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The Phox Homology (PX) Domain-dependent, 3-Phosphoinositide-mediated Association of Sorting Nexin-1 with an Early Sorting Endosomal Compartment Is Required for Its Ability to Regulate Epidermal Growth Factor Receptor Degradation*

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Recent studies have shown that phox homology (PX) domains act as phosphoinositide-binding motifs. The majority of PX domains studied show binding to phosphatidylinositol 3-monophosphate (PtdIns(3)P), an association that allows the host protein to localize to membranes of the endocytic pathway. One issue, however, is whether PX domains may have alternative phosphoinositide binding specificities that could target their host protein to distinct subcellular compartments or allow their allosteric regulation by phosphoinositides other than PtdIns(3)P. It has been reported that the PX domain of sorting nexin 1 (SNX1) specifically binds phosphatidylinositol 3,4,5-trisphosphate (PtdIns(3,4,5)P_3) (Zhong, Q., Lazar, C. S., Tronchere, H., Sato, T., Meerloo, T., Yeo, M., Songyang, Z., Emr, S. D., and Gill, G. N. (2002) *Proc. Natl. Acad. Sci. U. S. A.* 99, 6767–6772). In the present study, we have shown that whereas SNX1 binds PtdIns(3,4,5)P_3 in protein:lipid overlay assays, in liposomes-based assays, binding is observed to PtdIns(3)P and phosphatidylinositol 3,5-bisphosphate (PtdIns(3,5)P_2) but not to PtdIns(3,4,5)P_3. To address the significance of PtdIns(3,4,5)P_3 binding, we examined the subcellular localization of SNX1 under conditions in which plasma membrane PtdIns(3,4,5)P_3 levels were significantly elevated. Under these conditions, we failed to observe association of SNX1 with this membrane. However, consistent with the binding to PtdIns(3)P and PtdIns(3,5)P_2 being of more physiological significance was the observation that the association of SNX1 with an early endosomal compartment was dependent on a 3-phosphoinositide-binding PX domain and the presence of PtdIns(3)P on this compartment. Finally, we have shown that the PX domain-dependent/early endosomal association of SNX1 is important for its ability to regulate the targeting of internalized epidermal growth factor receptor for lysosomal degradation.

The membrane phospholipid phosphatidylinositol is the precursor for a family of lipid second messengers, known collectively as phosphoinositides, that differ solely in the phosphorylation status of their inositol head group (1, 2). Phosphoinositides are ideally suited to function as spatially restricted membrane second messengers because: the synthesis and turnover of phosphoinositides from the relatively abundant phosphatidylinositol precursor can be rapid and highly concentrated within discrete membrane micro-domains; the ratio of phosphoinositide to binding partner can be relatively large, which makes it possible to target a large number of distinct proteins to a particular membrane without saturating the binding sites; structurally distinct phosphoinositides can activate distinct downstream effectors; and the potential for rapid, sequential interconversion between phosphorylated forms means that phosphoinositides can confer processivity to membrane signaling events (reviewed in Refs. 3–5).

A major advance in our understanding of phosphoinositide signaling has been the identification of a number of highly conserved modular protein domains that bind various phosphoinositides. To date, a number of distinct phosphoinositide-binding motifs have been identified and characterized including the epsin amino-terminal homology (ENTH) domain, Fab1, YOTB, Vac1, and EEA1 (FYVE) domain, band 4.1, ezrin, radixin, and moesin (FERM) domain, pleckstrin homology domain, and more recently, the phox homology (PX)[1] domain (reviewed in Refs. 4–8). Such "cut and paste" modules are found in a diverse array of multidomain proteins and recruit their host protein to specific subcellular compartments via their interaction with phosphoinositides. They may also serve as allosteric regulators of enzyme activity and protein-protein interactions.

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[1] The abbreviations used are: PX, phox homology; EGF, epidermal growth factor; GFP, green fluorescent protein; EGFP, enhanced GFP; ARNO, ARF-nucleotide exchange factor; PtdIns, phosphatidylinositol; PtdIns(3)P, phosphatidylinositol 3-monophosphate; PtdIns(3,4,5)P_3, phosphatidylinositol 3,4,5-trisphosphate; PtdIns(3,5)P_2, phosphatidylinositol 3,5-bisphosphate; PtdIns(4)P, phosphatidylinositol 4-monophosphate; PtdIns(5)P, phosphatidylinositol 5-monophosphate; PtdIns(3,4)P_2, phosphatidylinositol 3,4-bisphosphate; PtdIns(4,5)P_2, phosphatidylinositol 4,5-bisphosphate.

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 (9). PX domains can be found in a wide variety of proteins involved in cell signaling (phospholipase D1 and D2, class II phosphoinositide 3-kinases), control of yeast bud emergence and polarity (Bem1p and Bem3p), and vesicle trafficking (human sorting nexins, yeast Vam7p). Currently, at least 57 human and 15 yeast proteins that contain PX domains have been identified (reviewed in Refs. 5 and 10–14).

