Sorting nexin 5 is localized to a subdomain of the early endosomes and is recruited to the plasma membrane following EGF stimulation

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Summary

Sorting nexins are a large family of proteins that contain the phosphoinositide-binding Phox homology (PX) domain. A number of sorting nexins are known to bind to PtdIns(3)*P*, which mediates their localization to membranes of the endocytic pathway. We show here that sorting nexin 5 (SNX5) can be recruited to two distinct membrane compartments. In non-stimulated cells, the PX domain was independently targeted to endosomal structures and colocalized with full-length SNX5. The membrane binding of the PX domain was inhibited by the PI 3-kinase inhibitor, wortmannin. Although SNX5 colocalized with a fluid-phase marker and was found predominantly within a PtdIns(3)*P*-rich endosomal domain, very little colocalization was observed between SNX5 and the PtdIns(3)*P*-binding protein, EEA1. Using liposome-based binding assays, we have shown that the PX domain of SNX5 interacts not only with PtdIns(3)*P* but also with PtdIns(3,4)P_2. In response to EGF stimulation, either the SNX5-PX domain or full-length SNX5 was rapidly recruited to the plasma membrane. The localization of SNX1, which does not bind PtdIns(3,4)P_2, was unaffected by EGF signalling. Therefore, SNX5 is localized to a subdomain of the early endosome distinct from EEA1 and, following EGF stimulation and elevation of PtdIns(3,4)P_2, is also transiently recruited to the plasma membrane. These results indicate that SNX5 may have functions not only associated with endosomal sorting but also with the phosphoinositide-signalling pathway.

Key words: Sorting nexins, Phox homology domain, Early endosomes, EGF signalling, Phosphoinositides

Introduction

The mammalian endosomal system consists of a heterogeneous set of compartments, including early endosomes, recycling endosomes, late endosomes and lysosomes (Mellman, 1996). Endocytic pathways function not only to internalize extracellular components but are also involved in signal-transduction pathways (Di Fiore and De Camilli, 2001; McPherson et al., 2001). A major family of second messengers are the phosphoinositides, membrane lipids that regulate a wide variety of cellular processes (Rameh and Cantley, 1999; Czech, 2000). Phosphoinositides are localized to discrete regions within membranes and their synthesis and turnover is highly regulated. The 3-phosphorylated phosphoinositides in particular, regulate endocytic membrane trafficking, signal transduction and cytoskeletal organization by interacting with lipid binding domains of a variety of effectors (Sato et al., 2001; Simonsen et al., 2001; Vanhaesebroeck et al., 2001). A number of conserved protein domains have now been identified which bind specifically to phosphoinositides, and these domains act to recruit their host protein to specific membrane compartments/domains via interactions with these membrane lipids (Lemmon, 2003).

Sorting nexins (SNXs) have emerged as a large and diverse family of hydrophilic cytoplasmic and membrane-associated proteins considered to be involved in endocytosis (Teasdale et al., 2001; Worby and Dixon, 2002). The hallmark of the SNX family of ~24 members is the presence of a modestly conserved Phox (PX) domain, a sequence of 70-120 residues that has been shown to bind various phosphoinositides (Xu et al., 2001b; Worby and Dixon, 2002). PX domains are present in many yeast/mammalian membrane and vesicle-associated proteins involved in a variety of cell functions (Ponting, 1996; Schultz et al., 2000). Proteins can associate intimately and specifically to membrane compartments through their PX domain, by binding phosphoinositide lipids present in the membrane (Wishart et al., 2001). In most cases studied so far PX domains can bind the endosomal lipid phosphatidylinositol 3-phosphate, PtdIns(3)*P* (Xu et al., 2001b; Yu and Lemmon, 2001; Lemmon, 2003).

From our studies based on BLAST searches and

phylogenetic analysis, it is clear that the PX domains of SNXs represent a distinct subgroup of the PX superfamily (Teasdale et al., 2001). Therefore, the PX domains of SNX proteins (SNX-PX) may have evolved unique characteristics/functions compared with other PX domains. Within the SNX family, we identified sub-groups based on the sequence similarity of the SNX-PX domain and the overall domain structure of each protein. The members of one sub-group, which includes human SNX1, SNX2, SNX4, SNX5 and SNX6 and the yeast Vps5p and YJL036W, all contain coiled-coil regions within their large C-terminal domains, and are found distributed in both membrane and cytosolic fractions, typical of hydrophilic peripheral membrane proteins (Teasdale et al., 2001; Worby and Dixon, 2002).

Members of the SNX family have been shown to have a role in protein sorting within the endosomal pathway (Worby and Dixon, 2002). In particular, a number of SNXs interact with various membrane receptors (Haft et al., 1998; Parks et al., 2001). Significantly, SNX1, SNX2 and Vps5p have been identified to be part of a membrane-associated complex, termed the retromer, which contains Vps26p, Vps29p and Vps35p (Seaman et al., 1998; Haft et al., 2000). First identified in yeast and now in mammalian cells, the retromer is considered to function as a membrane coat complex for vesicular transport in the endocytic pathway (Nothwehr et al., 2000). The analysis of mice lacking SNX1 and SNX2 has confirmed that SNXs perform essential functions in mammals (Schwarz et al., 2002).

As phosphoinositides are known to play a key role in the regulation of endosomal traffic (Simonsen et al., 2001), the lipid binding properties of the SNX-PX domains are likely to be fundamental for understanding their function. Early endosomes are enriched for PtdIns(3)*P* (Gillooly et al., 2000), therefore the phosphoinositide binding properties of the PX domain are likely to partially account for the endosomal localization of sorting nexins of mammals and yeast. Nonetheless, it is clear that not all PtdIns(3)*P* binding proteins are localized to the same membrane domain and furthermore, it is possible that different SNXs operate in different classes of endosomes (Hettema et al., 2003). Significantly recent studies have shown that the PX domain of SNX1 binds with a similar affinity to both PtdIns(3)*P* and PtdIns(3,5)P_2, a specificity which distinguishes this SNX-PX domain from other PX domains that have been characterized (Cozier et al., 2002) and from the PtdIns(3)*P* binding FYVE domain of the early endosomal protein, EEA1 (Stenmark et al., 2002). The latter study of SNX1 also demonstrated the importance of the PX domain in the membrane binding property of SNX1 and the ability of SNX1 to regulate EGF receptor degradation (Cozier et al., 2002). In view of this finding, a highly relevant issue is whether the PX domains of other SNX molecules may have different phosphoinositide binding specificities that could target their host protein to distinct subcellular compartments or domains and/or allow their regulation by phosphoinositides other than PtdIns(3)*P*. This possibility is particularly pertinent in view of the fact that the sequence similarity between the SNX-PX domains is only modest (50% similarity) (Teasdale et al., 2001).

In view of the number of SNXs, it is possible that they have a range of functions, unified by their ability to interact with phosphoinositide intermediates. More information is clearly required on the precise phosphatidylinositol binding specificity of the PX domains of the different SNX molecules, and their relative intracellular localization. Here we have analysed the phosphoinositide binding specificity and localization of another member of the SNX1-subfamily, namely SNX5, in detail. Compared with SNX1-4, SNX5 contains an insert in its PX domain (Teasdale et al., 2001; Worby and Dixon, 2002) and therefore may have subtle differences in its phosphoinositide binding specificity that could impart distinct functional properties to SNX5. Indeed, here we show that the PX domain of SNX5 can bind PtdIns(3)*P* as well as PtdIns(3,4)P_2, a specificity that differs from the PX domains of other SNXs characterized. Furthermore, the ability to bind these two phosphoinositides is consistent with a PX domain-dependent association of SNX5 with endosomes in unstimulated cells, and the subsequent movement of SNX5 to the plasma membrane following EGF stimulation and elevation of PtdIns(3,4)P_2. As these results indicate that localization of SNX5 is responsive to EGF signalling, the family of sorting nexins may have broader functions than originally envisaged.

