An Essential Role for SNX1 in Lysosomal Sorting of Protease-activated Receptor-1: Evidence for Retromer-, Hrs-, and Tsg101-independent Functions of Sorting Nexins

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Sorting nexin 1 (SNX1) and SNX2 are the mammalian homologues of the yeast Vps5p retromer component that functions in endosome-to-Golgi trafficking. SNX1 is also implicated in endosome-to-lysosome sorting of cell surface receptors, although its requirement in this process remains to be determined. To assess SNX1 function in endocytic sorting of protease-activated receptor-1 (PAR1), we used siRNA to deplete HeLa cells of endogenous SNX1 protein. PAR1, a G-protein-coupled receptor, is proteolytically activated by thrombin, internalized, sorted predominantly to lysosomes, and efficiently degraded. Strikingly, depletion of endogenous SNX1 by siRNA markedly inhibited agonist-induced PAR1 degradation, whereas expression of a SNX1 siRNA-resistant mutant protein restored agonist-promoted PAR1 degradation in cells lacking endogenous SNX1, indicating that SNX1 is necessary for lysosomal degradation of PAR1. SNX1 is known to interact with components of the mammalian retromer complex and Hrs, an early endosomal membrane-associated protein. However, activated PAR1 degradation was not affected in cells depleted of retromer Vps26/Vps35 subunits, Hrs or Tsg101, an Hrs-interacting protein. We further show that SNX2, which dimerizes with SNX1, is not essential for lysosomal sorting of PAR1, but rather can regulate PAR1 degradation by disrupting endosomal localization of endogenous SNX1 when ectopically expressed. Together, our findings establish an essential role for endogenous SNX1 in sorting activated PAR1 to a distinct lysosomal degradative pathway that is independent of retromer, Hrs, and Tsg101.

INTRODUCTION

Mammalian sorting nexins (SNXs) are a group of highly diverse cellular proteins defined by the presence of a phospholipid-binding domain termed the phox homology (PX) domain (Worby and Dixon, 2002). SNX1 and SNX2 are the mammalian homologues of the yeast vacuole protein-sorting molecule Vps5p (Haft *et al.*, 1998). Vps5p interacts with Vps17p and forms part of a retromer complex comprised of five distinct proteins, including Vps35p, Vps29p, and Vps26p. The yeast retromer complex is required for retrieval of Vps10p receptor from prevacuolar endosomes back to the *trans*-Golgi network (TGN) (Horazdovsky *et al.*, 1997; Nothwehr and Hinds, 1997; Seaman *et al.*, 1998). The retrograde trafficking of Vps10p is essential for delivery of newly synthesized hydrolases to the vacuole, an organelle equivalent

to the mammalian lysosome. The mammalian homologues of the retromer subunits have now been identified (with the exception of Vps17) and appear to have distinct functions in various cell types. Several recent studies indicate that mammalian retromer subunits Vps26, Vps35, and SNX1 are essential for retrieval of the cation-independent mannose 6-phosphate receptor (CI-MPR), the functional homologue of Vps10p, in HeLa cells (Arighi *et al.*, 2004; Carlton *et al.*, 2004; Seaman, 2004), suggesting that retromer function in retrograde trafficking of a lysosomal hydrolase receptor remained conserved in mammalian cells. However, the retromer Vps35-Vps29-Vps26 subcomplex has also been shown to regulate pIgR-pIgA transcytosis in MDCK cells (Verges *et al.*, 2004), indicating a role for retromer in protein sorting in polarized epithelial cells. Moreover, our recent work in mice demonstrates that mammalian retromer complexes, containing SNX1 and SNX2, have an essential function in embryonic development that does not involve regulation of CI-MPR trafficking (Griffin *et al.*, 2005). Thus, retromer activity appears to have evolved considerably from yeast and regulates complex and distinct cellular processes in different mammalian tissues.

