrinsic monolayer penetrating power, incorporation of 3 mol % PtdIns(4)P into the monolayer (i.e. POPC/POPE/PtdIns(4)P (77:20:3)) further increased its monolayer penetration, allowing it to penetrate the monolayer with surface pressure up to 35 dynes/cm (Fig. 4A). This increase was a PtdIns(4)P-specific effect because 3 mol % PtdIns(3)P, PtdIns(5)P, or POPS in the monolayer did not have detectable effects. We also measured the effect of PtdIns(4)P on the monolayer penetration of the PH domains of OSBP and FAPP1 (Fig. 4B). Both OSBP and FAPP1 PH domains displayed much lower monolayer penetration than Bem1p-PX in the absence of PtdIns(4)P (i.e. POPC/POPE (80:20)), but showed a significant increase in penetration when PtdIns(4)P was present in the monolayer (i.e. POPC/POPE/PtdIns(4)P (77:20:3)). Thus, as is the case with other PIs, PtdIns(4)P promotes the membrane penetration of its effector proteins, allowing them to penetrate densely packed bilayers, including cell membranes. However, the Bem1p PX domain has higher membrane penetrating activity than other PtdIns(4)P-binding domains in both the absence and presence of PtdIns(4)P.

To elucidate the structural determinant of the high membrane penetrating activity of the Bem1p PX domain, we measured the monolayer penetration of Bem1p PX domain mutants. As shown in Fig. 5A, R369A with abrogated PtdIns(4)P binding had significantly lower penetration into the POPC/POPE/PtdIns(4)P (77:20:3) monolayer compared with wild-type Bem1p-PX; it penetrated the POPC/POPE/
PtIns(4)P (77:20:3) monolayer only as well as the wild type penetrated the POPC/POPE (80:20) monolayer. This verifies the notion that PtIns(4)P binding to its pocket in Bem1p-PX specifically enhances the monolayer penetration of Bem1p-PX. In contrast, K338A and R349A behaved similarly to the wild type. Furthermore, W346A and Y360A had greatly reduced penetration into both POPC/POPE (80:20) (Fig. 5B) and POPC/POPE/PtIns(4)P (77:20:3) (Fig. 5A) monolayers, indicating that these residues are directly involved in the monolayer penetration of the Bem1p PX domain.

It may seem contradictory that Bem1p-PX was able to penetrate the POPC/POPE (80:20) monolayer with surface pressure up to 30 dyne/cm yet showed very low (>30 mm) affinity for POPC/POPE (80:20) and POPC/POPE/POPS (60:20:20) vesicles in SPR measurements. It should be noted, however, that the surface pressure of large (i.e. 100-nm diameter) unilamellar vesicles is estimated to be >30 dyne/cm (83–85). Therefore, despite its relatively high intrinsic membrane penetrating power, Bem1p-PX still cannot effectively bind to and penetrate large vesicles used in SPR studies without PtIns(4)P-mediated penetration.

Electrostatic Potential Calculations—To account for the unique membrane binding properties of the Bem1p PX domain, we calculated the electrostatic potentials of the domain in the absence and presence of bound PtIns(4)P. The results are illustrated in Fig. 6. In the absence of PtIns(4)P and PS, the PI-binding pocket and the cationic patch have a strong positive electrostatic potential due to the presence of multiple cationic residues. This strong positive potential is similar to that seen for other PX domains, including p40phox-PX and p47phox-PX (78), which was shown to contribute to the initial nonspecific absorption of the domains to the anionic membranes. Likewise, the positive electrostatic potential should drive the initial membrane adsorption of Bem1p-PX, which would then facilitate the specific PtIns(4)P binding by the domain through lateral diffusion on the membrane surface. Interestingly, the side chain of Tyr360 in the PP1–α2 loop near the PI-binding pocket protrudes from the positive electrostatic potential surface. This is an unusual finding because most hydrophobic side chains on the membrane-binding surfaces of PI-binding proteins have been found buried in the positive electrostatic potential in the absence of their cognate PI molecules (7). This unique structural feature explains how Bem1p-PX penetrates the membrane in the absence of PtIns(4)P. When PtIns(4)P binds to the domain, the positive electrostatic potential surrounding the membrane-binding surface is greatly reduced, which exposes another hydrophobic residue (Trp346) and facilitates its further membrane insertion, accounting for the enhanced monolayer penetration in the presence of PtIns(4)P. Fig. 6 also shows that the effect of PS on the electrostatic potential is not significant, which is consistent with the fact that PS and phosphatidic acid do not influence the monolayer penetration of the Bem1p PX domain, although they increase the affinity of Bem1p-PX for PtIns(4)P-containing membranes.