Recent simultaneous studies from a number of laboratories have now shown that several PX domains act as specific phosphoinositide-binding motifs (15–24). The majority of PX domains studied so far show binding selectivity to phosphatidylinositol 3-monophosphate (PtdIns(3)P); indeed, all yeast PX domains have been reported to recognize this lipid (23). In those cases in which it has been studied, the ability of the PX domain to bind PtdIns(3)P results in the association of the host protein with components of the endocytic pathway. In this respect, PX domains resemble FYVE domains, although the two are structurally distinct (25–27).

One key issue, however, is whether PX domains may have alternative phosphoinositide binding specificities that could target their host protein to distinct subcellular compartments or allow their allosteric regulation by phosphoinositides other than PtdIns(3)P. It has recently been reported that the PX domain of sorting nexin 1 (SNX1) does not bind PtdIns(3)P but rather specifically binds phosphatidylinositol 3,4,5-trisphosphate (PtdIns(3,4,5)P_3) (28). In this present study, we have shown that SNX1 may not bind PtdIns(3,4,5)P_3 *in vivo*; rather, its PX domain binds with similar affinities PtdIns(3)P and phosphatidylinositol 3,5-bisphosphate (PtdIns(3,5)P_2). Such a conclusion is entirely consistent with the PX domain-dependent, phosphoinositide-induced association of SNX1 with an early endosomal compartment, an association that is important for the ability of SNX1 to regulate EGF receptor traffic.

EXPERIMENTAL PROCEDURES

Antibodies—Anti-SNX1 and anti-EEA1 mouse monoclonal antibodies were from BD Biosciences. Anti-ARNO mouse monoclonal was from Sigma. Anti-CD63 mouse monoclonal antibody was from Biogenesis. Mouse anti-EGF receptor monoclonal antibody and rabbit anti-LAMP1 polyclonal antibody were kind gifts from Professor Peter Parker (Cancer UK, London, England) and Professor Colin Hopkins (Imperial College, London, England).

Cloning and Site-directed Mutagenesis—Eukaryotic expression plasmids carrying the entire coding region of SNX1 were as described previously (30). For bacterial expression, the SNX1 coding region was cloned into pGEX4T-3 (30). Site-directed mutagenesis was performed as described previously (29) using the Transformer kit (Clontech).

Protein-Lipid Overlay Assay—Protein-lipid overlay assays were based on the procedure described by Dowler *et al.* (31). Membrane arrays were generated by spotting the required mass of the indicated phosphoinositide (Cell Signals, Inc.) onto a Hybond-C extra membrane. After air drying and blocking in 3% (w/v) fatty acid-free bovine serum albumin in 10 mM Tris/HCl, pH 8.0, 150 mM NaCl, and 0.1% (v/v) Tween 20 (Tris-buffered saline), the membrane was incubated overnight at 4 °C in the same solution containing 0.5 μg/ml of the relevant recombinant protein. The membrane was extensively washed in Tris-buffered saline and then incubated with either an anti-SNX1 or an anti-ARNO monoclonal antibody. The membrane was washed as before, prior to being incubated with anti-mouse horseradish peroxidase conjugate. Finally, the membrane was again extensively washed, and protein associated with the various phosphoinositides was visualized using enhanced chemiluminescence.

Sucrose-loaded Liposome Assays—Lipid mixtures of phosphatidylethanolamine, phosphatidylcholine, and phosphatidylserine were supplemented with the required ratio of phosphatidylinositol and the relevant diC_{16} phosphoinositide (Cell Signals, Inc) prior to being dried down to form a film in a 0.5-ml minifuge tube (Beckman Coulter Inc.). The subsequent formation of sucrose-loaded liposomes and the analysis of recombinant SNX1 binding were performed as described previously (29).

Transient Transfection and Cell Imaging—HeLa and PC12 cells, cultured as described (29, 32), were plated on glass coverslips and transfected with vector DNA at 50–60% confluence by lipofection using LipofectAMINE (Invitrogen) at a ratio of 0.15 μg of DNA/μl of cationic lipid. At the designated period after transfection, cells were either fixed using paraformaldehyde (4% (w/v) for 5 min at room temperature) or used live for biochemical or imaging studies. Indirect immunofluorescence was performed on fixed cells permeabilized with 0.1% Triton X-100 for 10 min at room temperature using a Leica TCS-NT confocal microscope equipped with a krypton/argon laser as described previously (33). Live cell imaging was performed using an UltraView MultiUser Confocal Optical Scanner (PerkinElmer Life Sciences) as described previously (32).

EGF Receptor Degradation Assays—HeLa cells transiently transfected with the relevant SNX1 construct were serum-starved overnight prior to addition of EGF (100 ng/ml). At the required time point, cells were fixed with 4% (w/v) paraformaldehyde for 5 min at room temperature and permeabilized by incubation with 0.1% Triton X-100 for 10 min at room temperature. Endogenous EGF receptor was visualized by incubation with anti-EGF receptor antibody. The amount of EGF receptor within a single cell was quantified by confocal imaging. In blind experiments, groups of cells were imaged, and the pixel intensity of the EGF receptor fluorescent signal within an area defined by the outer limits of the plasma membrane was calculated using the Leica TCS-NT4 software.