SNX1 was originally identified in a yeast two-hybrid screen using the cytoplasmic tail of the epidermal growth factor receptor (EGFR) (Kurten *et al.*, 1996). A function for SNX1 in endosome-to-lysosome trafficking was then suggested based on studies in which SNX1 overexpression enhanced EGFR degradation and SNX1 deletion mutants inhibited EGFR degradation (Kurten *et al.*, 1996; Zhong *et al.*, 2002). Moreover, endogenous SNX1 localizes predominantly

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Abbreviations used: CI-MPR, cation-independent mannose 6-phosphate receptor; EGFR, epidermal growth factor receptor; GPCR, G protein-coupled receptor; Hrs, hepatocyte growth factor-regulated tyrosine kinase substrate; LAMP1, lysosomal-associated membrane protein-1; PAR1, protease-activated receptor-1; PX, phox homology domain; siRNA, small interfering RNA; SNX, sorting nexin; TGN, *trans*-Golgi network; Tsg101, tumor susceptibility gene 101; Vps, vacuolar protein sorting.

to early endosomes by binding to PtdIns(3)P, a phospholipid highly enriched in early endosomal membranes (Cozier *et al.*, 2002; Zhong *et al.*, 2002). SNX1 also interacts with Hrs, an early endosomal membrane-associated protein (Chin *et al.*, 2001; Raiborg *et al.*, 2001). Hrs associates with Tsg101 and is essential for lysosomal sorting of EGFR (Bishop *et al.*, 2002; Lu *et al.*, 2003). Other cell surface integral membrane receptors, including nutrient receptors and receptor tyrosine kinases, also associate with SNX1 when heterologously expressed (Haft *et al.*, 1998). However, we and others have recently shown that neither endogenous SNX1 nor SNX2 is required for lysosomal degradation of EGFR (Carlton *et al.*, 2004; Gullapalli *et al.*, 2004). Thus, whether endogenous SNX1 function is essential for endosome-to-lysosome sorting of cell surface receptors in mammalian cells remains to be determined.

Intracellular trafficking of G protein-coupled receptors (GPCRs), which comprises the largest family of cell surface receptors in the mammalian genome (Pierce *et al.*, 2002), controls the temporal and spatial aspects of receptor signaling. However, the mechanisms that mediate trafficking of GPCRs through the endocytic system remain poorly defined. A protein-protein interaction screen using SNX1 and a library of 59 GPCR carboxyl terminal tails revealed that SNX1 is capable of interacting with at least 10 distinct GPCRs in vitro (Heydorn *et al.*, 2004). We also previously demonstrated that SNX1 associates with protease-activated receptor-1 (PAR1), a GPCR for thrombin, and that a deletion mutant of SNX1 blocked lysosomal degradation of activated PAR1 (Wang *et al.*, 2002). Because activated PAR1 is rapidly internalized, sorted predominantly to lysosomes, and degraded with remarkable efficiency (Trejo *et al.*, 1998; Trejo and Coughlin, 1999), it is a useful model for dissecting the molecular mechanism(s) responsible for GPCR lysosomal sorting. Toward elucidating the molecular basis of GPCR trafficking and toward determining whether SNX1 functions in endosome-to-lysosome sorting in mammalian cells, we examined whether endogenous SNX1 functions in lysosomal degradation of activated PAR1. Our studies reveal for the first time that endogenous SNX1 is essential for sorting activated PAR1 to a distinct lysosomal degradative pathway that does not require retromer, Hrs, or Tsg101 activity.