DISCUSSION

As part of our continuing effort to understand the structural basis of the variable PI specificity of PX domains, we determined the crystal structure of the PtIns(4)P-binding Bem1p PX domain and characterized its membrane binding properties in this study. High resolution structures of a number of PX domains that bind PtIns(3)P, PtIns(3,4)P2, or PtIns(4,5)P2 have been determined as either free proteins or complexes with PIs (46,47,51–55,77). However, no structural information on PtIns(4)P-binding PX domains has been reported. This study provides new insight into not only the origin of stereospecific PtIns(4)P recognition by Bem1p-PX, but also the mechanism by which this PX domain interacts with membranes and thereby mediates the function of Bem1p in establishing a new bud in S. cerevisiae.

A recently determined crystal structure of the PI3K-C2α PX domain (46) that specifically binds PtIns(4,5)P2 revealed why the PX domain does not bind PtIns(3)P, which most PX domains prefer. In PI3K-C2α-PX, a canonical D3-phosphate ligand (i.e. Arg58 in p40phox-PX) is substituted with Thr, and an acidic residue (Asp148) replaces a D1-phosphate ligand (i.e. Arg60 in p40phox-PX). Similarly, in Bem1p-PX, the consensus D3-phosphate ligand is substituted with Tyr117, and the D1-phosphate ligand is replaced by a buried residue (Pro357). As is the case with PI3K-C2α-PX, these substitutions would not allow PtIns(3)P to favorably interact with the PI-binding pocket of Bem1p-PX. Other specific structural features of the PI-binding pocket of Bem1p-PX would also prevent productive interaction with all PIs but PtIns(4)P. Collectively, this negative selection confers high PtIns(4)P specificity on Bem1p-PX. The difficulty encountered in co-crystallization of Bem1p-PX with its PtIns(4)P ligand hampered our effort to directly determine the positive structural selection through which the
domain achieves stereospecific recognition of the D4-phosphate of PtdIns(4)P. However, sequence alignment, modeling, and our mutational analysis strongly suggest that Arg369 in Bem1p-PX directly interacts with the D4-phosphate, whereas Lys297 is involved in binding to the D1-phosphate.

PX domains have a varying number of cationic residues on their membrane-binding surfaces that promote their nonspecific electrostatic interactions with anionic membranes. For p47phox-PX (47) and PLD1-PX (49), some of these cationic residues have been proposed to form secondary lipid-binding pockets on the membrane-binding surface that are separate from the primary PI-binding pockets. The crystal structure of Bem1p-PX suggests that the cationic residues Lys338 and Arg349 may also form a lipid pocket. However, membrane binding measurements of the wild type and mutants show that this potential pocket is too shallow to accommodate a polar head group and that, consequently, Lys338 and Arg349 interact nonspecifically with anionic membrane surfaces.

Our previous studies on the FYVE (68, 86), PX (46, 78), and ENTH (81) domains indicated that PI binding specifically induces the membrane penetration of surface hydrophobic/aromatic residues surrounding the PI-binding pocket, presumably by causing local conformational changes in proteins and/or by attenuating the positive electrostatic potential surrounding hydrophobic residues. The crystal structure of Bem1p-PX shows the presence of two prominent aromatic residues, Tyr360 and Trp346, located in the PPII-α2 loop and the α1-PP1 loop, respectively. Our monolayer and SPR measurements indicated that PtdIns(4)P binding specifically (i.e. not induced by other PIs) enhances the membrane penetration of Bem1p-PX. Bem1p-PX also has unusually high PtdIns(4)P-independent monolayer penetrating power, with πc30 = 30 dynes/cm. Our mutational analysis showed that Tyr360 and Trp346 are largely responsible for the membrane penetrating activity of Bem1p-PX both in the presence and absence of PtdIns(4)P. Our electrostatic calculation suggests that the PtdIns(4)P-independent membrane penetration depends more on Tyr360, whereas the PtdIns(4)P-dependent penetration may involve both Tyr360 and Trp346. This is because Tyr360 in Bem1p-PX is not embedded in a positive electrostatic potential contour and, consequently, can readily penetrate the membrane without having to pay the hefty desolvation penalty (7, 87) and because PtdIns(4)P binding attenuates the positive electrostatic potential surrounding Trp346 in a favorable way.

