View metadata, citation and similar papers at core.ac.uk
brought to you by CORE
provided by Elsevier - Publisher Connector

Cell, Vol. 105, 817–820, June 29, 2001, Copyright ©2001 by Cell Press

Phoxy Lipids: Revealing PX Domains as Phosphoinositide Binding Modules

Minireview

Matthew J. Wishart,[2] Gregory S. Taylor,[2] and Jack E. Dixon[1]
Department of Biological Chemistry
University of Michigan Medical School and
Life Sciences Institute
Ann Arbor, Michigan 48109

The coordination of cellular signaling events requires both spatial and temporal regulation. A recurring theme among eukaryotic organisms is the use of phosphoinositide-specific binding domains to direct proteins to discrete sites within cells where their function(s) are required. To date, a number of distinct, highly conserved phosphoinositide binding motifs have been identified, the most well-characterized of which include the C2 (PKC conserved region 2), ENTH (epsin N-terminal homology) (Ford et al., 2001; Itoh et al., 2001), FYVE (Fab1, YOTB, Vac1, and EEA1), and PH (pleckstrin homology) domains (Schultz et al., 2000; see references in reviews by Gillooly et al. 2001; Hurley and Meyer, 2001; and Odorizzi et al., 2000). These domains recruit proteins to specific regions in cells via their interactions with inositol lipids and may also serve as allosteric regulators of enzyme activity or protein-protein interactions.

The PX domain was initially identified as a conserved motif of ~130 residues within the p40*phox* and p47*phox* subunits of the neutrophil NADPH oxidase superoxide generating complex (Ponting, 1996). The PX motif can also be found in a wide variety of proteins involved in cell signaling pathways (phospholipases D1 and D2, PI 3-kinase, and Spo14p), vesicular trafficking and yeast vacuolar morphology (human sorting nexins; yeast Vps5p, Vps17p, Vam7p, and Mvp1p), and control of yeast bud emergence and cell polarity (Bem1p and Bem3p) (Ponting, 1996). Currently, at least 57 human and 15 yeast proteins that contain PX domains have been identified (Schultz et al., 2000).

Although the function of PX domains has remained unclear, previous studies have implied a possible role in regulating the subcellular localization of their corresponding proteins. For example, a significant number of PX proteins are localized to membranes or vesicular structures within cells (Schultz et al., 2000). In the case of the NADPH oxidase complex, translocation from the cytosol to membranes occurs upon activation of the neutrophil respiratory burst response (see references in Babior, 1999). In addition, many of the PX proteins involved in membrane trafficking processes do not contain recognizable membrane targeting motifs (Schultz et al., 2000). Taken together, these observations have provided a strong rationale for investigating whether PX domains might function as membrane targeting modules. In a series of papers in *Nature Cell Biology* this month, the PX domains of the NADPH oxidase components, p40*phox* and p47*phox*, the yeast vacuolar SNARE, Vam7p, and the sorting nexin, SNX3, are shown to function as D3-phosphorylated phosphoinositide binding modules (Cheever et al. 2001; Ellson et al., 2001; Kanai et al., 2001; Xu et al. 2001). Moreover, the solution structure of the p47*phox* PX domain published in *Nature Structural Biology* offers intriguing insights into possible mechanisms for the regulation of PX domain function (Hiroaki et al., 2001). This review will focus on structural and functional insights into the PX domain as a novel phosphoinositide binding module.

PX Domains as Regulators of Membrane and Protein Trafficking

The mechanisms underlying membrane trafficking and vesicular fusion have been intensively studied using the vacuolar sorting pathway of the budding yeast, *Saccharomyces cerevisiae*, as a model system. Vam7p, a PX-containing protein that is an essential component of vacuolar sorting, is related to the SNAP-23/25 mammalian t-SNAREs (Schultz et al., 2000). SNARE proteins, which are found on both cytoplasmic donor vesicles (v-SNAREs) and the acceptor organelles to which they are targeted (t-SNAREs), serve to modulate the specificity of docking and fusion between donor and acceptor membranes. Vam7p has an N-terminal PX domain, a C-terminal coiled-coil t-SNARE motif (Schultz et al., 2000), and localizes to the vacuolar membrane as part of a vacuolar SNARE complex (Sato et al., 1998; Ungermann and Wickner, 1998). Vam7p has previously been shown to physically interact with Vam3p, which also localizes to vacuolar membranes (Sato et al., 1998; Ungermann and Wickner, 1998). Mutation of conserved residues within the Vam7p PX domain create synthetic vacuolar sorting defects when combined with a temperature-sensitive *vam3* allele, suggesting that the PX domain is necessary for Vam7p function (Sato et al., 1998). Further, Vam7p does not contain known membrane targeting motifs, prompting the question of how it associates with vacuolar membranes. In an effort to identify the role of the PX domain of Vam7p in vacuolar sorting, Cheever and colleagues have investigated whether it may serve as a targeting module to direct the localization of Vam7p (Cheever et al., 2001).