Materials and Methods

Antibodies

Human antibody against early endosome autoantigen 1 (EEA1) was a kind gift from J. Callaghan (University of Melbourne). Monoclonal antibodies to light and heavy chain clathrin and EEA1 were obtained from BD Transduction Laboratories (Australia), and to human Lamp-1 (CD107a) and SNX1 were obtained from Pharmingen International (San Jose, CA, USA). Monoclonal antibodies to the human transferrin receptor (OKT9) (Schneider et al., 1982) and the human epidermal growth factor receptor (Gill et al., 1984) were purified from hydridoma supernatants. Sheep anti-mouse Ig-FITC conjugate was from Silenus Laboratories (Melbourne, Victoria, Australia), and goat anti-mouse IgG-Alexa568, goat anti-human IgG-Alexa568, goat anti-rabbit-Alexa568 and Texas Red dextran conjugates were purchased from Molecular Probes (Eugene, Oregon, USA). Epitope-tagged proteins were detected with monoclonal antibody anti-FLAG (M2) purchased from Sigma (Castle Hill, NSW, Australia) and monoclonal anti-GFP from Roche Molecular Biochemicals (Indianapolis, USA). Horseradish peroxidase-conjugated anti-mouse Ig was obtained from DAKO (Carpinteria, CA).

DNA constructs

Green fluorescent protein (GFP) constructs used are described (Teasdale et al., 2001). Glutathione S-transferase (GST) fusion protein was constructed using pGEX-6P-3 (Pharmacia). GST-SNX5$_{PX}$ fusion protein was generated by subcloning the *Bam*HI/*Bgl*II fragment from pEGFP-SNX5$_{PX}$ construct directly into the *Bam*HI site of pGEX-6P-3. The construct GST-Hrs$_{FYVE}$ encoding the FYVE domain of Hrs corresponding to residues 147-223 (Komada and Soriano, 1999) inserted at the C-terminus of GST in pGEX-6P-3 was provided by Haydyn Mertons (Department of Biochemistry and Molecular Biology, The University of Melbourne). GFP-FYVE$_{EEA1}$ encodes the FYVE domain of EEA1, corresponding to residues 1252-1411, cloned into pEGFP (Hunyady et al., 2002) and was generously supplied by Tamas Balla (NIH, Bethesda).

Cell culture and transfection

HeLa cells were maintained in Dulbecco's modified Eagle's medium with 10% (v/v) foetal bovine serum, 100 units/ml penicillin, 100 μg/ml streptomycin and 2 mM L-glutamine in a humidified

air/atmosphere (10% CO_2) at 37°C. Cells were transiently transfected with 1 μg plasmid DNA per well of a 12-well dish using Fugene 6 transfection Reagent (Boehringer Mannheim, Germany) according to the manufacturer's instructions. The generation of the Flp-In^{TM}-HEK293 cell line expressing GFP-SNX5 was performed as follows. The entire GFP-SNX5$_{FL}$ coding region was subcloned from pEGFP-C1-GFP-SNX5$_{FL}$ (Teasdale et al., 2001) into pcDNA5/FRT (Invitrogen) to generate pcDNA5/FRT-GFP-SNX5$_{FL}$. Flp-In^{TM}-293 cells were maintained in culture containing 100 μg/ml Zeocin. pcDNA5/FRT-GFP-SNX5$_{FL}$ and the Flp recombinase expression vector, pOG44, were co-transfected at a 1:9 ratio into 70% confluent cells using the LF2000 system (Invitrogen). 24 hours after transfection, the cells were washed with PBS and cultured for another 24 hours in fresh media. Cells were again washed in PBS as before and then cultured in media containing 50 μg/ml hygromycin B.

Immunofluorescence

12 hours after transfection, cell monolayers were washed twice with PBS and processed for immunofluorescence as described previously (Kjer-Nielsen et al., 1999). Cells were incubated with the primary antibody for 45 minutes at room temperature, washed three times with PBS and incubated with the appropriate secondary antibody for 30 minutes at room temperature. Cells were examined by confocal microscopy using the Bio-Rad MRC-1024 imaging system and Lasersharp 3.1 software. For dual labelling, images were collected independently to ensure there was no spill-over of fluorescence between channels. For quantitative analysis, at least 100 transfected cells were scored.

Wortmannin treatment

HeLa cells were transfected with GFP-SNX5$_{PX}$ and 12 hours later cells were serum starved for 3 hours at 37°C. Serum-starved transfected HeLa cells, or stably transfected HEK293 cells were incubated at 37°C in the presence or absence of 100 nM wortmannin (Calbiochem, USA) for the indicated times. Following treatment, cells were washed three times in PBS, fixed and processed for immunofluorescence.

Fluid-phase uptake of dextran

Cell monolayers were incubated with unlabeled dextran (5 mg/ml) (10 kDa, Amersham) in serum-free DMEM, for 15 minutes at 37°C, washed in PBS, then incubated with 100-200 μg/ml Texas Red dextran, in serum-free DMEM, for 15 minutes either on ice or at 37°C. After the incubation, monolayers were immediately washed twice with ice-cold PBS then fixed in 4% paraformaldehyde (PFA) for 15 minutes and washed in PBS.

EGF stimulation

Cell monolayers were serum starved for 3 hours and stimulated with 20-100 ng/ml human EGF (Gibco, California), in serum-free medium for 0-30 minutes at 37°C. Cells were washed in PBS and fixed in 4% PFA.

Immunoprecipitation experiments

HeLa cells, co-transfected with GFP-SNX5$_{PX}$ and FLAG-SNX5$_{FL}$, were harvested 12 hours after transfection and resuspended in lysis buffer (1% Nonidet P-40 in PBS) containing a protease inhibitor cocktail (Roche Diagnostics, Germany) for 30 minutes at 4°C. Extracts were centrifuged at 14,000 ***g*** for 20 minutes at 4°C to remove insoluble debris. Aliquots (0.5 ml) of the supernatant were treated with 100 μl of 20% (w/v) protein G-Sepharose beads (Amersham Biosciences, Sweden) for 30 minutes and after removal of the beads, the supernatant was incubated with 2 μg anti-GFP or anti-FLAG antibody for 4 hours at 4°C with gentle agitation, followed by the addition of protein G-Sepharose beads for 30 minutes. Protein G-Sepharose was washed three times in lysis buffer and twice in PBS. Bound proteins were eluted by boiling the beads in SDS-PAGE loading buffer.

Immunoblotting

Proteins were resolved on 10% polyacrylamide gels in the presence of SDS and transferred onto PVDF membranes (Immobilon-P, Millipore) according to the manufacturer's instructions and immunoblotting performed as described (Gleeson et al., 1996). Immunoprecipitated proteins were visualized with either anti-GFP or anti-FLAG monoclonal antibodies followed by horseradish peroxidase-conjugated anti-mouse secondary antibody. Antibodies were diluted in of PBS containing 0.05% Tween-20. Blots were developed with enhanced luminol reagent (NEN, Boston, MA, USA) and visualized by chemiluminescence on Biomax MR film (Eastman Kodak Co.).

Protein expression and purification

Bacterial expression of GST-SNX5$_{PX}$ and GST-Hrs fusion protein was induced by addition of 0.1 mM isopropyl β-D-thiogalactoside (IPTG) to recombinant DH5α cultures for 3 hours at 30°C. Cells were pelleted at 8000 ***g*** for 10 minutes at 4°C and stored at –20°C until required. Cell pellets were resuspended in STE buffer, pH 8.0 (150 mM Tris pH 8.0, 150 mM NaCl, 5 mM EDTA, 5 mM DTT), plus 100 μg/ml lysozyme and 5 mM DTT. Bacterial suspensions were lysed by sonication and N-lauroylsarcosyl was added to a final concentration of 1.5% and proteins were extracted for 30 minutes at 4°C. Insoluble material was removed by centrifugation at 14,000 ***g*** for 20 minutes. Triton X-100 (1% final concentration) was added to the soluble fraction and this was subjected to affinity chromatography using glutathione-Sepharose 4B (Amersham Bioscience, Sweden) according to the manufacturer's instructions. Bound fusion proteins were eluted from the matrix with 10 mM glutathione in column buffer (50 mM Tris pH 8.0, 150 NaCl, 5 mM DTT) with 1% Triton X-100. Finally, purified fusion proteins were desalted on a PD10 column (Pharmacia Biotech) and fraction aliquots stored at –70°C until use.

