Phox homology (PX) domains are named for a 130-amino acid region of homology shared with part of two components of the phagocyte NADPH oxidase (phox) complex. They are found in proteins involved in vesicular trafficking, protein sorting, and lipid modification. It was recently reported that certain PX domains specifically recognize phosphatidylinositol 3-phosphate (PtdIns-3-P) and drive recruitment of their host proteins to the cytoplasmic leaflet of endosomal and/or vacuolar membranes where this phosphoinositide is enriched. We have analyzed phosphoinositide binding by all 15 PX domains encoded by the Saccharomyces cerevisiae genome. All yeast PX domains specifically recognize PtdIns-3-P in protein-lipid overlay experiments, with just one exception (a significant sequence outlier). In surface plasmon resonance studies, four of the yeast PX domains bind PtdIns-3-P with high (micromolar range) affinity. Although the remaining PX domains specifically recognize PtdIns-3-P, they bind this lipid with only low affinity. Interestingly, many proteins with “low affinity” PX domains are known to form large multimeric complexes, which may increase the overall avidity for membranes. Our results establish that PtdIns-3-P, and not other phosphoinositides, is the target of all PX domains in S. cerevisiae and suggest a role for PX domains in assembly of multiprotein complexes at cell membrane surfaces.

The phox homology (PX) domain (1) was first identified in 1996 as a 130-amino acid region of homology present in two components (p47<sub>phox</sub> and p40<sub>phox</sub>) of the phagocyte NADPH oxidase (phox) complex plus a wide variety of other proteins with diverse functions. Many PX domain-containing proteins are involved in vesicular trafficking, protein sorting, and lipid modification. The presence of a central PXXP motif in most (but not all) PX domains stimulated the initial suggestion that they represent binding partners for SH3 domains (1) and therefore may direct inter- and/or intramolecular protein-protein interactions. In support of this, the PX domain from p47<sub>phox</sub> was recently shown to interact, albeit weakly, with the C-terminal SH3 domain from the same protein (2).

A different class of PX domain ligand was recently reported by several groups who described binding of PX domains to phosphoinositides. Specifically, the PX domains from the yeast vacuolar t-SNARE Vam7p (3, 4), sorting nexin-3 (SNX3) (5), and p40<sub>phox</sub> (6, 7) were all shown to bind selectively to phosphatidylinositol 3-phosphate (PtdIns-3-P), which is enriched in endosomal and vacuolar membranes (8). Isolated PX domains from p40<sub>phox</sub> and SNX3 were found to be independently capable of localizing to endosomal structures in vivo in a manner that depends upon their ability to bind PtdIns-3-P and on PI-3-kinase activity (5–7). Similarly, analysis of deletion mutants indicated that the Vam7p PX domain is sufficient for localization of that protein to vacuoles and endosomes in yeast (3). Other phosphoinositides have been reported as the preferred ligands for certain PX domains. The PX domain from the C2-containing PtdIns kinase phosphatidylinositol 3-kinase (PI-3-K) appeared to recognize PtdIns(4,5)<sub>P2</sub> (4), the p47<sub>phox</sub> PX domain showed a preference for PtdIns(4,5)<sub>P2</sub> (6), and the PX domain from cytokine-independent survival kinase bound equivalently to PtdIns(4,5)<sub>P2</sub> and PtdIns(3,5)<sub>P2</sub> and PtdIns(3,4,5)<sub>P3</sub> (9).

The structure of the p40<sub>phox</sub> PX domain was determined using NMR (2) and was found to have a novel α + β fold with no resemblance to other known phosphoinositide-binding domains (10, 11). Analysis of chemical shift perturbations upon PtdIns-3-P binding to the Vam7p PX domain (3) indicated that the phosphoinositide-binding site is close to the PXXP motif at which SH3 domain binding is likely and further implied that communication between the two binding sites may occur (12). Thus, phosphoinositide binding and SH3 domain binding by PX domains may be coordinated in some manner to regulate membrane recruitment of PX domain-containing proteins.