In this report, several lines of evidence corroborate that the PX domain functions to localize Vam7p to vacuolar membranes (Cheever et al., 2001). First, either a PX domain point mutation or deletion results in mislocalization of Vam7p to the cytoplasm. Because a Vam7p mutant lacking the C-terminal coiled-coil motif required for its interaction with Vam3p still localizes to the vacuolar membrane, the authors conclude that the PX domain alone is both necessary and sufficient to direct membrane targeting of Vam7p. It is of note that a Vam7p mutant localizes to endosomes in a yeast strain in which endosome-to-vacuole sorting is blocked (*vps4Δ*). Here, they further observe that this localization is similar to that of FYVE-domain-containing proteins, which are known to target the membrane phosphoinositide, PI(3)P (see references in reviews by Gillooly et al. 2001; Hurley and Meyer, 2001; and Odorizzi et al., 2000), and suggest

[1] Correspondence: jedixon@umich.edu
[2] These authors contributed equally to this work.

that the PX domain might utilize a similar targeting mechanism.

In support of this hypothesis, Cheever et al. demonstrate that a recombinant Vam7p-PX domain protein specifically binds to PI(3)P in a protein-lipid overlay assay. *VPS34* encodes the sole PI 3-kinase found in budding yeast and PI(3)P is depleted in *vps34* null mutant strains or *vps34* temperature-sensitive yeast grown at the nonpermissive temperature. The mislocalization of Vam7p observed in yeast harboring *vps34* null or temperature-sensitive mutations provides further evidence of the requirement for PI(3)P in targeting Vam7p to vacuolar membranes.

Analysis of the Vam7p-PX domain by NMR spectroscopy has identified several structural elements necessary for its membrane targeting function and interaction with PI(3)P (Cheever et al., 2001). Specifically, binding of PI(3)P involves five basic regions and induces conformational changes, which suggests that the PX domain may form a pocket of positive charge into which the phosphorylated head group of phosphoinositide is inserted. In addition, the side chains of charged and hydrophobic residues in a loop region preceding the $\alpha 2$ helix are predicted to interact with both the surface and interior of a lipid bilayer. Together, the basic binding pocket and membrane attachment loop are likely to contribute significantly to the binding specificity and targeting function of the Vam7p PX domain. Collectively, these findings demonstrate that the PX domain of Vam7p is a PI(3)P-specific phosphoinositide binding motif that plays a key role in regulating the subcellular localization and vacuolar sorting function of Vam7p in yeast.

Further support for the role of the PX domain as a phosphoinositide binding/targeting module has been provided by Xu and colleagues in their analysis of the human sorting nexin, SNX3 (Xu et al., 2001). Sorting nexins are found in a variety of organisms from yeast to human and function as regulators of protein trafficking (see references in Kurten et al., 1996 and Haft et al., 1998). Although the exact mechanism by which they function has remained unclear, sorting nexins likely target candidate proteins such as growth factor receptors and vacuolar hydrolases to specific organelles for post-translational processing, recycling, or degradation. In their recent work, Xu et al. show that SNX3 colocalizes with the FYVE-domain protein EEA1 (early endosome autoantigen) and internalized transferrin receptor, suggesting that it is associated with early endosomes and recycling endosomal intermediates. This localization is disrupted by the PI 3-kinase inhibitor, wortmannin, indicating that D3-phosphorylated inositol lipids are required for proper targeting of SNX3. The authors also show that the PX domain of SNX3, like that of Vam7p, binds specifically to PI(3)P. Furthermore, overexpression of SNX3 disrupts trafficking of both transferrin and EGF receptors in a manner that is dependent on its ability to bind PI(3)P. Together, these findings implicate a role for SNX3 in regulating protein sorting through endosomal pathways and underscore the concept that targeting to PI(3)P by its PX domain is an essential component of this regulation.

