Phox Homology Domains Specifically Bind Phosphatidylinositol Phosphates†

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Received February 6, 2001; Revised Manuscript Received June 6, 2001

ABSTRACT: The recruitment of specific cytosolic proteins to intracellular membranes through binding phosphorylated derivatives of phosphatidylinositol (PtdIns) controls such processes as endocytosis, regulated exocytosis, cytoskeletal organization, and cell signaling. Protein modules such as FVYE domains and PH domains that bind specifically to PtdIns 3-phosphate (PtdIns-3-P) and polyphosphoinositides, respectively, can direct such membrane targeting. Here we show that two representative Phox homology (PX) domains selectively bind to specific phosphatidylinositol phosphates. The PX domain of Vam7p selectively binds PtdIns-3-P, while the PX domain of the CPK PI-3 kinase selectively binds PtdIns-4,5-P_2. In contrast, the PX domain of Vps5p displays no binding to any PtdInsPs that were tested. In addition, the double mutant (Y42A/L48Q) of the PX domain of Vam7p, reported to cause vacuolar trafficking defects in yeast, has a dramatically decreased level of binding to PtdIns-3-P. These data reveal that the membrane targeting function of the Vam7p PX domain is based on its ability to associate with PtdIns-3-P, analogous to the function of FYVE domains.

The Phox homology (PX) domain, consisting of ~100 amino acids, was first identified from the sequence analysis of two SH3 domain-containing cytosolic components of NADPH oxidase, $p47^{phox}$ and $p40^{phox}$ (*1*). NADPH oxidase is a multisubunit complex that generates the reactive oxygen compounds needed for the killing of phagocytosed microorganisms (*2*−*4*). Deficiency of the component subunits of NADPH oxidase leads to chronic granulomatous disease (CGDs) with predisposition to infection by fungi and bacteria (*5*, *6*). Other PX domain-containing proteins, such as Mvp1p (*7*), Vps5p (*8*−*10*), and Grd19p (*11*) in yeast, can bind vesicular trafficking machinery and regulate the proper sorting of carboxypeptidase Y to the vacuole. In addition, a family of sorting nexin molecules (SNX1−SNX5, SNX15), all containing a PX domain, can associate with receptors for insulin, EGF, PDGF, leptin, and HGF during their internalization and degradation in lysosomes (*12*−*15*). Recently, Vam7p, another PX domain-containing protein, was identified together with Vam3p, a syntaxin homologue, to function in yeast vacuolar protein trafficking. A double mutation (Y42A/L48Q) within the conserved region of the PX domain of Vam7p abolished the vacuolar protein sorting function of Vam7p (*16*−*18*).

A PX domain has also been identified at the C-terminal tail of the class II PI-3 kinase, between the catalytic domain and the C2 domain (*19*−*21*), and in phospholipase D1, an enzyme that is involved in membrane-associated phospholipid metabolism (*1*). Other PX domain-containing proteins include Fish, a protein involved in the tyrosine kinase signaling pathway (*22*), SH3PX1, a protein that interacts with the cytoplasmic domains of the matalloprotease disintergrins MDC9 and MDC15 (*23*), HS1BP3, a protein involved in lymphocyte activation (*24*), GASC1, a novel gene expressed in esophageal cancer cell lines (*25*), and CISK, a PX domain-containing serine/threonine protein kinase involved in cell survival (*26*). Therefore, PX domains represent a widely distributed module with potential functions in the regulation of vesicular trafficking machinery, growth factor receptor degradation in lysosomes, signal transduction mechanisms, and NADPH oxidase activity. Because most PX domain-containing proteins are associated with membranes, we tested the hypothesis that PX domains represent lipid-binding modules. We show here that this is indeed the case for two representative PX domains. The PX domain of Vam7p selectively binds PtdIns-3-P,[1] while that in the CPK PI-3 kinase binds PtdIns-4,5-P_2. Thus, phosphoinositides can direct the selective recruitment of PX domain-containing proteins to membranes.