Since PX domains appear to represent a novel phosphoinositide-binding module (13), we were interested to determine the range of their phosphoinositide-binding specificities and affinities to compare them with pleckstrin homology (PH) domains (which vary widely in affinity and specificity (14)) and FYVE domains (which all appear to recognize PtdIns-3-P with roughly comparable affinities (8)). The SMART database (15) identifies 57 PX domains in humans but only 15 in yeast. Therefore, for a genome-wide perspective, we chose to analyze all PX domains from S. cerevisiae. Our findings suggest that all
yeast PX domains specifically recognize PtdIns-3-P. Four of the 15 PX domains bind this phosphoinositide with high affinity, while the remainder bind with only low affinity indicating a requirement for additional components in their membrane recruitment. Most interestingly, proteins with low affinity PX domains appear most often to participate in the formation of large complexes that may be targeted only through multivalent PX domain interactions to specific cellular membranes.

**EXPERIMENTAL PROCEDURES**

**Production of GST-PX Domain Fusion Proteins**—DNA encoding protein fragments that correspond to the relevant PX domains were amplified (fused to GST) were: Mdm1p-(776–170), Vps17p-(88–241), Bem3p-(502–637), Mvp1p-(121–234), Ydr425wp-(112–908) SNX3/Grd19p-(1–214), Bem1p-(50–637), Mvp1p-(121–234), Ydr425wp-(112–908). GST fusion proteins produced from pGSTag contain a protein kinase A phosphorylation site (269–Ydl113cp-(150–258), Spo14p-(304–Vam7p-(1–498), Vps5p-(271–397), Ykr078wp-(108–397), Ydr423wp-(264–418), SNX4-(20–170), Vps1p-(88–234), Ydr425wp-(112–241), Ydl113cp-(150–316), and Ypr097wp-(264–508). GST fusion proteins were produced and reduced from pGSTag (16) using standard procedures. The protein fragments expressed from yeast genomic DNA and subcloned into pGStag (17) using standard procedures. The protein fragments expressed (fused to GST) were: Mdm1p-(776–908), SNX3-GxGrd19p-(1–162), Vam7p-(1–129), Yhr105wp-(65–214), Bem3p-(502–637), Mvp1p-(121–258), Spo14p-(304–498), Vps1p-(271–397), Ykr078wp-(108–258), Bem1p-(269–418), SNX4-(20–170), Vps1p-(88–234), Ydr425wp-(112–241), Ydl113cp-(150–316), and Ypr097wp-(264–508). GST fusion proteins produced from pGSTag contain a protein kinase A phosphorylation site between GST and the PX domain. GST fusion proteins were produced and (where required) labeled with [32P] using protein kinase A as described (17).

**Dot-Blot “Overlay” Assays to Determine Phosphoinositide-Binding Specificity**—[32P]labeled GST-PX domain proteins (at 0.5 μg/ml) were used to probe bovine serum albumin-blocked nitrocellulose filters on which had been spotted phosphoinositides (1 μl at 2 mg/ml) in the pattern shown in the legend to Fig. 3. Dot-blots were then processed exactly as described (17), and bound radioactivity was visualized with a phosphorimaging device.

**Surface Plasmon Resonance (SPR) Analysis of Phosphoinositide Binding**—Dioleoylphosphatidylcholine (DOPC) vesicles with or without (mock) PtdIns-3-P (Matreya Inc, Pleasant Gap, PA) were prepared by extrusion through a 0.1-μm polycarbonate membrane. A vesicle suspension (at 1.5 mM total lipid) was passed over an L1 sensor chip surface (BIAcore) in a BIAcore X instrument, resulting in a signal of 5000 to 7000 response units (RU). Binding of GST-PX domain fusion proteins was measured by passing protein at a range of concentrations over both a surface bearing PtdIns-3-P-containing-vesicles and a surface bearing pure DOPC vesicles. The signal from the DOPC surface was subtracted from that measured simultaneously for the PtdIns-3-P surface to correct for nonspecific binding and refractive index effects. Experiments were all performed at 25°C in 10 mM HEPES, pH 7.5, containing 150 mM NaCl. A 10-μl injection of 100 mM NaOH was used to regenerate the lipid surface after each experimental injection. Positive control experiments demonstrated that this regeneration step did not compromise the surface detectability. A new sensor chip surface was generated each day, since signals obtained with positive control proteins (HRS1-FYVE, PLCδ-PH) were found to decay over a period of 24 h. New sensor chip surfaces gave responses within 10% of one another using standard positive control FYVE or PH domain proteins.