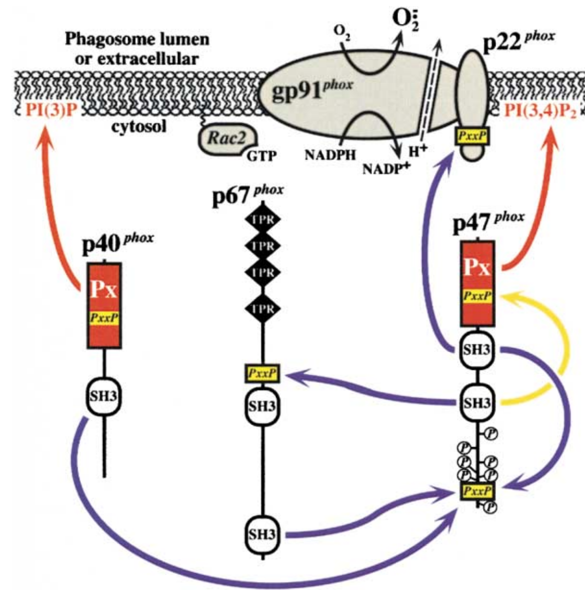

Figure 1. Model of Phosphoinositide and SH3:PxxP Interactions for PX Domain-Containing Proteins Involved in Targeted Membrane Assembly of the Phagocyte NADPH Oxidase

Selected regions of NADPH oxidase subunits gp91^{phox}, p22^{phox}, p40^{phox}, p67^{phox}, p47^{phox}, and p21$^{Rac1/2}$ are schematically represented as follows: phox domain, PX (red); src-homology 3 domain, SH3; proline-rich motif, PxxP (yellow); tetratricopeptide repeat, TPR; phosphorylation site, P. Specific phosphoinositides (orange) are depicted within a lipid bilayer. Blue arrows denote SH3:PxxP interactions previously identified for p47^{phox}. Red and gold arrows indicate lipid and protein interactions involving p40^{phox}- and p47^{phox}-PX domains, as described in the text.

PX Domains Target Membrane Translocation of NADPH Oxidase Components

In neutrophils and other professional phagocytic cells, NADPH oxidase catalyzes the production of superoxide (O_2^-) as a precursor to other reactive oxygen species (for example, hydrogen peroxide), which function to kill invading microorganisms (Babior, 1999). Generation of O_2^- is important to pathogen removal and genetic loss, or inactivation of individual oxidase subunits results in a heightened susceptibility to infection, clinically defined as inherited chronic granulomatous diseases (CGD) (Burg and Pillinger, 2001). Although PX domains were first identified as a region of sequence homology between the p40^{phox} and p47^{phox} subunits of phagocyte NADPH oxidase (phox), their role in oxidase function has remained obscure.

In their resting state, the subunits of NADPH oxidase are differentially localized to a membrane-bound flavocytochrome b_{558} (gp91^{phox} and p22^{phox}), or the cytoplasm (p40^{phox}, p67^{phox}, p47^{phox}, and p21$^{Rac1/2}$). Upon activation, the cytosolic components translocate and become part of a functional membrane oxidase complex (Figure 1). Because inappropriate O_2^- production can cause damage to host tissues, activation of the NADPH oxidase complex is subject to strict spatial and temporal constraints. In unstimulated cells, p40^{phox}, p67^{phox} and p47^{phox} exist as an inactive cytoplasmic complex held together by intra- and intermolecular protein-protein interactions that must rearrange to permit membrane translocation

and cytochrome activation to occur (Figure 1). Phosphorylation appears to modulate at least some of these protein interactions (Babior, 1999), however, recent work by two groups provides new insights into the signals responsible for targeted translocation of the p40^{phox}:p67^{phox}:p47^{phox} complex to the phagosomal membrane (Ellson et al., 2001; Kanai et al., 2001).