RESULTS

In Protein-Lipid Overlay Assays, the PX Domain of SNX1 Specifically Binds PtdIns(3,4,5)P_3—Given the precedent set by the specific binding of PtdIns(3)P to the PX domain of SNX3 (15), we examined whether SNX1 was also capable of interacting with phosphoinositides. To assess the phosphoinositide binding properties of recombinant full-length SNX1, we initially made use of a protein-lipid overlay (33–35). This assay, which is being increasingly used as a standard method for analyzing phosphoinositide binding profiles (*e.g.* see Refs. 20, 21, 23, and 28), relies upon spotting varying amounts of pure lipid onto a nitrocellulose membrane. Once dry, the membrane is incubated with the protein of interest and, after extensive washing, the interaction of the protein with any phosphoinositide is detected by immunoblotting. As shown in Fig. 1*A*, under these conditions, full-length recombinant SNX1 bound PtdIns(3,4,5)P_3 with no significant binding being observed to any other phosphoinositide. Such a phosphoinositide binding profile is similar to that observed for the well characterized PtdIns(3,4,5)P_3-binding pleckstrin homology domain of the ARF6 exchange factor ARNO (Fig. 1*B*; see also Ref. 36). Indeed, the affinity for PtdIns(3,4,5)P_3 appears similar for each protein (data not shown).

Sequence homology between PX domains is generally low, highlighted by the identity between $p40^{phox}$ and SNX1 PX domains being just 13%. However, a comparison of the $p40^{phox}$ and SNX1 PX domains sequences shows that all but one of the residues required for PtdIns(3)P binding to $p40^{phox}$ is conserved (or has a structurally equivalent alternative) in the SNX1 PX domain (Fig. 1*C*). The major residues in the binding site of the $p40^{phox}$ PX domain-PtdIns(3)P complex that interact with the PtdIns(3)P are Tyr-59 and the basic residues Arg-58, Arg-60, Lys-92, and Arg-105 (25). Tyr-59 forms a stacking interaction with the PtdIns(3)P ring, whereas the basic residues interact with the phosphates and hydroxyls (Arg-58 and Arg-60 with the 3-phosphate, Lys-92 with the 1-phosphate, and Arg-105 with the 4- and 5-hydroxyls). Only Arg-60 is not conserved (Ser-188 in SNX1); Tyr-59 of $p40^{phox}$ is replaced with Phe-187 in SNX1 and therefore retains the aromatic ring structure. Also, a proline-rich region and a structurally important arginine (Arg-57 in $p40^{phox}$ and Arg-185 in SNX1) are conserved. To examine the importance of the PX domain in the association of SNX1 with PtdIns(3,4,5)P_3, we therefore mutated the lysine residues at position 214 to alanine (K214A). As

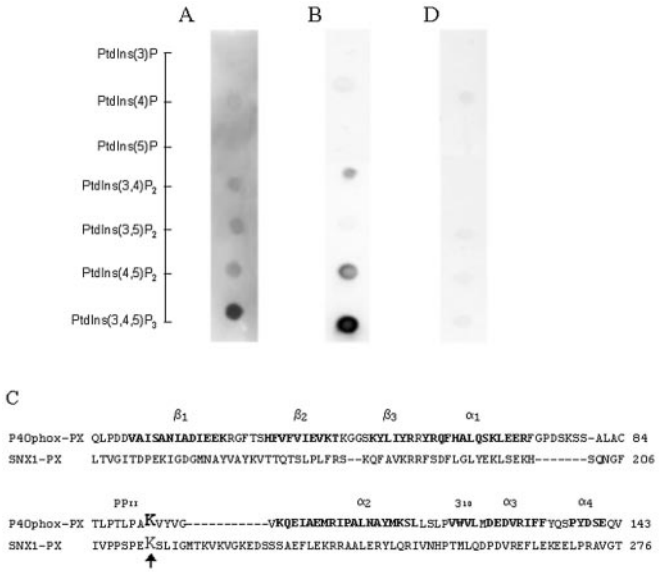

FIG. 1. **SNX1 binds specifically PtdIns(3,4,5)P_3 in lipid-overlay assays.** The ability of SNX1 (*A*), ARNO (*B*) , and SNX1(K214A) (*D*) to bind a variety of phosphoinositides was analyzed using a protein-lipid overlay assay. 100 pmol of the relevant phosphoinositide was spotted onto a nitrocellulose membrane, which was then incubated with the purified proteins. The membranes were washed, and proteins bound to the membrane by virtue of their interaction with lipid were detected using specific antibodies. A representative of at least three separate experiments is shown. *C*, the sequence homology between the $p40^{phox}$ and SNX1 PX domains, highlighting the highly conserved nature of the residues required for PtdIns(3)P binding to $p40^{phox}$ within the SNX1 PX domain and the lysine residue mutated in this study. The secondary structural elements from $p40^{phox}$ are shown in *bold*.