Liposome binding assays

L-3-phosphatidyl[N-methyl-^{3}H]choline 1,2-dipamitoyl (85 Ci/mmol) was obtained from Amersham. Phosphatidylcholine (PC), phosphatidylethanolamine (PE) and phosphatidylserine (PS) were obtained from Sigma (USA). Phosphatidylinositol 3-phosphate [PtdIns(3)*P*], phosphatidylinositol 3,4-bisphosphate [PtdIns(3,4)P_2], phosphatidylinositol 3,5-bisphosphate [PtdIns(3,5)P_2], phosphatidylinositol 3,4,5-trisphosphate [PtdIns(3,4,5)P_3] were obtained from Echelon (Salt Lake City, USA). Liposome binding assay was performed as follows and as described (Gaullier et al., 2000). Liposomes were prepared at 0.35 mg/ml containing 63-65% PC, 20% PS, 15% PE, 2% phosphatidylinositol (PI) and a trace of [^{3}H]PC, suspended in liposome buffer (20 mM Tris-HCl, pH 7.5, 60 mM NaCl), and sonicated on ice. GST fusion proteins were desalted into the liposome buffer and 20 μg GST or a molar equivalent of GST-SNX5$_{PX}$ or GST-Hrs fusion proteins immobilized on 50 μl of 50% (v/v) glutathione-Sepharose beads by rotation for 30 minutes at room temperature. The beads were washed three times and resuspended in 100 μl liposome buffer. 100 μl sonicated ^{3}H-labelled liposomes were added and the mixture incubated for 30 minutes with gentle rotation at room temperature. The beads were washed twice in liposome buffer, the supernatants collected and radioactivity determined. The percentage of ^{3}H-labelled liposomes bound to the Sepharose beads

was calculated as follows: [c.p.m. in pellet/total c.p.m. (test beads)] – [c.p.m. in pellet/total c.p.m. (beads alone)]. All experiments were carried out in triplicate.

Yeast two-hybrid analysis

Saccharomyces cerevisiae EGY48 (a-trp, ura3-52, his3, leu2), the pYESTrp and pLexA expression vectors were obtained from Clontech and yeast two-hybrid analysis performed using the standard protocol supplied by the manufacturer. The full-length cDNAs for SNX1, SNX5 and hVps35 were subcloned into both of the yeast expression vectors and transformed into EGY48. Each expression construct was initially assayed, using the liquid β-galactosidase assay, for autoactivation of the reporter gene and constructs that displayed levels of autoactivation above that of the empty vector were not used. Six independent colonies transformed with the expression plasmids were assayed to reduce the impact of clonal variation. The expression of the full-length fusion protein product was confirmed by immunoblotting of whole cell extracts.

Results

SNX5 PX domain is localized to a similar endosomal compartment as full-length SNX5

SNX5 is a 404-residue hydrophilic protein that contains a central PX domain and two coiled-coil regions within the large C-terminal domain (Teasdale et al., 2001). The full-length SNX5 molecule was epitope tagged with FLAG (FLAG-$SNX5_{FL}$), and in transfected HeLa cells was associated with punctate cytoplasmic structures indicative of endosomes (Fig. 1). The PX domains of some proteins have been shown to be sufficient for targeting to intracellular membranes. To determine if the PX domain of SNX5 can be independently recruited to endosomes via PI binding, we generated a construct containing the PX domain (residues 64-166) of SNX5 fused to GFP. After 12 hours of transfection, GFP-$SNX5_{PX}$ fusion protein was detected on endosomal-type structures dispersed throughout the cytoplasm (Fig. 1). Approximately 80% of transfected cells showed a punctate staining pattern similar to that depicted in Fig. 1 whereas the remaining 20% of transfected cells showed a low level of cytoplasmic staining. Furthermore, in cells co-transfected with GFP-$SNX5_{PX}$ and FLAG-tagged full-length SNX5, substantial colocalization of GFP-$SNX5_{PX}$ fusion protein with the FLAG-$SNX5_{FL}$ was observed (Fig. 1). The cytoplasmic staining patterns in cells co-transfected with both constructs was similar to the individual constructs alone (not shown). Therefore, these results indicate that the localization of the PX domain of SNX5 reflects the specific localization of the full-length molecule.

One possibility for the colocalization of the individual SNX domains with FLAG-$SNX5_{FL}$ is that the two molecules may associate physically. To determine if $SNX5_{PX}$ can form oligomers with full-length SNX5, HeLa cells were transfected with expression vectors encoding both GFP-$SNX5_{PX}$ and FLAG-$SNX5_{FL}$ and extracts of transfected cells were immunoprecipitated with antibodies either to the FLAG epitope or to GFP. The immunoprecipitates were immunoblotted and no association was detected between GFP-$SNX5_{PX}$ and FLAG-$SNX5_{FL}$ (not shown). Although the disruption of an interaction between GFP-$SNX5_{PX}$ and FLAG-$SNX5_{FL}$ during solubilization cannot be excluded, these results indicate that the localization studies above reflect independent targeting of GFP-$SNX5_{PX}$ molecules to endosomal structures.

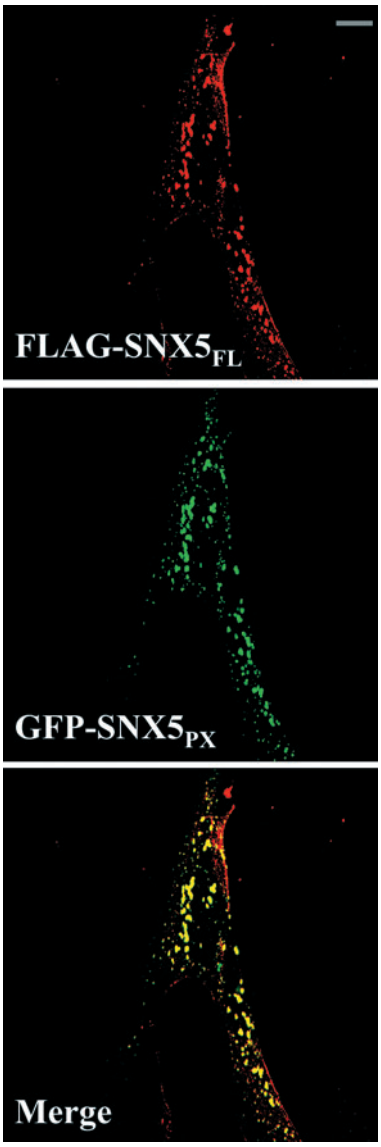

Fig. 1. SNX5 PX domain colocalizes with the full-length SNX5 protein. HeLa cells were co-transfected with the full-length SNX5 protein (FLAG-$SNX5_{FL}$) and a construct of SNX5 containing the PX domain (GFP-$SNX5_{PX}$). After 12 hours of transfection, cells were fixed, permeabilized and stained with anti-FLAG monoclonal antibody followed by goat anti-mouse IgG-Alexa568 conjugate. Confocal images were collected with identical iris settings. Superimposed images (Merge) reveal regions of colocalization. Plasma membrane staining is due to non-specific staining with the anti-FLAG monoclonal antibody. Bar, 10 μm.

To identify the GFP-$SNX5_{PX}$-positive structures, transfected HeLa cells were labelled with antibodies to various markers. Only very modest levels of colocalization were observed between the GFP-$SNX5_{PX}$ fusion protein and either the early endosome marker EEA1 or the late endosome marker LAMP1 (Fig. 2A,B). Furthermore, very little colocalization was detected between GFP-$SNX5_{PX}$ and endogenous SNX1 (Fig. 2A). By contrast, and as expected, endogenous SNX1 shows considerable colocalization with EEA1 (Fig. 2B). These results indicate that the PX domain of SNX5 may be recruited to a membrane domain distinct from the PX domain of SNX1.