Binding data for GST-PX domain proteins are plotted in Figs. 4 and 5 as the percent of maximal binding against protein concentration injected (estimated from Coomassie-stained SDS-PAGE gels with albumin standards; see Fig. 2). For high affinity PX domains, percent maximal binding was determined at each concentration (c) as (RU/RU sat) × 100, where RU is the response at concentration, and RU sat is the maximum response measured at saturation. RU sat ranged from ~2000 RU to 4000 RU for the high affinity PX domains in different experiments, and a mean value of RU sat was used to calculate percent saturation for the low affinity PX domains shown.
RESULTS AND DISCUSSION

The 15 PX domains encoded by the S. cerevisiae genome are aligned in Fig. 1 alongside the elements of the PX domain secondary structure defined by solution NMR studies (2, 3). The predicted PX domain from Ypr097wp is a clear outlier since it lacks several conserved features and has large (>45 amino acids) insertions in both the presumed α1/α2 and α2/α3 loops. To analyze phospholipid binding we expressed all 15 of the yeast PX domains in Escherichia coli as GST fusion proteins (see "Experimental Procedures"). Most of the fusion proteins were readily and solubly expressed (Fig. 2) and could be obtained in milligram quantities. Exceptions to this were the Spo14p, Mvp1p, and Ykr078wp PX domains that were only expressed readily at low levels. These proteins (and Ypr097wp-PX) were used only for dot-blot studies.

To compare phosphoinositide-binding specificities of the 15 PX domains we employed a dot-blot overlay assay, previously used to study lipid binding by PH and FYVE domains (17, 18). In this qualitative assay, nitrocellulose filters on which different phosphoinositides have been immobilized are probed with 32P-labeled GST fusion proteins. As shown in Fig. 3, all yeast PX domains displayed a strong preference for PtdIns-3-P (top left spot in each blot) over other phosphoinositides. The exception was Ypr097wp, the significant sequence outlier noted above. In at least 8 of the cases, PtdIns-3-P was the only phospholipid detectably recognized. In other cases such as Vps17p and Ydr113cp, phosphoinositide binding appeared more promiscuous, but PtdIns-3-P was clearly preferred. The data therefore appear to define PtdIns-3-P as a target of all PX domains in yeast. This situation contrasts with that for PH domains, which vary significantly in both the extent and nature of their phosphoinositide-binding specificity (14). Instead, yeast PX domains are more similar as a group to FYVE domains, which all bind PtdIns-3-P (8).

For a more quantitative view of PtdIns-3-P recognition by yeast PX domains, we next used SPR to analyze their binding to immobilized vesicles containing 3% (mol/mol) PtdIns-3-P in a phosphatidylincholine backbone. SPR studies were performed with the highly expressed GST fusion proteins shown in the top part of Fig. 2. As shown in Fig. 4, four of these PX domains (those from Mdm1p, SNX3/Grd19p, Vam7p, and Yhr105wp) bound to PtdIns-3-P with relatively high affinity (apparent K_d values for GST fusion proteins of 0.15 to 0.5 μM; see below). The remaining PX domains bound to the same vesicles with only low affinity (apparent K_d values ≥ 100 μM).

The distinction between high affinity and low affinity PX domains is clearly illustrated in Fig. 4 by the fact that SNX3-PX saturates a PtdIns-3-P-containing-sensor surface when injected at less than 1 μM, while Bem1p-PX and SNX4-PX show only low-level binding even when injected at 50-70 μM (2-3 mg/ml). Since many of the highly expressed and most well behaved PX domains fall into the low affinity category (see Fig. 2), we are confident that weak binding does not simply reflect the quality of our protein preparations. Our data therefore suggest the existence of at least two categories of S. cerevisiae PX domains: those that bind PtdIns-3-P with relatively high affinity (the four grouped at the top of Figs. 1 and 3), and those that bind specifically to PtdIns-3-P but with low affinity (the remainder). Additionally, we find that specific recognition of PtdIns-3-P does not necessarily coincide with high binding affinity.