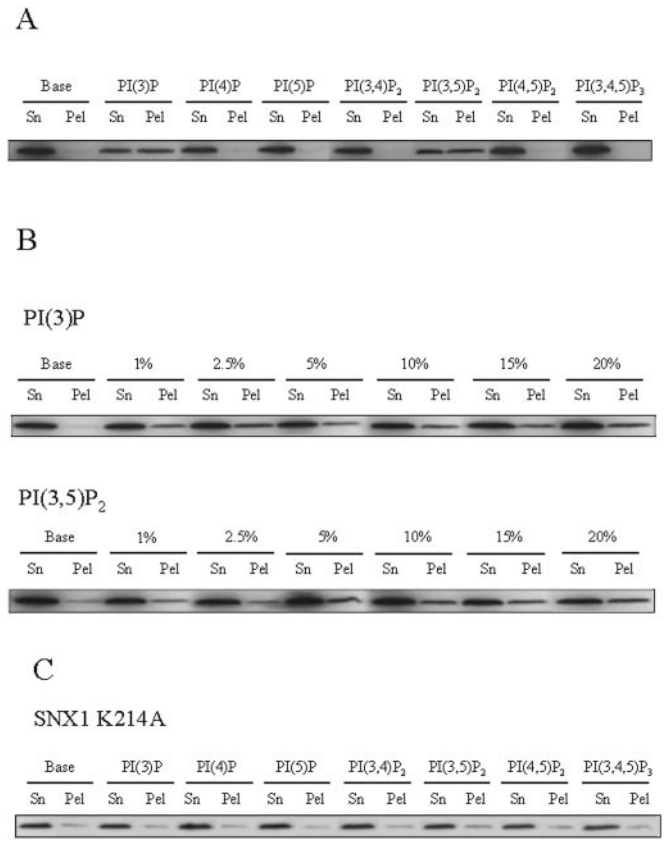

FIG. 2. **SNX1 binds both PtdIns(3)P (*PI(3)P*) and PtdIns(3,5)P_2 (*PI(3,5)P_2*) in liposome-based assays.** In *A*, to dried-down phosphatidylserine, phosphatidylcholine, and phosphatidylethanolamine (each at 26.3% (w/w)) was added 20% (w/w) of the relevant phosphoinositide (total lipid content within the assay of 70 μM). Sucrose-loaded liposomes, formed as described under 'Experimental Procedures,' were incubated with 250–500 ng of recombinant SNX1 for 4 min at 30 °C. The resultant SNX1-lipid complexes were pelleted by centrifugation, and the resultant supernatants (*S*) and pellets (*P*) were separated prior to being resolved by SDS-PAGE and Western blotting using SNX1-specific antisera. In *B*, to dried-down phosphatidylserine, phosphatidylcholine, and phosphatidylethanolamine (each at 26.3% (w/w)) was added the relevant amount of PtdIns(3)P or PtdIns(3,5)P_2. The total lipid content within the assay was maintained at 70 μM by the addition of PtdIns. PtdIns(3)P and PtdIns(3,5)P_2 binding was quantified from Western blots by volume integration using ImageQuaNT software (version 3.3, Molecular Dynamics Inc). Data from three separate experiments were used to determine the association constants of SNX1 for the two phosphoinositides (see 'Results'). In *C*, to dried-down phosphatidylserine, phosphatidylcholine, and phosphatidylethanolamine (each at 26.3% (w/w)) was added 20% (w/w) of the relevant phosphoinositide (total lipid content within the assay of 70 μM). Sucrose-loaded liposomes, formed as described above, were incubated with 250–500 ng of recombinant SNX1(K214A) for 4 min at 30 °C. The resultant SNX1/lipid complexes were pelleted by centrifugation, and the resultant supernatants (*S*) and pellets (*P*) were separated prior to being resolved by SDS-PAGE and Western blotting using SNX1-specific antisera.

shown in Fig. 1*D*, SNX1(K214A) failed to associate with PtdIns(3,4,5)P_3, an observation entirely consistent with a role for the PX domain in the binding to this phosphoinositide.

In Liposome-based Assays, the PX Domain of SNX1 Does Not Bind PtdIns(3,4,5)P_3, Rather Association with PtdIns(3)P and PtdIns(3,5)P_2 Is Observed—To perform a more physiologically relevant phosphoinositide binding analysis, we generated sucrose-loaded liposomes composed of a mixture of phosphatidylserine, phosphatidylethanolamine, and phosphatidylcholine (each at 26.3% (w/w)) to which was added 20% (w/w) of PtdIns, PtdIns(3)P, PtdIns(4)P, PtdIns(5)P, PtdIns(3,4)P_2, PtdIns(4,5)P_2, PtdIns(3,5)P_2, or PtdIns(3,4,5)P_3 (total lipid content within the assay of 70 μM). Under these conditions, although binding of ARNO to PtdIns(3,4,5)P_3-containing liposomes was clearly detected (data not shown), we failed to observe any binding of recombinant SNX1 to liposomes supplemented with PtdIns(3,4,5)P_3 (Fig. 2*A*). Rather, binding was clearly detectable to liposomes that had been supplemented with PtdIns(3)P or PtdIns(3,5)P_2. No significant binding was detected to any of the other phosphoinositides (Fig. 2*A*). To obtain the relative affinity of SNX1 for these two phosphoinositides, we sequentially lowered PtdIns(3)P and PtdIns(3,5)P_2 levels but maintained the same total lipid content by increasing the mass of PtdIns (Fig. 2*B*). Under these conditions, SNX1 displayed a similar affinity for PtdIns(3)P and PtdIns(3,5)P_2, having an apparent association constant (assuming an even distribution of the lipids within the aqueous environment) of 1.1 ± 0.3 and 3.1 ± 0.7 μM, respectively. Binding was via the SNX1 PX domain since SNX1(K214A) failed to associate with either PtdIns(3)P or PtdIns(3,5)P_2 (Fig. 2*C*).