EXPERIMENTAL PROCEDURES

Materials. PtdIns, PtdCho, PtdSer, and PtdEth were purchased from Avanti Polar Lipids. PtdIns-4-P, PtdIns-5-P, and PtdIns-3,5-P_2 were purchased from Echelon Research Laboratories Inc. PtdIns-3-P, PtdIns-3,4-P_2, PtdIns-4,5-P_2, and PtdIns-3,4,5-P_3 were purchased from Matreya, Inc. All

† This work was supported by a research grant from the American Diabetes Association (G.W.Z.), an ARF grant from the Worcester Foundation (G.W.Z.), and NIH Program Project Grant DK60564 (M.P.C.).

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[1] Abbreviations: PtdIns, phosphatidylinositol; PtdIns-3-P, phosphatidylinositol 3-phosphate; PtdIns-4-P, phosphatidylinositol 4-phosphate; PtdIns-5-P, phosphatidylinositol 5-phosphate; PtdIns-3,4-P_2, phosphatidylinositol 3,4-diphosphate; PtdIns-3,5-P_2, phosphatidylinositol 3,5-diphosphate; PtdIns-4,5-P_2, phosphatidylinositol 4,5-diphosphate; PtdIns-3,4,5-P_3, phosphatidylinositol 3,4,5-triphosphate; PtdSer, phosphatidylserine; PtdEth, phosphatidylethanolamine; PtdCho, phosphatidylcholine.

10.1021/bi0155100 CCC: $20.00
Published on Web 07/06/2001

the phospholipids were dissolved in chloroform, methanol, or a chloroform/methanol/water mixture as directed. For PCR cloning, human Quick cDNA was purchased from Clontech. Yeast genomic DNA was a gift from A. Virbasius. Expression vector pGEX-4T-1 was purchased from Pharmacia Biotech. Ni−NTA−agarose beads were purchased from Qiagen, Inc. Glutathione−agarose beads were purchased from Sigma.

Expression and Purification of His-Tag and GST Fusion Proteins. The expression constructs for His-tag fusion proteins were obtained by cloning the PCR-amplified fragments corresponding to the respective PX domain from either human Quick cDNA or yeast genomic DNA into the pXL1α vector (modified from pET15b), using the standard PCR cloning technique. GST-Vam7p was obtained by subcloning the DNA fragment corresponding to Vam7p from the His-tag vector into the pGEX-4T-1 vector, using restriction enzymes *Bam*HI and *Sal*I. The double mutant (Y42A/L48Q) of the Vam7p PX domain was prepared using two oligonucleotide primers corresponding to the mutation sites (5′-CGC CTT TAC AAA AGG GCT TCC GAG TTT TGG AAA CAG AAG ACA CGA TTG GAG-3′ and 5′-CTC CAA TCG TGT CTT CTG TTT CCA AAA CTC GGA AGC CCT TTT GTA AAG GCG-3′) and two primers previously used for cloning the wild-type PX domain. The corresponding fragment was then cloned into the pGEX-4T-1 vector. All the constructs were confirmed by sequencing. All fusion proteins were expressed from *Escherichia coli* strain BL21(DE3) at room temperature overnight, after induction by IPTG from 50 μM to 0.5 mM. The His-tagged PX domains were purified from bacterial lysate with Ni−NTA beads, using standard protocols. The GST fusion proteins were purified with glutathione−agarose beads. Protease inhibitors were added during the purification.

Liposome Binding Assays. A previously published liposome binding assay protocol was used for the binding experiments described in this report (*28*). The procedure is summarized as follows. The appropriate phospholipid mixture (100 μg per reaction) was dried in a speed vacuum centrifuge. The dried mixture was then resuspended in 100 μL of liposome buffer [50 mM Hepes and 100 mM NaCl (pH 7.2)], sonicated in a bath sonicator for 15 min, and spun for 10 min at 14 000 rpm and 4 °C. The supernatant was discarded, and the liposomes were resuspended in binding buffer [50 mM Hepes, 100 mM NaCl, and 1 mM $MgCl_2$ (pH 7.2)] at a concentration of 1 mg/mL. The liposomes were then incubated with 8 μg of purified protein for 15 min at room temperature, and centrifuged as before at 14 000 rpm and 4 °C for 10 min. The supernatant was transferred to another tube, and the pellet was resuspended into 100 μL of binding buffer. Twenty microliters of both the supernatant and pellet were used for analysis with SDS−PAGE and Coomassie blue staining. The intensity of the stained bands was quantitatively determined on a densitometry scanner.