Previous studies have implicated the products of PI 3-kinase in stimulating neutrophil O_2^- production (see references in Kanai et al., 2001 and Wymann et al., 2000), but until recently, their mechanism of action has remained unknown. Using a cell free assay, Ellson and colleagues report that recombinant PI 3-kinase or its purified lipid products, PI(3,4,5)P_3 or PI(3,4)P_2, independently stimulate the production of O_2^- from a mixture of neutrophil membranes and cytosol. Ellson et al. use chromatographic fractionation and sequencing of neutrophil cytosolic proteins to identify two lipid phosphatases, SHIP-1 and PI(3,4)P_2 4-phosphatase type 1α, as well as a complex of p40^{phox}:p67^{phox}, that are independently capable of mediating PI(3,4,5)P_3-dependent O_2^- production. Because these phosphatases could possibly act to dephosphorylate PI(3,4,5)P_3 and PI(3,4)P_2 at the D5 and D4 positions, respectively, and catalytic activity is essential for their effects, the authors surmise that the ultimate PI(3)P product could be the O_2^- generating signal. Indeed, PI(3)P alone is capable of activating a relipidated mixture of purified cytochrome *b*, p40^{phox}:p67^{phox}, p47^{phox}, and GTP-rac. Importantly, activation by PI(3)P requires the presence of p40^{phox}. Moreover, isolated p40^{phox} bound to immobilized lipid vesicles in a PI(3)P-dependent and -specific manner, suggestive of a direct protein-lipid interaction. Recombinant versions of the PX domain of p40^{phox} exhibit lipid vesicle binding but are unable to activate cytochrome b, indicating that the PX domain facilitates PI(3)P-mediated activation in vitro only in the context of full-length p40^{phox}. Finally, as seen with the SNX3 protein (Xu et al., 2001), wortmannin disrupts the colocalization of p40^{phox}-PX domains with endosomal EEA1, suggesting that membrane targeting of endogenous p40^{phox} is likely mediated by PX:PI(3)P interactions (Figure 1).

In a structure-function analysis of the PX domains of p40^{phox} and p47^{phox}, Kanai et al. demonstrate both the PI(3)P-specificity of p40^{phox}-PX interactions in vitro, and the wortmannin-sensitive localization in cultured cells as seen by Ellson (Kanai et al., 2001). They also demonstrate that p40^{phox}-PX colocalization with EEA1 occurs at sites of PI(3)P-specific, EEA1-FYVE domain interaction. In contrast to p40^{phox}-PX specificity, however, Kanai et al. also observe p47^{phox}-PX binding with different affinities toward immobilized D3-phosphorylated inositol lipids in vitro (PI(3,4)$P_2 > $ PI(3)P $>>$ PI(3,5)$P_2 =$ PI(3,4,5)P_3). Moreover, differential binding of p47^{phox}-PX to liposomes containing either PI(3,4)P_2, PI(4,5)P_2, or no phosphoinositides indicates that in addition to sites of high affinity PI-lipid binding, p47^{phox}-PX contains determinants for general membrane interaction as seen in the NMR structure of Vam7p (Cheever et al., 2001). In an attempt to identify amino acids critical for PX function, Kanai et al. have mutated two arginines whose identity or charge are conserved in the majority of PX-domain-containing proteins (Ponting, 1996). Mutation of p47^{phox}-PX Arg90 to Leu significantly reduces binding to all immobilized

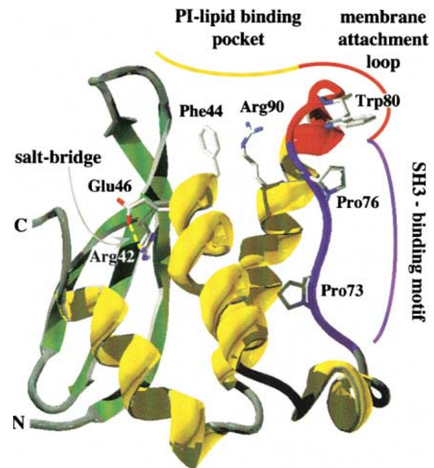

Figure 2. NMR Solution Structure and Putative Functional Regions of the p47^{phox}-PX Domain

Coordinates of the p47^{phox}-PX structure (PDB 1gd5_No1) were kindly provided by D. Kohda. Segments of α helical (yellow) and β strand (green) conformation are indicated. Selected amino acids and regions implicated in PX domain functions are provided as described in the text.