3-Phosphoinositide Binding to the PX Domain Is Necessary for the Targeting of SNX1 to an Early/Sorting Endosomal Compartment—To examine the relationship between phosphoinositide binding and the resultant subcellular localization of SNX1, we initially examined the localization of endogenous SNX1 in HeLa cells. This revealed that SNX1 was predominantly localized to punctate cytoplasmic structures (Fig. 3*A*). A similar subcellular localization was also observed in HeLa cells transiently transfected with wild-type SNX1 tagged with green fluorescent protein (GFP) (Fig. 3*B*). To more precisely define the nature of the SNX1-positive compartment, we probed HeLa cells expressing GFP-SNX1 with antibodies against various markers of the endocytic pathway, including early endosomal antigen 1 (EEA1) and the late endosomal/lysosomal markers LAMP1/lgp120 and CD63. Under these conditions, SNX1

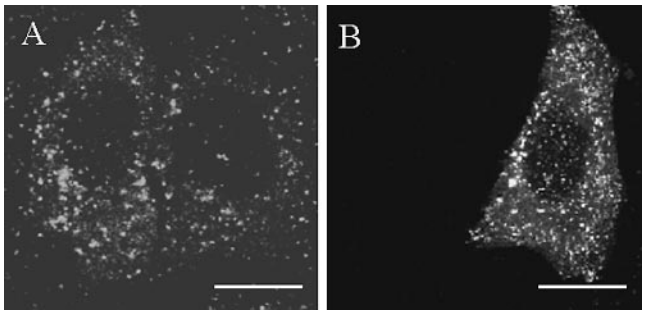

FIG. 3. **SNX1 is localized to punctate cytoplasmic structures.** HeLa cells were stained for endogenous SNX1 (1:200 primary antibody) (*A*) or transiently transfected with pEGFP-SNX1 (*B*). Cells were fixed and mounted, and the localization of SNX1 was visualized by confocal microscopy. The *scale bar* is 10 μm.

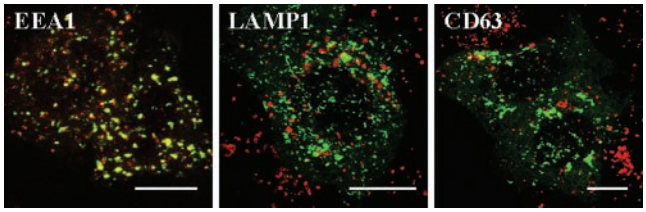

FIG. 4. **SNX1 is localized to an early sorting endosomal compartment.** HeLa cells were transiently transfected with pEGFP-SNX1 and incubated for 12 h prior to fixation. Permeabilized cells were subsequently stained for the presence of EEA1 (1:200), CD63 (1:200), and Lamp1 (1:200) using a Cy3-conjugated secondary antibody prior to visualization of the degree of co-localization by confocal microscopy. The *scale bar* is 10 μm.

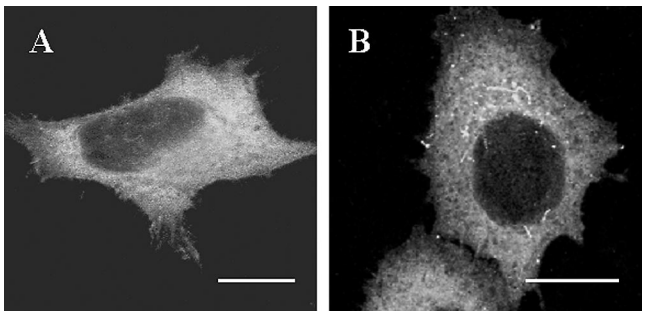

FIG. 5. **Association of SNX1 with the early sorting endosomal compartment requires a 3-phosphoinositide-binding PX domain and the activity of a wortmannin-sensitive phosphatidylinositol 3-kinase.** In *A*, HeLa cells were transiently transfected with pEGFP-SNX1(K214A) and incubated for 12 h prior to fixation. In *B*, HeLa cells, transiently transfected with pEGFP-SNX1, were cultured for 12 h and then incubated for 15 min with 100 nM wortmannin prior to fixation. The *scale bar* is 10 μm.

showed substantial, but not complete, co-localization with EEA1 (Fig. 4). There was substantially less co-localization with late endosomal and lysosomal markers. Thus, as documented previously (24, 30, 37–39), SNX1 appears to be partially localized to the early endosomal compartment.

In contrast to GFP-SNX1, GFP-SNX1(K214A) was entirely present within the cytoplasm (Fig. 5*A*). Given that wild-type SNX1 is capable of binding phosphoinositides, a function not retained by SNX1(K214A), these data are entirely consistent with a requirement for 3-phosphoinositide binding to the PX domain for the targeting of SNX1 to the early endosomal compartment. Further evidence in favor of this came from the observation that following the addition of wortmannin (100 nM for 15 min), wild-type SNX1 underwent dissociation from this compartment (Fig. 5*B*).