We next compared PtdIns-3-P binding by high affinity PX domains with phosphoinositide binding by well characterized PH and FYVE domains. Since GST dimerization can affect binding studies that employ SPR (19), we compared GST-PX domains with GST fusion proteins of the Hrs1 FYVE domain (Hrs1-FYVE) and the phospholipase C-δ1 PH domain (PLC-δ1-PH), which specifically recognize PtdIns-3-P (8) and PtdIns(4,5)P2 (20), respectively. As shown in Fig. 5, PtdIns-3-P binding by a high affinity PX domain (apparent K_d ~ 0.3 μM for Mdm1p-PX) is quantitatively indistinguishable from PtdIns-3-P binding by Hrs1-FYVE or from PtdIns(4,5)P2 binding by PLC-δ1-PH. We have previously reported using other techniques that monomeric Hrs1-FYVE binds equivalent PtdIns-3-P-containing vesicles with a K_d of 2.5 μM (18) and that a PLC-δ1-PH monomer binds equivalent PtdIns(4,5)P2-containing vesicles with a K_d of 1.7 μM (20). SPR studies of these monomeric proteins gave identical results (not shown). Therefore, a reasonable estimate of K_d values for PtdIns-3-P binding by monomeric PX domains (without fusion to GST) from the high affinity category is ~2–3 μM. Fig. 5 also shows that Mdm1p-PX does not bind detectably to PtdIns-4-P or PtdIns(4,5)P2 in SPR studies. Very similar results were obtained in identical experiments for the PX domains from SNX3/Grd19p, Vam7p, and Yhr105wp (Fig. 4 and data not shown).

Our SPR data argue that only four of the 15 yeast PX domains (the high affinity PX domains) might be independently capable of driving localization of their host proteins to endosomes by binding PtdIns-3-P as reported for the isolated PX domain from mammalian p40phox (6, 7). These PX domains therefore have properties analogous to those of FYVE domains, which recognize membranes of the endosomal trafficking system. Two of the high affinity yeast PX domains occur in proteins that consist of little more than a PX domain: the sorting proteins that consist of little more than a PX domain: the sorting
nexins SNX3/Grd19p (162 amino acids) and Yhr105wp (214 amino acids). Yeast SNX3/Grd19p is predominantly cytoplasmic but localizes to a prevacuolar endosome-like organelle that accumulates in \textit{vps27}-mutant yeast (21). Mammalian SNX3 (also 162 amino acids) specifically recognizes PtdIns-3-P and associates with endosomes in a PI-3-kinase-dependent manner (5). Yhr105wp is most closely related in sequence and architecture to SNX3/Grd19p and may function similarly to regulate endosomal trafficking. The high affinity Vam7p PX domain comprises the N-terminal half of a 317-amino acid protein, and in \textit{vivo} studies of deletion mutants indicate that it is sufficient for targeting Vam7p to the yeast vacuole, where it acts as a t-SNARE (3). The remaining yeast protein with a high affinity PX domain is Mdm1p, a 51-kDa protein that functions in nuclear and mitochondrial inheritance and localizes to punctate structures that are distributed throughout the yeast cytoplasm (22). Although not previously implicated in Mdm1p function, our finding that PtdIns-3-P binds strongly and specifically to the Mdm1p PX domain plus a report that several mutations in the Mdm1p PX domain affect nuclear and/or mitochondrial inheritance (23) suggest an important role for PtdIns-3-P binding in Mdm1p action.

Excluding Ypr097wp, all remaining yeast PX domains appear to bind PtdIns-3-P specifically, but with such low affinity that they cannot possibly drive membrane localization independently. Instead, these low affinity PX domains may only contribute to membrane association together with other domains in the same protein or multi-protein complex. For example, the PX domain of Spo14p and Bem3p is followed immediately by a PH domain, with which it may cooperate in driving membrane association. Alternatively, homo- and/or hetero-oligomerization of proteins containing low affinity PX domains could enhance their effective PtdIns-3-P binding affinity or avidity. For example, Bem1p is a scaffolding protein that forms complexes with several proteins, some of which have PH domains (24) at sites of bud emergence. Perhaps the clearest illustration of this point, however, is seen with the sorting nexins that regulate membrane traffic. Several of the 16 mammalian sorting nexins have been studied biochemically and most form both homo- and hetero-
oligomers (25–28). The exception to this rule is SNX3 (26, 27), which contains little more than just the PX domain and (in both yeast and mammals) lacks the coiled-coil regions proposed to drive sorting nexin oligomerization. Yhr105wp is the only other yeast-sorting nexin that lacks substantial sequence outside the PX domain and is also the only example other than SNX3 that has a high affinity PX domain. Thus, all of the yeast sorting nexins that cannot oligomerize seem to have high affinity PX domains. By contrast, all of the yeast sorting nexins with low affinity PX domains have the capacity to increase their PtdIns-3-P binding avidity through self-association and/or interaction with other related proteins. For example, SNX1 appears to form homo-oligomers through C-terminal coiled-coil motifs (25) that are critical for the endosomal localization of SNX1 (29). Yeast SNX4 was found in high-throughput two-hybrid screens to interact with both Ydr425wp (30) and Ydl113cp (31), both of which have the sequence characteristics of sorting nexins and contain low affinity PX domains. Vps5p, the S. cerevisiae ortholog of mammalian SNX1 (32, 33), forms large oligomers (34) and has been localized to an endosomal compartment in vivo (25, 33). Vps5p forms heteromeric complexes with Vps17p, which also has a low affinity PX domain, and this interaction is necessary for membrane association of Vps5p (32). Along with Vps35p, Vps25p, and Vps26p (which do not have PX domains), Vps5p and Vps17p assemble to form the “retromer” on prevacuolar membranes (34). A Vps35p/Vps29p/Vps26p subcomplex is believed to recognize cargo for retrieval from this endosomal compartment to the Golgi, and a Vps5p/Vps17p complex is then thought to be recruited to membranes bearing this subcomplex to complete retromer formation. A requirement for cooperative PtdIns-3-P binding by the Vps5p and Vps17p PX domains, coincident with their heteromeric assembly, would ensure that the retromer only forms at endosomal membranes containing PtdIns-3-P.