To further define the localization of SNX5, and to ensure that overexpression of constructs had not influenced the intracellular location of the SNX5 products, we generated stable HEK293 cells expressing full-length SNX5 fused to GFP. The use of the Flp-In™ system ensured equivalent levels of expression within the cell population. The GFP fusion protein was localized on endosomal structures as well as being distributed in the

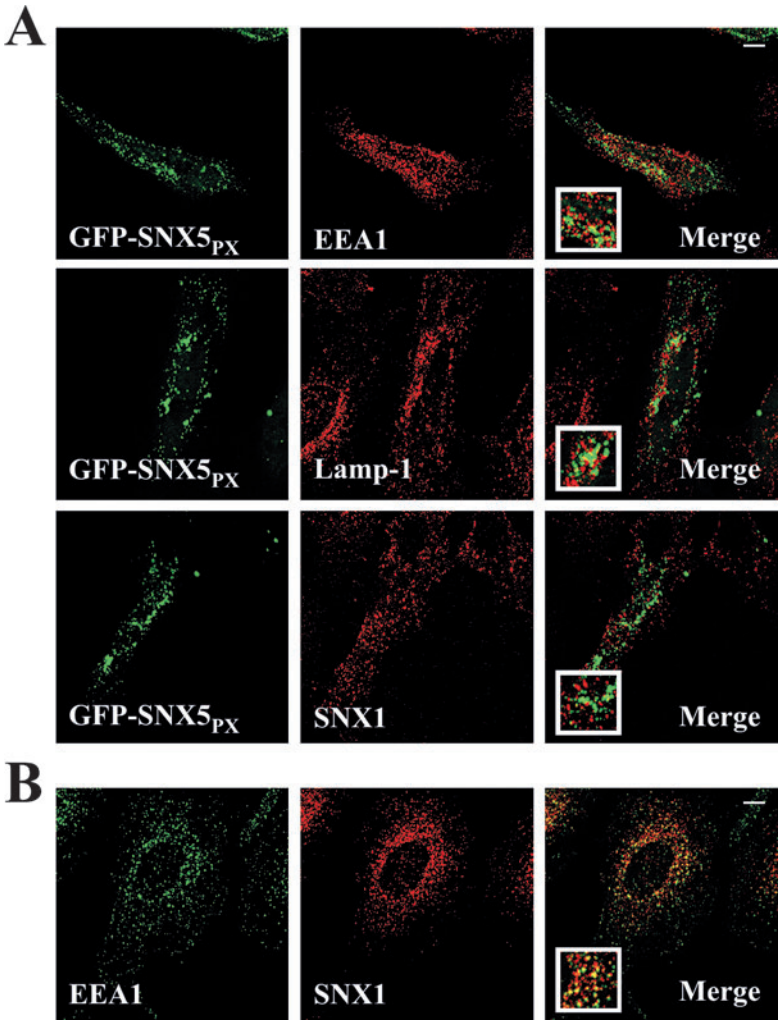

Fig. 2. Intracellular localization of human SNX5 PX domain in transfected HeLa cells. (A) HeLa cells were transiently transfected with GFP-SNX5$_{PX}$ and after 12 hours of transfection, cells were fixed in 4% paraformaldehyde, permeabilized with 0.1% Triton-X-100, and co-stained for EEA1, Lamp-1 and SNX1. EEA1 was detected with human anti-EEA1 antibodies followed by human anti-Ig Alexa568, and Lamp-1 and SNX1 were detected with mouse monoclonal antibodies and goat anti-mouse IgG-Alexa568 conjugate. (B) EEA1 was detected with human anti-EEA1 antibodies and FITC-goat anti-human IgG and SNX1 as for A. Control incubations demonstrated no cross-reactivity between the anti-Ig conjugates and the irrelevant primary antibody. Confocal images were collected with identical iris settings. Superimposed images (Merge) reveal regions of colocalization. Inserts show regions of the cell at higher magnification. Bar, 10 μm.

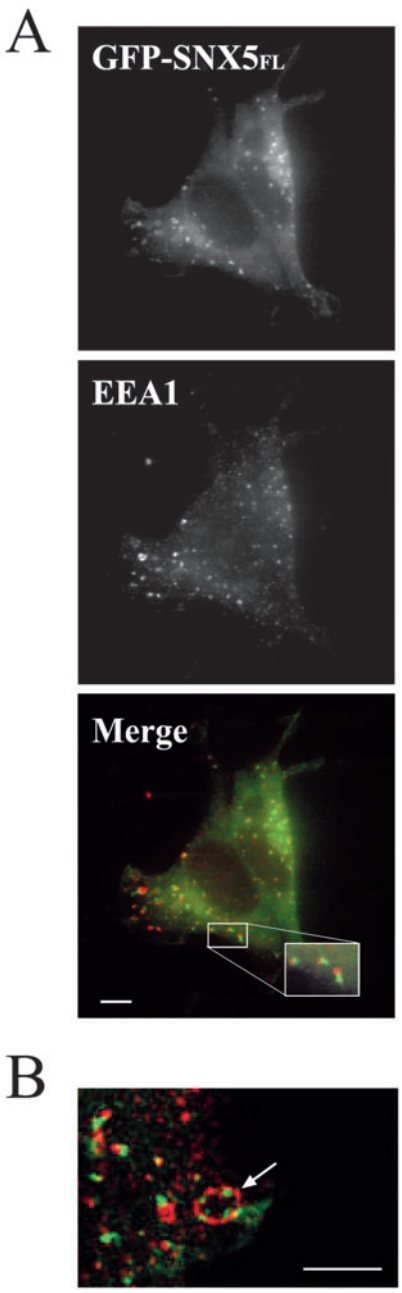

Fig. 3. Localization of GFP-SNX5$_{FL}$ fusion protein in stably transfected HEK293 cells. (A) HEK293 cells stably expressing a GFP-SNX5$_{FL}$ fusion protein were fixed in 4% paraformaldehyde, permeabilized with 0.1% Triton-X-100, and co-stained for EEA1 with human anti-EEA1 antibodies followed by human anti-Ig Alexa568. (B) High power image showing enlarged GFP-SNX5$_{FL}$-positive structures that also stain for EEA1. Note the lack of colocalization of the two molecules within these structures. Confocal images were collected with identical iris settings. Superimposed images (Merge) reveal regions of colocalization. Bar, 10 μm (A) and 5 μm (B).

cytoplasm of stable HEK293 cells, consistent with the results observed in transiently transfected HeLa cells (Fig. 3A). Only a low level of colocalization of GFP-SNX5 and EEA1 was observed (Fig. 3A shows a representative example) again consistent with the localization of SNX5 constructs in transiently transfected HeLa cells. However, GFP-SNX5-labelled structures were frequently found closely juxtaposed to EEA1 stained structures (Fig. 3A), suggesting that SNX5 was localized to a region of the early endosomes distinct from EEA1. Often, GFP-SNX5 was associated with enlarged endocytic structures, with profiles typical of enlarged early endosomes that have been described as a consequence of the expression of components of the endocytic machinery, such as rab5 mutants (Stenmark et al., 1994; Chen and Wang, 2001; Gillooly et al., 2003). These GFP-SNX5-positive enlarged

structures were also positively labelled for EEA1, however, GFP-SNX5 was often found on regions distinct from EEA1 (Fig. 3B).

The location of GFP-SNX5 within the endocytic pathway was further investigated by co-staining experiments with an endocytic fluid-phase marker. Texas-Red dextran was incubated with the stable HEK293 cells for 10 minutes at 37°C to allow fluid-phase uptake in endocytic compartments. GFP-SNX5 showed extensive colocalization with the endosomal compartments defined by the fluid-phase marker (Fig. 4). In addition, often distinct regions of the same structures were labelled with either GFP-SNX5 or Texas Red dextran suggesting that SNX5 may be located in membrane regions of the early endosome with a low luminal volume.

It has recently been shown that SNX1 can physically interact with the retromer component, Vps35p using a yeast two-hybrid assay (Haft et al., 2000). In view of the distinct endosomal locations of SNX1 and SNX5 we have compared their ability to interact with retromer. To determine if SNX5 also interacts with Vps35p we also used the yeast two-hybrid assay. As expected SNX1 showed a strong interaction with Vps35p, whereas SNX5 did not interact with Vps35 in this assay (not shown). This result demonstrates differences in protein-protein interactions of these two SNX molecules that may play an important role in their recruitment to specific membranes and their function.

Fig. 4. Colocalization of GFP-SNX5$_{FL}$ fusion protein in HEK293 cells with the fluid-phase marker, Texas-Red dextran. HEK293 cells stably expressing the GFP-SNX5$_{FL}$ fusion protein were incubated in Texas-Red dextran (100-200 μg/ml) for 15 minutes at 37°C as described in Materials and Methods. Cells were fixed in 4% paraformaldehyde. Confocal images were collected with identical iris settings. Superimposed images (Merge) reveal regions of colocalization. Inserts show regions of the cell at higher magnification. Bar, 10 μm.