RESULTS AND DISCUSSION

In initial experiments, the specificity of binding of each of three PX domains to different phosphoglycerides [phosphatidylserine (PtdSer), phosphatidylethanolamine (PtdEth), phosphatidylcholine (PtdCho), and phosphatidylinositol (PtdIns)] was examined using a liposome binding assay (*27*).

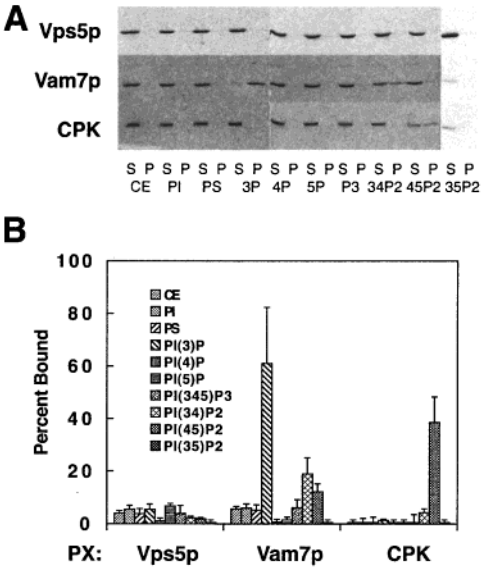

FIGURE 1: Liposome binding assays demonstrate the binding selectivity of the Vam7p PX domain for PtdIns-3-P and of the CPK PX domain for PtdIns-4,5-P. (A) Binding of fusion proteins containing the PX domain of Vps5p (amino acids 275−394), Vam7p (amino acids 13−125), and CPK (amino acids 1245−1360) to liposomes. The His-PX domain fusion proteins were generated using amplification by PCR of the Quick cDNA (Clontech). Liposomes were composed of 50 μg of phosphatidylcholine (PtdCho) and 50 μg of phosphatidylethylamine (PtdEth) or of 50 (PtdCho), 45 (PtdEth), and 5 μg of the tested phospholipids. The total amount of lipid per condition is 100 μg. Then 8 μg of PX domains of Vam7p, CPK, and Vps5p were added to the liposomes, and the mixture was incubated for 15 min. After centrifugation, liposome pellets (P) and supernatants (S) were analyzed by SDS−PAGE and Coomassie blue staining: PI, PtdIns; PS, phosphatidylserine; 3P, PtdIns-3-P; 4P, PtdIns-4-P; 5P, PtdIns-5-P; 345P3, PtdIns-3,4,5-P_3; 34P2, PtdIns-3,4-P_2; 45P2, PtdIns-4,5-P_2; and 35P2, PtdIns-3,5-P_2. (B) Percentage of different PX domains binding to liposomes containing 5% phosphatidylinositol phosphates. The results in panel B are the average of at least three experiments, while the gel shows a representative result.

We generated His-tagged PX domains derived from Vps5p, Vam7p, and the CPK PI-3 kinase and measured the level of PX domain binding to liposomes composed exclusively of each of the phosphoglycerides listed above (data not shown). The PX domains from Vam7p and the CPK PI-3 kinase strongly bound PtdIns and PtdSer, but exhibited little binding to PtdEth and PtdCho. The PX domain from Vps5p failed to associate with liposomes containing PtdEth, PtdCho, or PtdSer, and only weakly bound those containing PtdIns. These data indicate that all three PX domains do not bind PtdCho and PtdEth, while the PX domains of Vam7p and the CPK PI-3 kinase have higher affinities for PtdIns and PtdSer than the PX domain of Vps5p.

On the basis of these preliminary results, all additional experiments were performed with liposomes prepared with a PtdCho:PtdEth:tested phosphoinositide composition ratio of 50:45:5 unless otherwise indicated. Figure 1 depicts the presence of PX domain polypeptides in the supernatant and in the liposome pellets following sedimentation of the incubation mixtures. The PX domains of Vps5p, Vam7p, and the CPK PI-3 kinase were seen to display remarkably selective binding specificities for the liposomes containing 5% phosphoinositides.