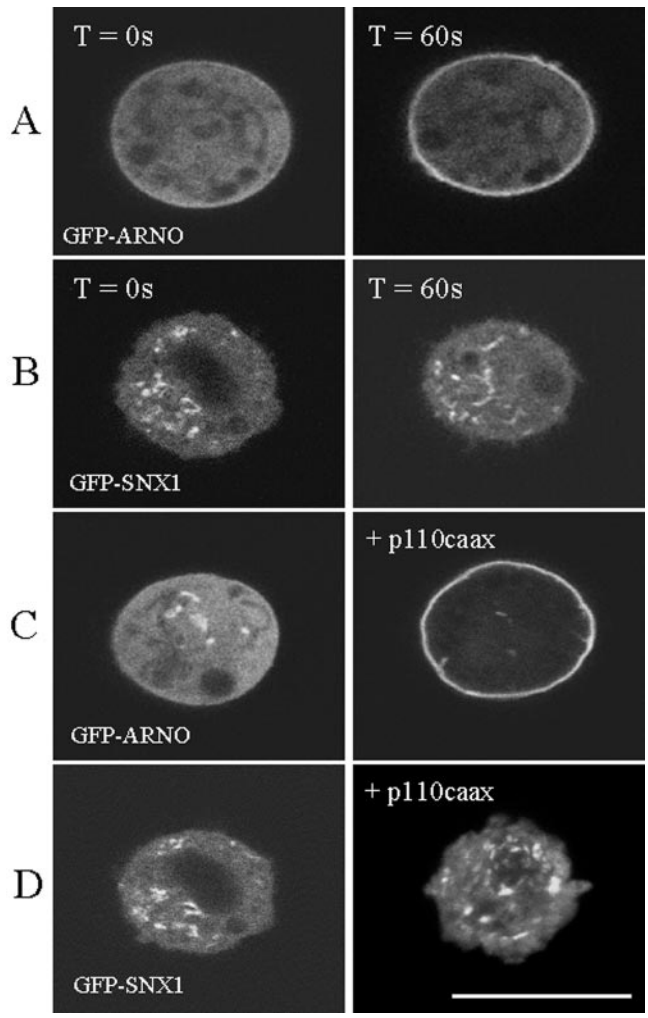

FIG. 6. **SNX1 does not associate with the plasma membrane following elevation of PtdIns(3,4,5)P_3.** PC12 cells were transiently transfected with pEGFP-ARNO (*A*) and pEGFP-SNX1 (*B*). In *C* and *D*, cells were transiently co-transfected with a vector encoding for $p110^{CAAX}$ and pEGFP-ARNO or pEGFP-SNX1 respectively. In all cases, cells were serum-starved for 2 h prior to treatment. In *A* and *B*, cells were stimulated with 100 ng/ml EGF immediately after capture of the $t = 0$ image. Images were captured every 10 s for a period of 5 min. For clarity, only the images collected after 60 s of stimulation are shown. Similar data were obtained from a further 15 imaged cells. In *C* and *D*, cells were imaged without stimulation. The *scale bar* is 10 μm.

SNX1 Does Not Associate with the Plasma Membrane under Conditions in which Plasma Membrane PtdIns(3,4,5)P_3 Is Elevated—Although the data described above highlight the importance of 3-phosphoinositide binding to the PX domain for the association of SNX1 with the early endosomal compartment, these data fail to address whether this association is because of the interaction with PtdIns(3)P, PtdIns(3,5)P_2, and/or PtdIns(3,4,5)P_3. Work form a number of groups, including ourselves, has shown that the major site of PtdIns(3,4,5)P_3 production is the inner leaflet of the plasma membrane (40–42). For example, in PC12 cells expressing a GFP-tagged diglycine ARNO chimera (GFP-ARNO), live cell confocal imaging has revealed that following stimulation with an agonist that elevates PtdIns(3,4,5)P_3, cytosolic GFP-ARNO undergoes a rapid recruitment to the plasma membrane (Fig. 6*A*). This recruitment results from the pleckstrin homology domain of ARNO directly binding PtdIns(3,4,5)P_3 generated at the inner plasma membrane leaflet (36). Thus, if the binding of the SNX1 PX domain to PtdIns(3,4,5)P_3 observed in lipid overlay assays is physiologically relevant, one would predict that SNX1 should

FIG. 7. **The SNX1-positive compartment partially co-localizes with a probe for PtdIns(3)P.** HeLa cells were transiently co-transfected with pEGFP-2xFYVE^{hrs} and pCIneo-SNX1 and incubated for 12 h prior to fixation. Permeabilized cells were subsequently stained for the presence of SNX1 (1:200) prior to visualization of the degree of co-localization by confocal microscopy. The *scale bar* is 10 μm.