Membrane binding of PX domain is inhibited by wortmannin

To determine if the binding of the GFP-SNX5$_{PX}$ to endosomes was dependent on lipid, transfected cells were treated with the PI 3-kinase inhibitor wortmannin. The addition of wortmannin (100 nM) resulted in the dissociation of the GFP-SNX5$_{PX}$ fusion protein from endosomal structures, and the appearance of weak fluorescence dispersed throughout the cytoplasm (Fig. 5). Analysis of transfected cells treated with wortmannin showed that approximately 80% of treated cells displayed exclusively cytosolic fluorescence whereas the remaining 20% of cells still displayed endosomal staining. The dissociation of GFP-SNX5$_{PX}$ from endosomes in the presence of wortmannin clearly shows a phosphoinositide-dependent binding of SNX5$_{PX}$ to this endosomal compartment

The lipid dependence of the endosomally localized GFP-SNX5 in the stable HEK293 cells was also assessed. Within 20 minutes of treatment with 100 nM wortmannin, the GFP-SNX5 fusion protein was found exclusively in the cytosol of the entire cell population, demonstrating the importance of PtdIns(3)*P* binding to the localization of the full-length molecule (Fig. 5B).

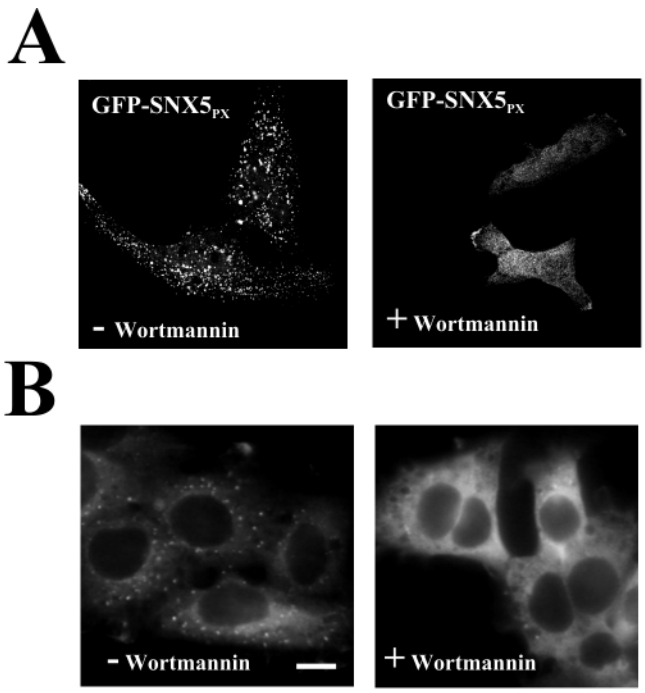

Fig. 5. Endosomal localization of PX domain of SNX5 requires the activity of a wortmannin sensitive PI 3-kinase. (A) HeLa cells were transfected with GFP-SNX5$_{PX}$, and after 12 hours cell monolayers were serum-starved for 3 hours. Cells were either untreated or treated with 100 nM wortmannin for 2 hours at 37°C and fixed in 4% paraformaldehyde. (B) HEK293 cells expressing GFP-SNX5$_{FL}$ were either untreated or treated with 100 nM wortmannin for 20 minutes at 37°C and fixed in 4% paraformaldehyde. Bar, 10 μm.

SNX5 is localized within PtdIns(3)*P* microdomains

To determine the relationship of PtdIns(3)*P* and SNX5 localization, we transfected HeLa cells with both FLAG-$SNX5_{FL}$ and GFP-$FYVE_{EEA1}$, a probe for PtdIns(3)*P*. Significantly, both SNX5 and the FYVE construct showed considerable colocalization, demonstrating that SNX5 is indeed localized to the early endosomes and, in particular, to PtdIns(3)*P*-containing membrane domains (Fig. 6A). However, SNX5 and the FYVE construct showed only partial colocalization, a result that indicates the presence of PtdIns(3)*P* sub-domains devoid of SNX5. As expected GFP-$FYVE_{EEA1}$ also showed considerable overlap with EEA1 (Fig. 6B), consistent with previous findings (Gillooly et al., 2003).

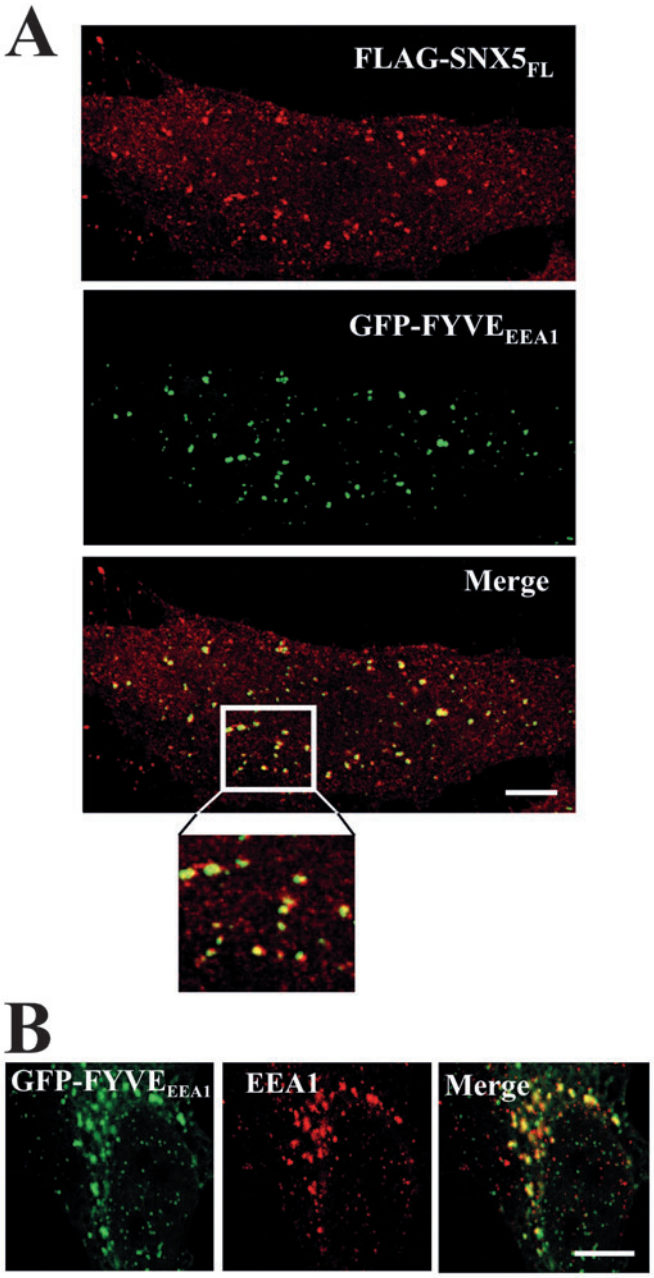

Fig. 6. SNX5 partially colocalizes with the PtdIns(3)*P* probe, GFP-$FYVE_{EEA1}$. (A) HeLa cells were transfected with FLAG-$SNX5_{FL}$ and GFP-$FYVE_{EEA1}$. After 24 hours, cell monolayers were permeabilized and stained with anti-FLAG monoclonal antibody followed by goat anti-mouse IgG-Alexa568 conjugate. (B) HeLa cells transfected with GFP-$FYVE_{EEA1}$ as above and stained for EEA1 with mouse anti-EEA1 antibodies followed by anti-Ig Alexa568. Confocal images were collected with identical iris settings. Superimposed images (Merge) reveal regions of colocalization. Bar, 10 μm.