The PX domain of Vam7p displayed strong binding for PtdIns-3-P only. About 60% of the Vam7p PX domain was observed in the liposome pellets containing 5% PtdIns-3-P. Only around 20% of the Vam7p PX domain was observed in the liposome pellets containing PtdIns-3,4-P_2 and PtdIns-4,5-P_2, and less than 5% of the Vam7p PX domain was observed in the pellet liposomes containing other phosphoinositides. The second class of tested PX domains, the PX domain from the CPK PI-3 kinase, however, showed selective affinity for PtdIns-4,5-P_2. About 50% of this PX domain selectively bound liposomes containing 5% PtdIns-4,5-P_2, whereas less than 10% of the PX domain bound liposomes containing other PtdInsPs. The third tested PX domain, the PX domain from Vps5p, however, showed little specific affinity for all of the tested PtdInsPs. Less than 5% of the PX domain was observed in the liposome pellets containing the tested PtdInsPs. All the liposome binding assays were carried out in the presence of 1 mM $MgCl_2$. When 5 mM EDTA was added to the liposome binding assay, the above PX domains displayed increased levels of nonspecific binding to liposomes containing the different lipids (data not shown). Overall, our data show that the PX domains from Vps5p, Vam7p, and the CPK PI-3 kinase have remarkably divergent binding specificity for phosphoinositides.

Among the three tested PX domains, the Vam7p PX domain was the only one that specifically bound PtdIns-3-P. To further characterize the binding ability for PtdIns-3-P of the Vam7p PX domain, we quantified the three PX domains present in the supernatant and in the liposome pellets after incubation with liposomes containing different concentrations of PtdIns-3-P, using SDS−PAGE analysis (Figure 2A). Figure 2B shows the percentage of each of the three PX domains present in the liposomes containing between 1 and 20% PtdIns-3-P. The PX domain of Vam7p, as expected, strongly bound PtdIns-3-P compared to the other PX domains. About 50% of the PX domain of Vam7p bound to liposomes containing 5% PtdIns-3-P, like those observed in Figure 1. However, more than 80% of the PX domain of Vam7p bound liposomes containing 10% PtdIns-3-P. The binding for PtdIns-3-P of the PX domain of Vam7p is saturable under the experimental conditions used in these experiments (Figure 2). Under the same conditions, less than 20% of the PX domains of Vps5p and of the CPK PI-3 kinase bound these liposomes. Addition of 5 mM EDTA only slightly increased the apparent affinity of the Vam7p PX domain for liposomes containing PtdIns-3-P. These data further support the concept that the PX domain of Vam7p selectively binds PtdIns-3-P.

The FYVE domain is another protein module that selectively binds PtdIns-3-P (*27*−*30*). It is a double-zinc finger motif of around 70 amino acids and is conserved in several proteins involved in vesicular trafficking. The binding of the FYVE domain to PtdIns-3-P serves to help localize proteins such as EEA1 to endosomes (*31*). Thus, the EEA1 FYVE domain serves as a reference for comparing the binding ability of the Vam7p PX domain to PtdIns-3-P. Binding assays with the FYVE domain of EEA1 were performed with liposomes prepared with a PtdSer:PtdIns:PtdIns-3-P composition ratio of 50:49:1 (*27*), and thus differ from our assay conditions using liposomes prepared with a PtdCho:PtdEth: tested phosphoinositide composition ratio of 50:45:5. Therefore, we compared the binding percentage for PtdIns-3-P of both the Vam7p PX domain (His-tagged protein) and the FYVE domain of EEA-1 (GST fusion protein) under identical conditions. These experiments were carried out with liposomes composed of only PtdCho and PtdEth, and with liposomes containing either 1 or 5% PtdIns-3-P (Figure 3). Both the Vam7p PX domain and the FYVE domain of EEA1 displayed little binding to liposomes composed of PtdEth and PtdCho or those containing 1% PtdIns-3-P. However, about 80% of the FYVE domain of EEA1 and of the Vam7p PX domain was observed to bind liposomes containing 5% PtdIns-3-P (Figure 3). These results indicate that the PX domain from Vam7p binds PtdIns-3-P with an apparent affinity that is similar to that of the FYVE domain of EEA1.