PI-lipids. Similarly, mutation of p47^{phox}-PX Arg42 to Gln, which mimics an endogenous mutation identified in a subset of CGD patients (Noack et al., 2001), or the analogous mutation of p40^{phox}-PX (Arg57 to Gln), completely abolishes PI-lipid interaction in vitro. In addition, the p40^{phox}-PX Arg57 to Gln mutation causes a reduction in the endosomal localization observed with wild-type p40^{phox}-PX. Although the relative contribution of lipid binding by either p40^{phox} or p47^{phox} to membrane translocation remain to be determined, the Ellson and Kanai reports provide a mechanism for targeting the cytoplasmic p40^{phox}:p67^{phox}:p47^{phox} complex to the phagosomal membrane and further establish PI-lipid binding as a general function of the PX domain family.

SH3 Domain Binding Alters PX Domain Structure

Binding of src homology-3 (SH3) domains to proline-rich motifs (PxxP) constitutes the major interaction between p47^{phox} and the other NADPH oxidase subunits (Figure 1) (Babior, 1999). In the resting state, intramolecular interactions within p47^{phox} are thought to prohibit its two SH3 domains from binding other oxidase subunits. Mindful of the fact that a majority of known PX domains contain a PxxP motif in the center of their conserved sequence (Ponting, 1996), Hiroaki and coworkers suggest a new mechanism for regulating p47^{phox} activity. They demonstrate that the PxxP motif within the p47^{phox}-PX serves as a specific target for the C-terminal p47^{phox}-SH3 domain (Figure 1) (Hiroaki et al., 2001). These authors also determine the NMR solution structure of the p47^{phox}-PX domain (Figure 2), and show that p47^{phox}-SH3 binding alters the conformation of the PxxP loop and hydrophobic contacts between helices in the structural core. These observations suggest that SH3 binding is a significant aspect of p47^{phox}-PX domain function.

The paper by Hiroaki et al. appeared concomitant with the reports of PI-lipid binding by p47^{phox}-PX (Kanai et al., 2001) and the NMR data from Vam7p-PX (Cheever

et al., 2001). Analysis of the collective work on SH3 and phospholipid binding makes it possible to draw additional insight from the PX domain solution structure. First, the p47*phox*-PX structure places the Phe44 and Arg90 residues in a surface accessible pocket (Figure 2). Mutation of these residues in p47*phox* or the corresponding residues in Vam7p (Tyr42 and Arg88, respectively) diminish PI-lipid binding (Kanai et al., 2001) and disrupt PX localization (Cheever et al., 2001). The NMR signal of Vam7p-Tyr42 (Phe44 in p47*phox*) undergoes a dramatic positional change in the presence of PI(3)P, suggesting that it may be a site of phosphoinositide binding. A flexible loop (shown in red in Figure 2) adjacent to this pocket has also been identified as the site of Vam7p membrane attachment (Cheever et al., 2001). Secondly, p47*phox*-Arg42 and Glu46 form a salt bridge which likely constrains the orientation of core PX structures, including Phe44, involved in specific ligand binding. As Kanai et al. observe, mutations in p47*phox*-Arg42 completely abolish PI-lipid binding, and disrupt the localization of p40*phox*. This structural role likely accounts for the inhibition of O_2^- production in cases of CGD where p47*phox*-Arg42 has been mutated to Gln (Noack et al., 2001). Finally, it appears that both PI(3)P and SH3 interactions result in interrelated NMR shifts of Phe44, Arg90, and residues within the PxxP motif. Although the magnitude of NMR changes may depend on the conditions employed, it is important to note that SH3 binding causes a shift in the NMR signals of residues within the PI(3)P binding pocket (Hiroaki et al., 2001), and that lipid binding causes NMR shifts of residues within the PxxP motif (Cheever et al., 2001). SH3 binding also significantly alters the position of p47*phox*-Trp80, which resides in the corresponding membrane attachment loop of Vam7p-PX (Figure 2, red loop). Thus, SH3 binding could modulate the accessibility of the phosphoinositide binding pocket and membrane attachment of the PX domain. This raises the possibility that SH3 interaction acts as a switch to regulate PX domain function.