PX domain of SNX5 binds to PtdIns(3)*P* and PtdIns(3,5)P_2

There is limited information on the precise phosphoinositide binding specificity of the PX domains of the different SNX molecules. Given the recent finding that SNX1 binds to both PtdIns(3)*P* and PtdIns(3,5)P_2 (Cozier et al., 2002), it is important to ascertain if the PX domains of different SNX family members have the same or a different phosphoinositide binding specificity as SNX1. This is particularly relevant as $SNX5_{PX}$ shows very little endosomal colocalization with SNX1 or EEA1. We used the physiologically relevant liposome-binding assay to examine the phosphoinositide specificity of SNX5. A GST fusion protein was constructed with the PX domain of SNX5. Liposomes generated were composed of a mixture of phosphatidylserine,

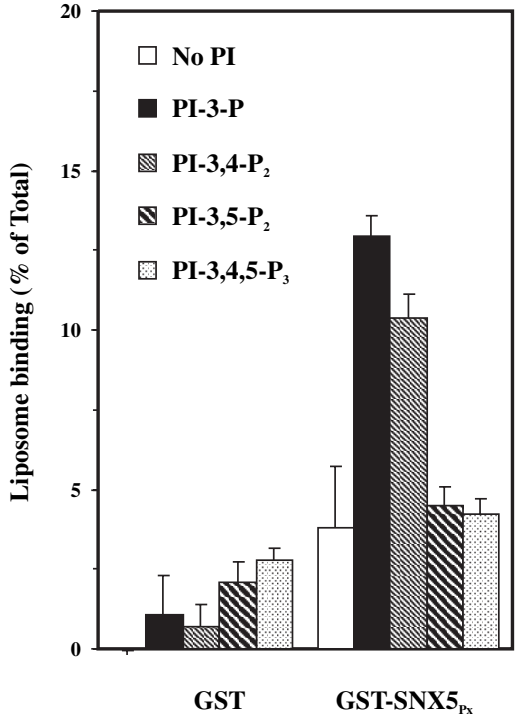

Fig. 7. The PX domain of SNX5 binds to PtdIns(3)*P* and PtdIns(3,4)P_2. The ability of $SNX5_{PX}$ domain to bind different phosphoinositides was analysed using a liposome binding assay. ^{3}H-labelled liposomes were prepared as described containing either PtdIns(3)*P*, PtdIns(3,4)P_2, PtdIns(3,5)P_2, PtdIns(3,4,5)P_3 or no PI, as indicated. Sonicated liposomes (100 μl) were incubated for 30 minutes at room temperature with 20 μg GST or purified GST-$SNX5_{PX}$ fusion protein immobilized on glutathione-Sepharose beads. After incubation, the beads were washed three times and collected by centrifugation. The radioactivity in both pellets and supernatant was determined. The assay was performed in triplicate and the standard deviations are shown.

phosphadtidylethanolamine and phosphatidylcholine to which was added either PtdIns(3)*P*, PtdIns(3,4)P_2, PtdIns(3,5)P_2 or PtdIns(3,4,5)P_3. Under these assay conditions, the FVYE finger domains of Hrs showed strong binding to PtdIns(3)*P*-containing liposomes, but not to the others, consistent with published data (not shown) (Gaullier et al., 1998). Using this liposome binding assay, the PX domain of SNX5 binds to PtdIns(3)*P* and PtdIns(3,4)P_2 with very little specific binding detected to the other phosphoinositides (Fig. 7), a specificity that distinguishes this PX domain from the PX domain of SNX1. Fig. 7 shows a representative assay of four separate liposome-binding experiments.

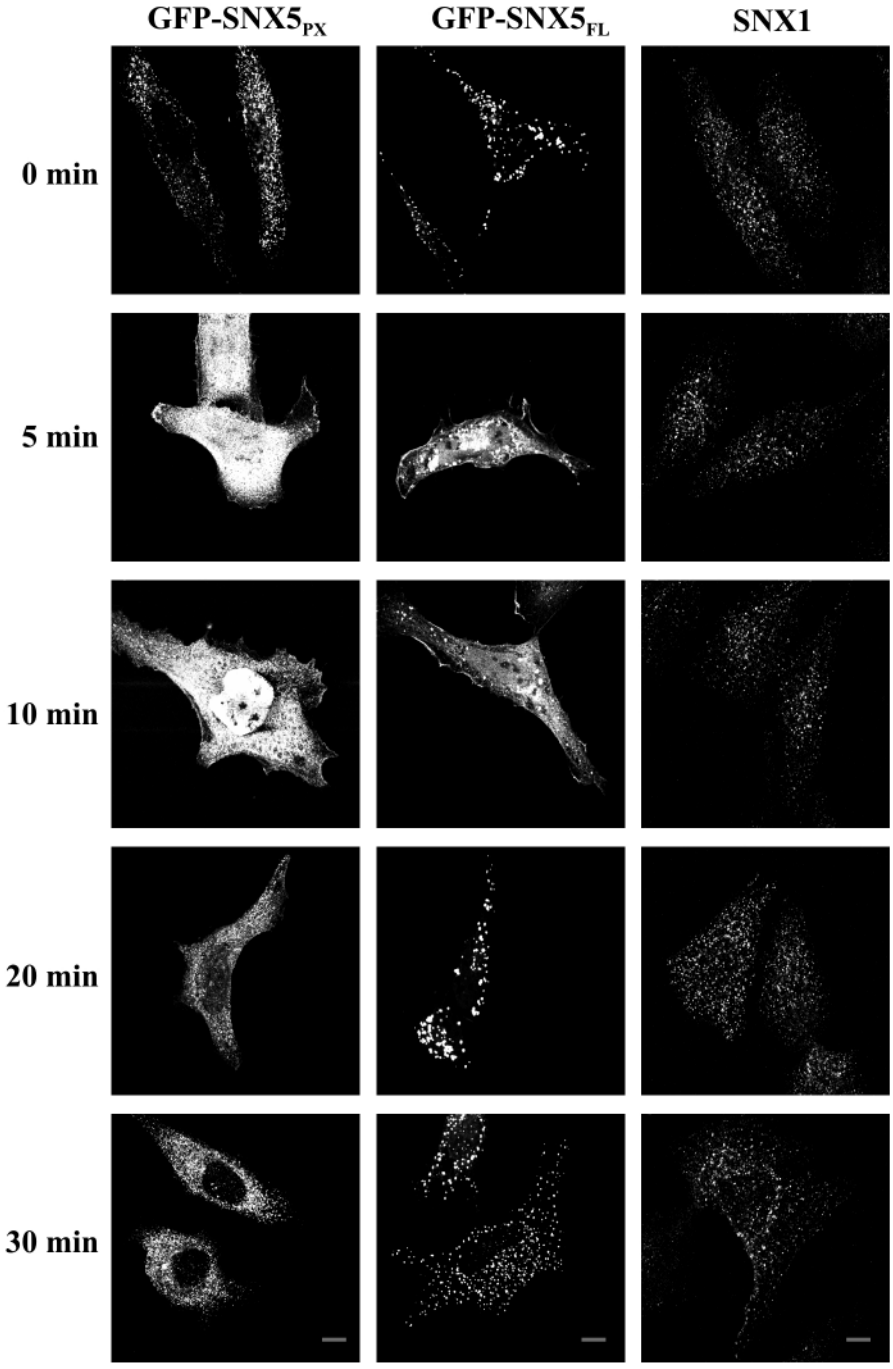

Fig. 8. SNX5 associates with the plasma membrane following EGF stimulation of transfected HeLa cells. HeLa cells were transfected with constructs encoding GFP-SNX5$_{PX}$ or GFP-SNX5$_{FL}$. Untransfected HeLa cells were also grown under the same conditions. After 12 hours, cell monolayers were serum-starved for 3 h and either left unstimulated or stimulated with human EGF (100 ng/ml) for the indicated times, fixed and processed for fluorescence. The distribution of GFP-SNX5$_{PX}$ and GFP-SNX5$_{FL}$ was directly visualized by confocal microscopy. Untransfected cells were stained with anti-SNX1 monoclonal antibody followed by goat-anti-mouse IgG conjugated to Alexa568. Bar, 10 μm.