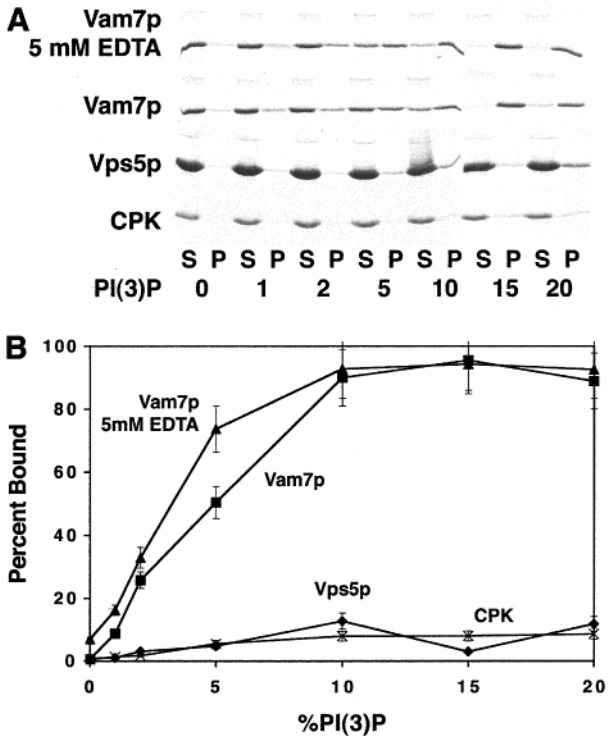

FIGURE 2: Liposome binding assays demonstrate that high-affinity binding of the PX domain of Vam7p to PtdIns-3-P is insensitive to EDTA. (A) Liposomes were composed of PtdCho and PtdEth, and PtdIns-3-P constituted 1, 2, 5, or 10% of the total lipid with equal percentage decreases of PtdEth. Eight micrograms of the PX domain of Vam7p, CPK, or Vps5p was added, and the mixture was incubated for 15 min at room temperature. Under another condition, 5 mM EDTA was added to Vam7p to examine its effect on PI-3-P binding. (B) The curve shows the percent binding of the PX domains to liposomes containing different amounts of PtdIns-3-P. The results in panel B are the average of three independent experiments, while the gel shows a representative result.

Recent studies have documented the biological importance of the PX domain of Vam7p. This protein, a SNAP-25-like molecule, functions together with Vam3p, a syntaxin homologue in yeast vacuolar protein trafficking. Vam7p and Vam3p pair with VAMP homologue Vti1 to form SNARE complexes during vesicle docking and/or fusion with the yeast vacuole (*16*−*18*). A double mutation (Y42A/L48Q) within the PX domain of Vam7p causes vacuolar trafficking defects in yeast (*17*). The mutated amino acids, Tyr^{42} and Leu^{48}, are located within the conserved region of the Vam7p PX domain that may be part of the binding pocket for PtdIns-3-P. Thus, this mutant Vam7p PX domain may be dysfunc-

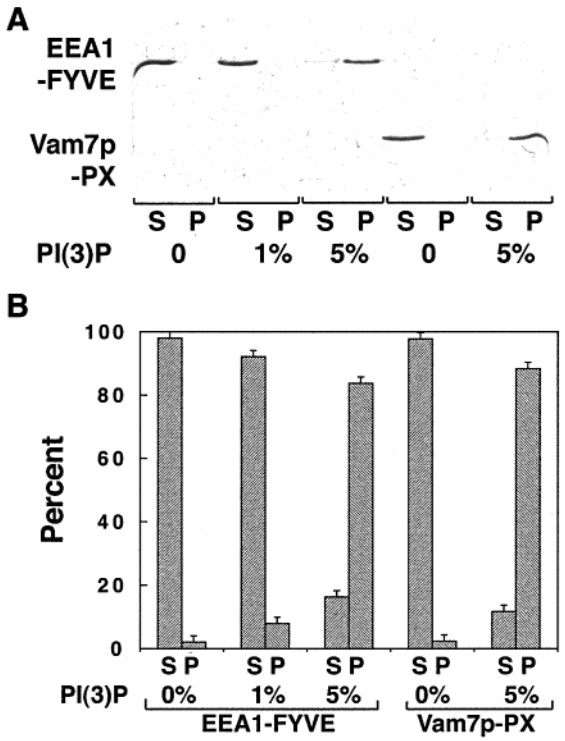

FIGURE 3: Comparison of the PX domain of Vam7p with that of the FYVE domain of EEA1 for binding PtdIns-3-P. (A) Liposomes were composed of PtdCho and PtdEth with the addition of PtdIns-3-P at 1 or 5%. The GST-fused FYVE domain of EEA1 was obtained using the published method, and the level of binding was determined according to the legend of Figure 2. (B) Percentage of the PX domains in the supernatant and pellet as determined from the densitometry scan. The results in panel B are the average of at least three experiments, while the gel shows a representative result.