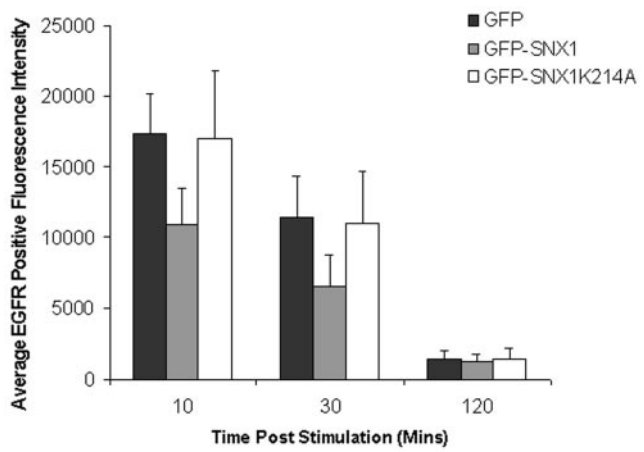

FIG. 8. **Overexpression of SNX1, but not SNX1(K214A), leads to a stimulation in the degradation of the EGF receptor (*EGFR*).** HeLa cells, transiently transfected with pEGFP, pEGFP-SNX1, or pEGFP-SNX1(K214A), were cultured for 24 h. Cells were serum-starved overnight prior to being stimulated with EGF (100 ng/ml) for the required time period. Cells were fixed and stained for EGF receptor. The amount of EGF fluorescent signal was determined as described under 'Experimental Procedures.' Data are averaged ± S.E. for not less then 75 cells imaged under each condition.

associate with the inner leaflet of the plasma membrane. Although in HeLa cells we have failed to observe any association of GFP-SNX1 to this membrane prior to or after stimulation with growth factors that elevate plasma membrane PtdIns(3,4,5)P_3 (data not shown), we have used the observation that in PC12 cells expressing GFP-SNX1, a significant proportion of the GFP-SNX1 is located in a cytoplasmic pool to address this issue more directly (Fig. 6*B*). Under these conditions, we failed to observe any plasma membrane recruitment of GFP-SNX1 following stimulation of PC12 cells with either maximal doses of EGF or maximal doses of nerve growth factor (Fig. 6*B*). Finally, in PC12 cells transiently co-transfected with GFP-SNX1 and p110^{CAAX}, a constitutively active version of phosphatidylinositol 3-kinase that induces a basal elevation in plasma membrane PtdIns(3,4,5)P_3 levels (36), GFP-SNX1 remained cytosolic, whereas in control experiments, GFP-ARNO was clearly constitutively associated with the plasma membrane (Fig. 6, *C* and *D*). When taken together, such data raise doubt as to the whether in an SNX1 does indeed bind PtdIns(3,4,5)P_3 *in vivo* context.

SNX1 Partially Co-localizes with a Specific PtdIns(3)P Probe—To assess the relationship between SNX1 and the subcellular distribution of PtdIns(3)P, we transiently transfected HeLa cells with constructs encoding for SNX1 and GFP-2xFYVE^{hrs}, a specific probe for PtdIns(3)P (43, 44). As shown in Fig. 7, confocal microscopy revealed a partial co-localization of SNX1 with the PtdIns(3)P probe. These data, when taken with the evidence from the liposome assays, strongly imply that the association of SNX1 with the early endosomal compartment results from the ability of its PX domain to bind PtdIns(3)P.

3-Phosphoinositide-dependent Association of SNX1 with an Early Endosomal Compartment Is Required for the Regulation of EGF Receptor Degradation—SNX1 was originally isolated in a yeast two-hybrid screen through its ability to interact with the lysosomal-targeting signal of the EGF receptor (37). This has led to the suggestion that SNX1 may play a role in targeting the EGF receptor for lysosomal degradation through the endocytic pathway. Indeed, overexpressed SNX1 decreases the amount of EGF receptor by enhancing the rate of ligand-induced degradation (28, 37, 38). In HeLa cells, within 10 min of stimulation with EGF, the EGF receptor is clustered and internalized into small vesicles corresponding to early endosomes (45). The receptor reaches larger perinuclear structures by 30 min with some loss of signal at 60 min as the receptor begins to reach the lysosomal compartment and is degraded (45). By 120 min, few EGF receptor-positive structures remain, owing to lysosomal degradation of the signal (45). To examine the functional consequence of 3-phosphoinositide-dependent association of SNX1 with the early endosomal compartment, we analyzed the degradation of endogenous EGF receptor using a single cell assay. In this assay, we quantified, as a function of time after EGF addition, the pixel intensity of the EGF receptor-positive signal within a region of interest that was defined by the plasma membrane. Using this assay, we could compare the effect of overexpressing SNX1 and SNX1(K214A) on EGF receptor degradation. In control cells, we observed a decrease in EGF receptor signal following the addition of EGF (Fig. 8). Furthermore, entirely consistent with previous studies on SNX1, in cells overexpressing wild-type SNX1, there was a small but clear enhancement in the rate of EGF receptor degradation (Fig. 8). In contrast, in those SNX1(K214A)-overexpressing cells, no increase in the rate of EGF receptor degradation was observed (Fig. 8). These data suggest, therefore, that the PX domain-induced, 3-phosphoinositide-dependent association of SNX1 with an early endosomal compartment is required to regulate the lysosomal traffic and degradation of the internalized EGF receptor.