SNX5 is recruited to the cell surface following EGF stimulation

As PtdIns(3,4)P_2 is predominantly found on the cytosolic face of the plasma membrane (Gray et al., 1999; Oatey et al., 1999), we investigated whether the PtdIns(3,4)P_2 binding by SNX5 detected in the in vitro assay was biologically relevant. The levels of plasma membrane PtdIns(3,4)P_2 are regulated by extracellular signals such as EGF, therefore, we investigated whether the intracellular location of SNX5 may be influenced by increases of this phosphoinositide intermediate following receptor stimulation. GFP-SNX5$_{PX}$-transfected HeLa cells were stimulated with EGF and the location of the GFP-SNX5$_{PX}$ fusion protein analysed over a period of 30 minutes (Fig. 8). Prior to stimulation, the fusion protein was found on endosomes structures in the majority of transfected cells (>80%). Within 5 minutes of EGF stimulation, GFP-SNX5$_{PX}$ was found within the cytosol and on patches on the plasma membrane (Fig. 8). Analysis of 50 transfected cells showed that more than 60% of GFP-positive cells had both cytosolic and plasma membrane staining. Very little endosomal staining by GFP-SNX5$_{PX}$ was observed in the transfected cells at either 5 or 10 minutes after EGF stimulation. The cellular distribution of the EGF receptors was also determined. Within 10 minutes of stimulation, EGF receptors had been internalized from the cell surface and were localized to intracellular endosomes (not shown); therefore, the generation of PtdIns(3,4)P_2 at the plasma membrane would be expected to be maximal by 5-10 minutes after EGF stimulation. By 20 minutes following receptor activation, the level of GFP-SNX5$_{PX}$ plasma membrane and cytosolic staining had receded and an endosomal location was now apparent (Fig. 8). By 30 minutes, the localization of GFP-SNX5$_{PX}$ had returned to an endosomal distribution

similar to that observed prior to stimulation with EGF. The time-course of the transient relocation of GFP-SNX5$_{PX}$ to the plasma membrane following EGF stimulation parallels the increase in levels of plasma membrane PtdIns(3,4)P_2 in other systems. For example, previous reports have indicated that PtdIns(3,4)P_2 levels increase within 2-10 minutes of stimulation by growth factors and return to basal levels by 20 minutes (Kimber et al., 2002). This dramatic redistribution of the PX domain of SNX5 following EGF stimulation has been observed in three separate experiments.

To determine whether the dynamic movement of GFP-SNX5$_{PX}$ reflected the behaviour of the full-length SNX5 molecule following EGF stimulation, the experiment was repeated using HeLa cells transfected with GFP-SNX5$_{FL}$. After 10 minutes of EGF stimulation the full-length SNX5 was found predominantly within the cytosol and also associated with regions of the plasma membrane (in more than 60% of transfected cells). As for the SNX5 PX domain, after 20 minutes of EGF stimulation, most of the full-length SNX5 was found again associated with endosomes (Fig. 8).

The localization of full-length SNX5 in stable HEK293 cells expressing GFP-SNX5 was also affected by EGF treatment. Within 3 minutes of EGF treatment SNX5 was readily detected at the plasma membrane and staining was very prominent by 12 minutes, and by 20 minutes the cell surface recruitment of SNX5 had disappeared (Fig. 9). The stable cell line maintained considerable endosomal staining throughout the EGF treatment period indicating that the recruitment of SNX5 to the plasma membrane may be mediated via the cytosol pool of SNX5. These results show that the recruitment of SNX5 to the plasma membrane can occur in different cell types.

In contrast to the movement of the SNX5-PX domain or the full-length SNX5 molecule, endogenous SNX1, which does not bind to PtdIns(3,4)P_2, showed no change in the intracellular endosomal location following EGF stimulation in either transiently transfected HeLa cells (Fig. 8) or stable HEK293 transfectants (not shown). Collectively these data suggest that the transient movement of SNX5 to the plasma membrane following EGF receptor stimulation is mediated by the ability of the PX domain of SNX5 to bind PtdIns(3,4)P_2.

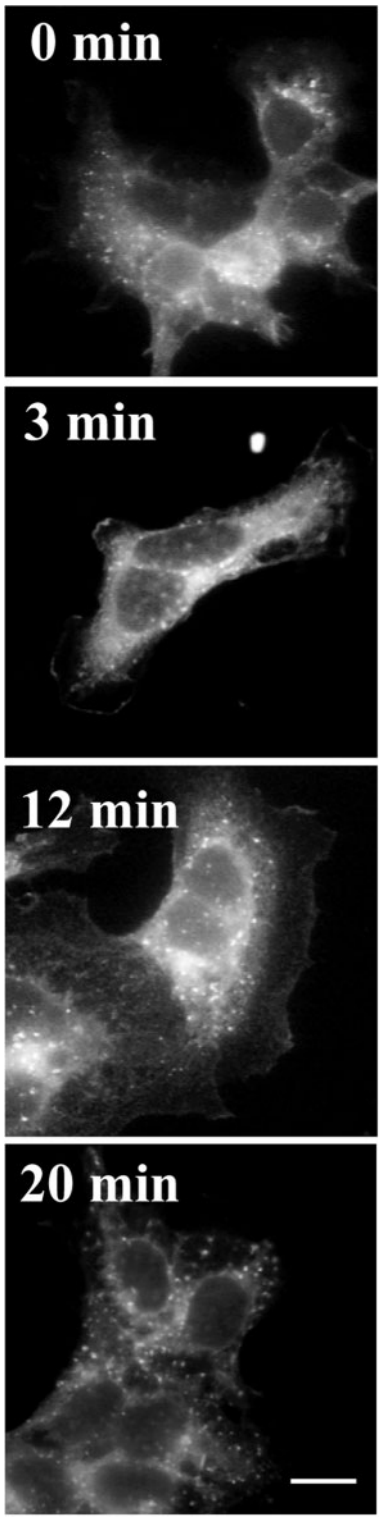

Fig. 9. SNX5 associates with the plasma membrane following EGF stimulation of HEK293 cells expressing GFP-SNX5$_{FL}$. Stable HEK293 cells stably expressing GFP-SNX5$_{FL}$ were serum-starved for 3 h and either left unstimulated or stimulated with human EGF (20 ng/ml) for the indicated times, fixed and processed for fluorescence. Bar, 10 μm.

Discussion

Members of the sorting nexin family have been implicated in the regulation of the trafficking of internalized receptors (Barr et al., 2000; Cozier et al., 2002; Stockinger et al., 2002; Hanson and Hong, 2003). In this study, we have analysed the localization and behaviour of SNX5, a sorting nexin with a PX domain and two coiled-coil regions. Based on structural and sequence relationships, SNX5 is closely related to SNX1, -2, -4 and -6 (Teasdale et al., 2001). However, the localization and behaviour of SNX5 differs from other sorting nexins characterized to date. First, SNX5 was localized to an early endosomal domain in non-stimulated cells distinct from a number of other markers of the early endosomes; second, the PX domain was able to independently target a GFP fusion to similar endosomal structures as the full-length molecule; third, unlike SNX1, SNX5 cannot interact with the retromer component Vps35p; fourth, the PX domain of SNX5 binds selectively to not only PtdIns(3)*P* but also PtdIns(3,4)P_2; and fifth, this dual PI binding specificity is associated with transient location of SNX5 to the cell surface following EGF stimulation. The ability of a sorting nexin to be recruited to the plasma membrane following a transient increase in phosphoinositide second messenger levels is novel and potentially broadens the functions of the family of SNX molecules. Furthermore, the regulated translocation of SNX5 to the cell surface, a process possibly mediated by PX domain-

lipid interactions, is likely to be crucial to the proper functioning of SNX5.

Numerous PX domains, including those from sorting nexins, have been shown to bind specifically to phosphoinositides with the majority interacting selectively with PtdIns(3)*P* (Xu et al., 2001a; Yu and Lemmon, 2001; Lemmon, 2003). Consistent with these earlier studies we have shown here that the isolated PX domain of SNX5 binds PtdIns(3)*P*. However, in addition to PtdIns(3)*P* the SNX5-PX domain also binds to PtdIns(3,4)P_2. The PI binding specificity of SNX5-PX domain contrasts with SNX1, which binds selectively to PtdIns(3)*P* and PtdIns(3,5)P_2 based on the liposome binding assay (Cozier et al., 2002), whereas SNX3 appears to interact exclusively with PtdIns(3)*P*. SNX16 binds predominantly to PtdIns(3)*P*, although the isolated SNX16 PX domain also shows very weak binding to PtdIns(3,4)P_2 based on protein-lipid overlays (Hanson and Hong, 2003). From these studies, it is clear that there are differences in the PI binding specificities of different sorting nexins. Detailed analyses of other SNX-PX domains are now warranted. Sequence comparison between the PX domains shows some interesting features of the SNX5-PX domain that may account for its unusual PI binding specificity. In particular, the SNX5-PX domain has an insert between the Pro-rich sequence and the α3 helix of the PX structure (Teasdale et al., 2001; Worby and Dixon, 2002). In addition, the highly conserved Arg58 and Arg105 of the $p40^{phox}$ domain, basic residues that interact directly with PtdIns(3)*P*, are replaced in SNX5-PX by Gln and Thr residues, respectively (Bravo et al., 2001). SNX6, which shares 66% sequence identity to SNX5, also contains an insert in the PX domain. Clearly, it will be interesting to resolve the structural basis for the PI binding specificity of SNX5-PX.