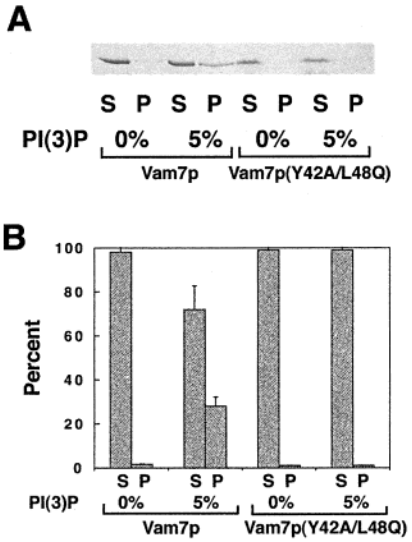

FIGURE 4: Binding of GST-Vam7p and GST-Vam7p(Y42A/L48Q) to liposomes containing 5% PtdIns-3-P. (A) GST-Vam7p was obtained by subcloning the DNA fragment corresponding to Vam7p from the His-tag vector into the pGEX-4T-1 vector, using restriction enzymes *Bam*HI and *Sal*I. The Y42A/L48Q double mutant was cloned into the pGEX-4T-1 vector by standard PCR techniques, using two primers corresponding to both strands of the mutation site and two primers previously used for cloning wild-type Vam7p. The GST fusion proteins were expressed in *E. coli* BL21(DE3) and purified using standard methods. Protease inhibitors were added during the purification. (B) Percentage of the PX domains in supernatants and pellets as determined from the densitometry scan. The results in panel B are the average of five experiments. The gel shows a representative result. The difference between the binding percentages of the GST- and His-Vam7p PX domain to liposomes containing 5% PtdIns-3-P (30% for GST-Vam7p and 90% for His-Vam7p) is likely due to the different molar concentrations of the proteins used in the binding experiments. Though 8 μg of proteins was used in the binding experiments, the molar concentration of the GST-Vam7p PX domain is ~3 times lower than that of the His-tagged protein, because of their different molecular weights.

tional due to its loss of binding affinity for PtdIns-3-P. To test this hypothesis, we generated both the double-mutant (Y42A/L48Q) and wild-type Vam7p PX domains as GST fusion proteins and examined their binding to liposomes containing 5% PtdIns-3-P. As shown in Figure 4, about 30% of wild-type GST-Vam7p bound PtdIns-3-P-containing liposomes under these experimental conditions. However, less than 5% of the mutated GST-Vam7p (Y42A/L48Q) bound to the PtdIns-3-P-containing liposome. Thus, the double-mutant Vam7p PX domain, as predicted, is dramatically impaired in its ability to bind PtdIns-3-P. Furthermore, the PtdIns-3-P binding function of the Vam7p PX domain appears to be critical to Vam7p function in vesicle trafficking.

In summary, we have shown that each of three representative PX domains displays a distinct capability to bind phosphorylated phosphatidylinositols. Most importantly, the PX domain from Vam7p is shown to selectively bind PtdIns-3-P with a binding affinity that appears to be similar to that of the FYVE domain of EEA1. In addition, the PX domain of the CPK PI-3 kinase is found to specifically bind PtdIns-4,5-P_2. In contrast, the PX domain from Vps5p displayed no specificity for the phosphorylated phosphatidylinositols tested in this study. Interestingly, phylogenetic analysis of PX domain-containing proteins based on multiple alignment of the PX domain sequences shows that the PX domains of Vps5p, Vam7p, and the CPK PI-3 kinase belong to different subgroups (*15*). The observed selective binding specificity for different phosphorylated phosphatidylinositols discovered here may reflect the lipid binding characteristics of individual subgroups of the PX domains.

ACKNOWLEDGMENT

We thank Jian Ni for generating the construct for the PX domain of vps5p and Lei Tang for generating the construct for the PX domain of CPK. We also thank Susan Hayes for giving us the purified GST-fused FYVE domain of EEA1.

NOTE ADDED IN PROOF

The interaction of PX domains with phosphoinositides has also been reported in *Nature Cell Biology* (32−35).

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BI0155100