The electrostatic attenuation of Bem1p-PX caused by PtdIns(4)P

FIGURE 6. Bem1p PX domain in the absence and presence of PS and PtdIns(4)P. A, C, and E show the electrostatic potential mapped to the membrane-binding surface of the PX domain. B, D, and F represent the PX domain as a C-α backbone and the electrostatic potential as a two-dimensional contour. The molecules are rotated 90° forward from A, C, and E, and the membrane-binding surfaces point downward in this orientation. Even in the absence of lipids (A and B), Tyr360 is exposed over the electrostatic potential surface, accounting for the high intrinsic membrane penetrating activity of Bem1p-PX. Upon binding to PS (C and D), the electrostatic potential of the membrane-binding surface of Bem1p-PX is relatively unchanged. Upon binding to PtdIns(4)P (E and F), the positive electrostatic potential of the membrane-binding surface of Bem1p-PX is greatly decreased, exposing Trp346, which will further penetrate into the membrane. PtdIns(4)P is colored yellow, and Trp346 and Tyr360 are colored green. PS is not shown.
unusually high PI-independent monolayer penetrating activity, this activity may not be sufficient to drive its binding to compactly packed membranes under normal conditions, judging from the low affinity of Bem1p-PX for POPC/POPE (80:20) and POPC/POPE/POPS (60:20:20) vesicles. Subsequent PtdIns(4)P binding at the membrane surface enhances the membrane penetration of Bem1p-PX and would allow for elongated membrane residence of the domain, which may be important for the physiological function of full-length Bem1p. The high intrinsic membrane penetrating activity of Bem1p-PX may also allow the domain to interact favorably with the local cell membrane with lower surface packing density prior to PtdIns(4)P binding. This type of interaction may be particularly important for keeping the protein on the membrane even after the local depletion of PtdIns(4)P.

It has been shown that Bem1p localizes to the plasma membrane and serves as an adaptor protein that links Cdc24p to other proteins during yeast budding and mating (35, 36). PS is rich in the inner leaflets of most plasma membranes. Also, the presence of PtdIns(4)P has been noted in the yeast plasma membrane (27). The affinity of Bem1p-PX for PtdIns(4)P- and PS-containing membranes is comparable with that of other PtdIns(4)P-binding PH domains under the same conditions. Thus, the interaction of the Bem1p PX domain with the PtdIns(4)P- and PS-containing yeast plasma membrane should promote the specific plasma membrane recruitment of the full-length Bem1p molecules. Protein-protein interactions at the membrane, in addition to PtdIns(4)P-mediated membrane binding of the PX domain, are also expected to contribute to the membrane localization of Bem1p. Bem1p forms complexes with several other proteins, some of which harbor PH domains (33), at sites of budding. Thus, interactions with other membrane-associated proteins could facilitate the assembly of a protein complex at the membrane, as seen with many signaling complexes at the membrane (3).

In summary, this study elucidates the structural basis of the specific PtdIns(4)P binding by the Bem1p PX domain and the mechanism by which this PX domain interacts with PtdIns(4)P-containing membranes. This, in conjunction with our previous work on other PX domains, shows that these small domains with similar molecular architecture achieve diverse PI specificity and distinct membrane binding properties through minor variation of non-conserved residues. This work thus contributes to our understanding of the structure and function of a large family of PX domains that serve as membrane and protein interaction modules during cell signaling and membrane trafficking. This study may also provide the basis of further systematic studies on the membrane recruitment and regulation of Bem1p.

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Structure of the Bem1p PX Domain