DISCUSSION

In the present study, we have analyzed the phosphoinositide binding profile of the PX domain from SNX1 using two distinct techniques, protein-lipid overlays, a simple and extremely rapid assay, and liposome binding assays, a more physiological relevant but technically demanding assay. Consistent with the study of Zhong *et al.* (28), we have shown that in the overlay assay, the PX domain of SNX1 appears to specifically bind PtdIns(3,4,5)P_3. However, when phosphoinositide binding was determined using the liposome-based assay, we failed to detect any binding to PtdIns(3,4,5)P_3; rather, high affinity binding was observed to PtdIns(3)P and PtdIns(3,5)P_2. Why the mode of presenting the phosphoinositide substrate should have such a dramatic bearing on the binding profile of the SNX1 PX domain is currently unclear. However, our data do raise an important issue. Evidence presented here would imply that using just the protein-lipid overlay assays to obtain a phosphoinositide binding profile for a given protein should not be viewed as an altogether reliable approach. Rather, evidence from at least two independent assays should be obtained to define a phosphoinositide binding profile.

In an attempt to address which interaction with 3-phosphoinositides may be more physiologically relevant, we examined the subcellular localization of SNX1. Consistent with other studies (28, 39), we observed that SNX1 was associated with

intracellular vesicles that showed a partial overlap with the EEA1-positive early endosomal compartment. We failed to observe any association of SNX1 with the plasma membrane, the major site of PtdIns(3,4,5)P_3 synthesis (40–42), either after stimulation with agonists that elevate PtdIns(3,4,5)P_3 or in experiments in which we elevated basal PtdIns(3,4,5)P_3 levels by co-expressing SNX1 with a constitutively active class I phosphatidylinositol 3-kinase. Under all of these conditions, however, we observed a clear plasma membrane association of the well characterized PtdIns(3,4,5)P_3-binding protein ARNO, the pleckstrin homology domain of which binds PtdIns(3,4,5)P_3 with a similar affinity to the SNX1 PX domain (32). These data would therefore argue against the idea that the binding of PtdIns(3,4,5)P_3 to the PX domain of SNX1 is required for its association with the SNX1-positive endosomal compartment. Furthermore, it raises the issue of whether the binding of PtdIns(3,4,5)P_3 to the PX domain of SNX1 observed using the overlay assays is of any physiological relevance.

The association of SNX1 to the early endosomal compartment was clearly dependent on the ability of its PX domain to bind 3-phosphoinositides since firstly, a point mutant, SNX1(K214A), incapable of binding 3-phosphoinositides localized to the cytosol, and secondly, wild-type SNX1 could be dissociated from the early endosomal compartment by incubation with the phosphatidylinositol 3-kinase inhibitor wortmannin. Such data, when taken together with the partial co-localization of SNX1 with a probe that reveals the subcellular localization of PtdIns(3)P, are certainly more easily explained by the binding of the SNX1 PX domain to endosomal PtdIns(3)P being necessary for the targeting of this protein to the early endosomal compartment.

The physiological importance of the interaction of SNX1 with 3-phosphoinositides has been highlighted through an analysis of the role of SNX1 in the regulation of EGF receptor degradation. We have shown that whereas wild-type SNX1 is capable of stimulating the rate of EGF receptor degradation, this is not achieved with the SNX1(K214A) mutant. Thus, the PtdIns(3)P-dependent, PX domain-mediated association of SNX1 with an early endosomal compartment is necessary for the ability of this protein to target the EGF receptor for lysosomal degradation through the endocytic pathway (28, 37).

One outstanding issue, however, concerns the physiological relevance of PtdIns(3,5)P_2 binding to the SNX1 PX domain. Studies in *Saccharomyces cerevisiae* have highlighted a role for PtdIns(3,5)P_2 in yeast membrane traffic (46–48). The major phenotypic characteristics resulting from inactivation of yeast *fab1*, whose gene product encodes for the PtdIns(3)P 5-kinase that produces PtdIns(3,5)P_2, include severe growth defects and extremely enlarged vacuoles that occupy the majority of the cell (48). Despite these defects, however, all transport pathways to the vacuole in Fab1p-deficient cells appear intact (49), suggesting that Fab1p kinase and PtdIns(3,5)P_2 function to maintain vacuolar size and membrane homeostasis by regulating recycling/turnover of membranes from the yeast vacuole surface to earlier compartments. Consistent with PtdIns(3,5)P_2 playing a role in endocytic membrane homeostasis has been the observation that overexpression of a kinase-deficient point mutant of PIKfyve, the mammalian ortholog of Fab1p (50–53), leads to the formation of multiple swollen endosomal vacuoles in a number of different mammalian cells (54). In contrast with our understanding of the role of PtdIns(3)P in endosomal traffic that has arisen from the identification of PtdIns(3)P effectors (55), our understanding of PtdIns(3,5)P_2 is hampered by the lack of identified effectors for this lipid. Indeed, at present, no PtdIns(3,5)P_2 effectors have been described. In light of the data presented here, it is tempting to speculate that SNX1 may constitute such an effector molecule, allowing PtdIns(3,5)P_2 to regulate endosomal to lysosomal membrane traffic.

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The Phox Homology (PX) Domain-dependent, 3-Phosphoinositide-mediated Association of Sorting Nexin-1 with an Early Sorting Endosomal Compartment Is Required for Its Ability to Regulate Epidermal Growth Factor Receptor Degradation

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