In summary, the identification of the PX domain as a phosphoinositide binding module provides significant insight into its function in targeting proteins to specific cellular membranes. In addition, the PX domain structure-function studies reveal key determinants of its lipid binding specificity as well as potential mechanisms by which its function may be regulated. Now that a role has been ascribed to the PX domain, there are several interesting aspects of its specificity and regulation which may be investigated further. For example, what phosphoinositides are the specific targets of other PX domain proteins and how might they affect the physiologic functions of these proteins? It is clear from the works reviewed herein that individual PX domains are capable of recognizing different phosphoinositides, which will likely effect the localization of PX proteins to distinct subcellular compartments. Another question of significant interest is how potential intra- or intermolecular interactions of the PxxP motif with SH3 domains affect lipid binding and thus localization of PX domain proteins. Are lipid binding and SH3 binding mutually exclusive events or does binding of one of these ligands enhance interactions with the other? Finally, the role of residues implicated in interactions between the PX domain and phosphoinositides will require further study to better understand both the specificity of lipid binding and regulatory mechanisms of PX function.

Selected Reading

Babior, B.M. (1999). Blood *93*, 1464–1476.

Burg, N.D., and Pillinger, M.H. (2001). Clin. Immun. *99*, 7–17.

Cheever, M.L., Sato, T.K., de Beer, T., Kutateladze, T., Emr, S.D., and Overduin, M. (2001). Nat. Cell Biol. *3*, 613–618.

Ellson, C.D., Gobert-Grosse, S., Anderson, K.E., Davidson, K., Erdjument-Bromage, H., Tempst, P., Thuring, J.W., Cooper, M.A., Lim, Z.-Y., Holmes, A.B., et al. (2001). Nat. Cell Biol. *3*, 679–682.

Ford, M.G., Pearse, B.M., Higgins, M.K., Vallis, Y., Owen, D.J., Gibson, A., Hopkins, C.R., Evans, P.R., and McMahon, H.T. (2001). Science *291*, 1051–1055.

Gillooly, D.J., Simonsen, A., and Stenmark, H. (2001). Biochem. J. *355*, 249–258.

Haft, C.R., de la Luz Sierra, M., Barr, V.A., Haft, D.H., and Taylor, S.I. (1998). Mol. Cell. Biol. *18*, 7278–7287.

Hiroaki, H., Ago, T., Ito, T., Sumimoto, H., and Kohda, D. (2001). Nat. Struct. Biol. *8*, 526–530.

Hurley, J.H., and Meyer, T. (2001). Curr. Opin. Cell Biol. *13*, 146–152.

Itoh, T., Koshiba, S., Kigawa, T., Kikuchi, A., Yokoyama, S., and Takenawa, T. (2001). Science *291*, 1047–1051.

Kanai, F., Liu, H., Field, S.J., Akbary, H., Matsuo, T., Brown, G.E., Cantley, L.C., and Yaffe, M.B. (2001). Nat. Cell Biol. *3*, 675–678.

Kurten, R.C., Cadena, D.L., and Gill, G.N. (1996). Science *272*, 1008–1010.

Noack, D., Rae, J., Cross, A.R., Ellis, B.A., Newburger, P.E., Curnutte, J.T., and Heyworth, P.G. (2001). Blood *97*, 305–311.

Odorizzi, G., Babst, M., and Emr, S.D. (2000). Trends Biochem. Sci. *25*, 229–235.

Ponting, C.P. (1996). Protein Sci. *5*, 2353–2357.

Sato, T.K., Darsow, T., and Emr, S.D. (1998). Mol. Cell. Biol. *18*, 5308–5319.

Schultz, J., Copley, R.R., Doerks, T., Ponting, C.P., and Bork, P. (2000). Nucleic Acids Res. *28*, 231–234.

Ungermann, C., and Wickner, W. (1998). EMBO J. *17*, 3269–3276.

Xu, Y., Hortsman, H., Seet, L., Wong, S.H., and Hong, W. (2001). Nat. Cell Biol. *3*, 658–666.

Wymann, M.P., Sozzani, S., Altruda, F., Mantovani, A., and Hirsch, E. (2000). Immunol. Today *21*, 260–264.