The characteristics of proteins with low affinity PX domains suggest that their primary role is to provide specificity but that other (possibly protein) targets are needed besides PtdIns-3-P to drive membrane association. Recognition of the well conserved PXPPX motif in PX domains by SH3 domains could provide this additional targeting in at least some cases. It is likely that the additional targets will only recruit PX domain-containing proteins when they occur alongside PtdIns-3-P in endosomal compartments. By contrast, it is possible that proteins with high affinity PX domains are targeted to all locations at which PtdIns-3-P is present. It might therefore be anticipated that proteins with low affinity PX domains will be found only in specific endosomal subcompartments, perhaps defined by non-lipid targets, while endosomal targeting of proteins with high affinity PX domains will be more general.

Analysis of PX domain sequences does not provide any clear clues as to what distinguishes high affinity from low affinity PX domains. All key conserved residues and most of those implicated in PtdIns-3-P binding (3) are maintained in both categories as expected since PtdIns-3-P specificity is maintained. In that sense, yeast PX domains differ starkly from PH domains. Whereas PX domains remain PtdIns-3-P-specific, but vary greatly in their affinity, specificity and high affinity usually coincide in the case of PH domains. Furthermore, unlike PX domains, PH domains that bind weakly to phosphoinositides are always quite promiscuous (14). It remains to be seen whether all or most mammalian PX domains will specifically recognize PtdIns-3-P or whether other phosphoinositides might be recognized. Song et al. (4) have reported that the PX domain from the C2-containing PtdIns kinase PI-3-kinase, which has no ortholog in S. cerevisiae, binds specifically to PtdIns(4,5)P2. Kanai et al. (6) reported that the p47phox PX domain prefers PtdIns(3,4,5)P3. Thus, the expansion of the PX domain family in going from yeast to humans may include additional phosphoinositide targets, although all S. cerevisiae PX domains specifically recognize PtdIns-3-P.

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Fig. 5. Comparison of PX, FYVE, and PH domain binding. The Mdm1p PX domain (as a GST fusion protein) bound to PtdIns-3-P vesicles in SPR studies with an affinity similar to PtdIns-3-P binding by the GST/Hrs1-FYVE domain and PtdIns(4,5)P2 binding by GST/PLC6-PH. By contrast, the Mdm1p PX domain did not bind to vesicles containing 3% PtdIns-4-P or PtdIns(4,5)P2 (Echelon). Essentially identical results for binding specificity were obtained in parallel experiments for the other high affinity PX domains, from SNX3/Grb19, Vam3p, and Yhr105wp. Experiments were performed as described under “Experimental Procedures”. In equivalent experiments, monomeric (non GST fusion) forms of the Hrs1 FYVE domain and the PLC-6 PH domain bound to PtdIns-3-P and PtdIns(4,5)P2 with KD values of ~2.5 μM and 2.0 μM respectively in agreement with those measured using other techniques (18, 20).
PtdIns-3-P Binding by Yeast PX Domains