The PX domain of SNX5 was localized to endosomal structures in transfected cells and the inhibition of membrane binding by wortmannin demonstrates the importance of PI 3-kinase activity in the membrane localization of SNX5-PX. Several PX domains when expressed in isolation are known to efficiently target PtdIns(3)*P*-containing membranes in vivo (Kanai et al., 2001), including SNX3 (Xu et al., 2001a). In other cases, such as SNX1, endosomal targeting requires both the PX domain and the C-terminal coiled coil regions (Teasdale et al., 2001; Zhong et al., 2002). The ability of SNX5-PX to target membranes in vivo indicates that this PX domain binds strongly to PtdIns(3)*P*. As the SNX5-PX domain colocalized with full-length SNX5, the membrane targeting of the isolated PX domain clearly reflects the full-length protein.

The intracellular distribution of SNX5 was subtly different to EEA1 and SNX1. Although colocalization with EEA1 was occasionally observed, careful quantitative analyses showed that in the majority of transfected cells there was very little direct overlap between either EEA1 or SNX1 and SNX5. The colocalization with the fluid-phase marker Texas-Red dextran, the extensive colocalization with the PtdIns(3)*P* probe, GFP-$FYVE_{EEA1}$, the inhibition of membrane binding by the PI 3-kinase inhibitor wortmannin, together with the physical proximity of the individual EEA1 and SNX5 decorated structures, strongly indicates that SNX5 is localized to a PtdIns(3)*P* subdomain of early endosomes. A recent study has also demonstrated that different PtdIns(3)*P* binding proteins differ in their microdomain distribution, in particular EEA1 and Hrs (Gillooly et al., 2003) and our results extend these observations to include sorting nexins. The localization of the SNX5 constructs to a different PtdIns(3)*P* subdomain from EEA1 may be due to interactions with both lipid and protein. Alternatively, the context in which the PtdIns(3)*P* lipid is presented within the membrane may differ between microdomains and limit the recruitment of the different PtdIns(3)*P* binding proteins to specific domains.

The endosomal localization of SNX5 also differs from other sorting nexins characterized to date. For example, many sorting nexins of the SNX1 subfamily show partial colocalization with early endosome markers, which is perhaps not surprising as SNX1, SNX2, SNX3 and SNX4 have been found to interact with each other and form heterooligomers (Haft et al., 1998). SNX15 and SNX16 show partial overlap with the late endosomal marker LAMP1 (Barr et al., 2000; Hanson and Hong, 2003), and the yeast t-SNARE Vam7 localizes to vacuole membranes (Cheever et al., 2001).

The C-terminal domain of SNX5 contains coiled-coil regions and is likely to be involved in multiprotein complexes. Previously, we showed that the C-terminal domain of SNX5 (residues 167-404) was localized to endosomal structure (Seaman et al., 1998; Teasdale et al., 2001), indicating the possible involvement of protein-protein interactions of the membrane-associated full-length molecule. Significantly, SNX1 sub-group molecules, SNX1, SNX2 and Vps5p have been identified to be part of a membrane-associated complex, termed the retromer, which contains Vps26p, Vps29p and Vps35p (Haft et al., 2000). First identified in yeast and now in mammalian cells, the retromer is considered to function as a membrane coat complex for vesicular transport (Seaman et al., 1998; Haft et al., 2000). The mammalian retromer is probably involved in one or more of the endosomal transport pathways. Our finding that SNX5 cannot interact with Vps35p, further distinguishes this sorting nexin from SNX1, and indicates that SNX5 does not interact with retromer components but rather may function in a different complex. SNX5 has been reported to bind the Fanconi anaemia complementation group A protein, detected in a yeast two-hybrid screen and by co-immunoprecipitation experiments (Otsuki et al., 1999). However, the significance of this interaction remains unclear.

PtdIns(3,4)P_2 is present at very low levels in non-stimulated cells but its concentration, along with PtdIns(3,4,5)P_3, increases dramatically at the plasma membrane after receptor stimulation (Gray et al., 1999; Oatey et al., 1999; Vanhaesebroeck et al., 2001). Based on the liposome binding assay, the PX domain of SNX5 recognizes PtdIns(3,4)P_2 but not PtdIns(3,4,5)P_3. Many proteins with the phosphoinositol recognition pleckstrin homology (PH) domains bind to either PtdIns(3,4,5)P_3 and PtdIns(3,4)P_2 or to PtdIns(3,4,5)P_3 only (Lemmon, 2003). The PX domain of p47phox appears to bind selectively to PtdIns(3,4)P_2, which is likely to account for the movement of the phagocyte NADPH oxidase to the plasma membrane after neutrophil stimulation and phagocytosis (Kanai et al., 2001). The SNX5-PX domain is unusual amongst PI binding domains in recognizing both PtdIns(3)*P* and PtdIns(3,4)P_2. The binding of the SNX5-PX domain to the plasma membrane of stimulated cells is probably due to a direct interaction with PtdIns(3,4)P_2 as first, the transient interaction with the plasma membrane is consistent with the transient wave of PtdIns(3,4)P_2 production (Kimber et al., 2002); second, the PX domain used in our studies represents

the minimal PX domain sequence and does not contain other domains; and third, the ability of SNX5 to bind to the plasma membrane is specific, as SNX1, which does not bind to PtdIns(3,4)P_2 or PtdIns(3,4,5)P_3 (Cozier et al., 2002) is not recruited to the plasma membrane after cell stimulation. Furthermore, we have demonstrated colocalization of SNX5 with actin-rich regions of the plasma membrane (our unpublished observations), consistent with the central role of PI-signalling molecules in the regulation of cytoskeletal-membrane linkages (Vanhaesebroeck et al., 2001). Thus the behaviour of SNX5 is more typical of PH domain proteins that respond to elevated second messenger molecules at the cell surface. Of interest is the recent finding that the signalling enzyme phospholipase D1 recycles between endosomes and the plasma membrane by coordinating interactions mediated by PI-binding PH and PX domains (Du et al., 2003). In view of these findings, the possibility that other sorting nexins may be able to traffic between distinct membrane compartments should be considered.

The production of PtdIns(3,4)P_2 or PtdIns(3,4,5)P_3, detected at the plasma membrane, is a key event during cell signalling as it allows the recruitment of proteins with PH domains which control the activity and subcellular localization of an array of signal transduction molecules (Lemmon and Ferguson, 2000). Attention has primarily focused on PtdIns(3,4,5)P_3 as it represents the major product of ligand-stimulated PI 3-kinases (Rameh and Cantley, 1999). The majority of PtdIns(3,4)P_2 is considered to be derived from phosphatase action on PtdIns(3,4,5)P_3, although recent data has suggested that PtdIns(3,4)P_2 can be synthesized independently of PtdIns(3,4,5)P_3 (Banfic et al., 1998a; Banfic et al., 1998b). The recent identification of a PH domain, from the protein TAPP1, which interacts selectively with PtdIns(3,4)P_2 (Kimber et al., 2002) indicates the possibility of PtdIns(3,4)P_2-specific signalling pathway. Our findings here on SNX5 raise the possibility that this sorting nexin is targeting a complex to the site of plasma membrane signalling. Using the TAPP1 PH domain as a probe for immunoelectron microscopic labelling, PtdIns(3,4)P_2 was not only detected at the plasma membrane of stimulated cells but also on intracellular membranes such as multivesicular endosomes (Watt et al., 2004), raising the possibility that the PtdIns(3,4)P_2 binding properties of SNX5 may impact on its intracellular localization.

In summary, SNX5 is recruited to a subdomain of the early endosome distinct from EEA1 and SNX1, and the PI binding PX domain plays a dominant role in its localization. It is likely that the SNX5-specific domain is associated with a role in protein sorting within the early endosome. In addition to an endosomal localization, SNX5 can shuttle from the cytoplasm to the plasma membrane as a consequence of EGF signalling. The localization of SNX5 to the plasma membrane is likely to be mediated by the binding of the PX domain to PtdIns(3,4)P_2, although interactions with proteins may also be involved. Although the function of SNX5 recruitment to the plasma membrane is not known, the behaviour of SNX5 reinforces the dynamic interplay between components of the endosome machinery and signalling events. Sorting nexins represent a very large family, and the results here highlight the need to examine closely the localization of individual sorting nexins not only in quiescent cells but also under a variety of stimuli.

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