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Second basic pockets contribute to the localization of PX domains by binding to phosphatidic acid

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Introduction

Physiological cellular events, such as growth, cytoskeletal reorganization, membrane trafficking, are mediated by numerous signaling molecules, of which subcellular localizations are regulated strictly. Recruitment of proteins to appropriate sites at correct timings is mediated by a number of protein modules whose functions are the interaction with membrane phospholipids (DiNitto et al., 2003). Following the discovery of phosphoinositides as ligands for pleckstrin homology (PH) domains (Harlan et al., 1994; Yagisawa et al., 1994; Garcia et al., 1995; Lemmon et al., 1995; Kanematsu et al., 1996; Takeuchi et al., 1997), numbers of protein domains that mediate protein-lipid interaction have been discovered, including the epsin N-terminal homologue (ENTH), FYVE (Fab1p/YOTP/Vac1p/EEA1) and phox homology (PX) domains (DiNitto et al., 2003). The PX domain is a protein module consisting of approximately 130 amino acids with three β-strands followed by three α-helices forming a helical subdomain, and is found in more than 50 human proteins related to membrane traffic and intracellular signaling (Ellson et al., 2002). Most of the PX domains examined to date specifically recognize phosphatidylinositol 3-phosphate [PI(3)P], which is predominantly found in early endosomes, yet there are some PX domains with binding specificities to different phospholipids (Ellson et al., 2002). Out of the PX domains showing a preference for phospholipids other than PI(3)P, the PX domain of $p47^{phox}$

Abbreviations: class III PI3K, class III phosphatidylinositol 3-kinase (E.C.2.7.1.137); DGK, diacylglycerol kinase (E.C.2.7.1.107); DMEM, Dulbecco's modified Eagle's Medium; EEA1, early endosomal antigen 1; ENTH, epsin N-terminal homologue; FYVE, Fab1p/YOTP/Vac1p/EEA1; EGFP, enhanced green fluorescent protein; LPAAT, lysophosphatidic acid acyltransferase (E.C.2.3.1.51); mTOR, mammalian target of rapamycin; PA, phosphatidic acid; PH domain, pleckstrin homology domain; PI(3)P, phosphatidylinositol 3-phosphate; PLC, phospholipase C (E.C.3.1.4.11); PLD, phospholipase D (E.C.3.1.4.4); PMA, phorbol 12-myristate 13-acetate; PX domain, Phox homology domain.

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($p47^{phox}$ PX) was found to have dual specificity to the phospholipids (Karathanassis et al., 2002; Yaffe, 2002), *i.e.*, there are two distinct pockets on the membrane-binding surface; one prefers $PI(3,4)P_2$ and the other binds anionic phospholipids such as phosphatidic acid (PA). A series of *in vitro* experiments showed that simultaneous occupancy of the second pocket by anionic phospholipids may synergistically increases affinity of the PX domain to the membrane containing the phosphoinositide specific for the main pocket (Karathanassis et al., 2002; Stahelin et al., 2003). However, the contribution of the second pocket to spatiotemporal regulation of the PX domain in cells has not been directly investigated in detail.

PA, a membrane phospholipid, is proposed to have intracellular messenger functions to activate a number of physiological events, including secretion, neutrophil superoxide generation, cytoskeletal reorganization and cell growth (English et al., 1996; Ktistakis et al., 2003), but in most cases the defined molecular targets remain to be identified. Three enzymes, phospholipase D (PLD), diacylglycerol kinase (DGK), and lysophosphatidic acid acyltransferase (LPAAT), are known to produce PA by hydrolyzing phosphatidylcholine (PC), phosphorylating diacylglycerol (DAG), and adding an acyl chain to lysophosphatidic acid (LPA), respectively. As the amount of PA produced by each enzyme compared to overall PA mass in cells is very little, the enzymes might contribute to cellular signaling by regulating only the localized levels of PA. Although the collection remains small so far, a growing number of putative downstream targets of PA which interact directly with PA have been reported, including the cyclic AMP-specific phosphodiesterase PDE4D3 (Grange et al., 2000), Raf-1 (Ghosh et al., 1996; Rizzo et al., 2000), the mammalian target of rapamycin (mTOR) (Fang et al., 2001), the catalytic subunit of protein phosphatase 1γ (Jones and Hannun, 2002; Jones et al., 2005) and Son of sevenless (Zhao et al., 2007). However, a PA-binding module has not yet been reported to date, in contrast to other lipid binding modules such as PH or FYVE domains for $PI(4,5)P_2$ or PI(3)P, respectively.

In the course of examining the specificity of phospholipid-binding of multiple PX domains, we found that the PX domains from two proteins, RPK118 and KIF16B, showed dual specificity to PI(3)P and PA. In the present study, we investigated the role of binding of the PX domains to two different phospholipids, with special reference to the function of these modules as downstream targets of PA.

Materials and methods

Materials

Phosphoinositides were obtained from Cell Signals (Lexington, KY) or Echelon Biosciences (Salt Lake City, UT) and other phospholipids were from Avanti Polar Lipids (Alabaster, AL). DGK from *Escherichia coli* and PLD from *Streptomyces chromofuscus* were purchased from Sigma–Aldrich (St Louis, MO), and phosphatidylinositol-specific PLC from *Bacillus cereus* were from BIOMOL (Enzo Life Science, Farmingdale, NY). Antibodies used in this study were as follows: anti-HA antibody (HA11; Babco, Richmond, CA), anti-EEA1 (early endosomal antigen 1, BD Biosciences, San Jose, CA) and Cy3-anti-mouse antibody (Jackson Immuno Research Laboratories, West Grove, PA). Wortmannin and phorbol 12-myristate 13-acetate (PMA) were from Sigma–Aldrich.

Cell culture, immunofluorescence and live-cell imaging

MDCK and COS-7 cells were obtained from the American Tissue Culture Collection (ATCC) and maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum. Transient transfection experiments were carried out with lipofectamine PLUS reagents (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. The procedures for immunofluorescence and live-cell imaging were described previously (Takeuchi et al., 2010).

Protein-lipid overlay assays

A protein-lipid overlay assay was performed using GST-fusion proteins exactly as described previously (Kanai et al., 2001). GST-tagged recombinant proteins were expressed using a bacterial