MATERIALS AND METHODS

Antibodies and Reagents

Agonist peptide SFLLRN was synthesized as the carboxyl amide and purified by reverse phase high-pressure liquid chromatography (UNC Peptide Facility, Chapel Hill, NC). The epidermal growth factor (EGF) ligand and leupeptin were purchased from Sigma (St. Louis, MO). Rabbit anti-PAR1 antibody was generated against the amino-terminal peptide sequence-YEPFWEDEEKNESGLTEYC, as previously described (Hung *et al.*, 1992). Anti-EGFR mouse monoclonal LA22 antibody was from Upstate Biotechnology (Lake Placid, NY). Monoclonal M1 and M2 anti-FLAG antibodies were purchased from Sigma. Mouse anti-SNX1 antibody was purchased from BD Transduction Laboratories (Lexington, KY). Anti-myc rabbit polyclonal antibody (A-14) and mouse anti-myc (9E10) were from Santa Cruz Biotechnology (Santa Cruz, CA). The rabbit polyclonal anti-SNX antibodies were generated against full-length SNX1 or SNX2 expressed as a glutathione S-transferase fusion protein (Haft *et al.*, 1998). Anti-actin antibody was obtained from Sigma. The rabbit polyclonal anti-Vps26 and anti-Vps35 antibodies were generated as described previously (Haft *et al.*, 2000). Anti-CI-MPR mouse monoclonal antibody was from Research Diagnostics. Monoclonal anti-Hrs antibody was purchased from Alexis Biochemicals and mouse anti-Tsg101-4A10 antibody was from GeneTex. Anti-lysosomal-associated membrane protein-1 (LAMP1) H4A3 mouse antibody was obtained from the Developmental Studies Hybridoma Bank (University of Iowa, Iowa City, IA). The secondary antibodies, goat anti-mouse- and anti-rabbit-conjugated to horseradish peroxidase, were from Bio-Rad (Richmond, CA). Alexa488- and Alexa594-conjugated goat anti-mouse and anti-rabbit antibodies were obtained from Molecular Probes (Eugene, OR).

Cell Lines and cDNAs

HeLa cells stably expressing an amino-terminal FLAG-tagged PAR1 were grown and maintained as described previously (Trejo *et al.*, 2000). The N-terminal myc-tagged SNX1 and SNX2 cDNAs were gifts from C. R. Haft (National Institute of Diabetes and Digestive and Kidney Diseases, National Institutes of Health) and have been described previously (Haft *et al.*, 1998). A myc-tagged SNX1 small interfering RNA (siRNA)-resistant mutant cDNA was generated by introducing a silent mutation at codon Ile-549 (ATC→ATT) using Quick Change site-directed mutagenesis kit (Stratagene, La Jolla, CA), and specific mutations were confirmed by dideoxy sequencing. Ile-549 is located within the region targeted by SNX1 siRNA3 oligo. To generate SNX1/2 chimeras, a *BsiWI* site was introduced in the myc-SNX1 and myc-SNX2 cDNAs just 5' to the sequence encoding the PX domain (SKPQRTYE for SNX1 and VIFDRTRE for SNX2, where the location corresponding to the *BsiWI* sites are underlined). A second site *BspEI* was then introduced just 3' to the PX domain (TQTLSCGAG for SNX1 and TQALSCGAG for SNX2, where the location corresponding to the *BspEI* sites are underlined). These *BsiWI* and *BspEI* sites were then used to exchange cDNA fragments encoding the N-terminus/PX domain or PX domain/C-terminus of SNX1 and SNX2 (see Figure 7). Mutations in all constructs were confirmed by dideoxy sequencing.

siRNAs Transient Transfection

HeLa cells plated at 5.0×10^5 cells per well of 6-well dishes or at 1.25×10^5 cells per well of 12-well dishes were grown overnight. Cells were then transiently transfected with 100 nM of specific siRNAs using LipofectAMINE 2000 according to the manufacturer's instructions (Invitrogen, Carlsbad, CA), and experiments were performed 48 h later or after two consecutive 72-h siRNA transfections with Vps26 siRNA as previously described (Arighi *et al.*, 2004). siRNAs were from Dharmacon (Lafayette, CO) and were designed to target the following specific mRNA sequences: SNX1 siRNA3 (5'-CCA CGU GAU CAA GUA CCU U-3'), SNX2 siRNA2 (5'-GAU AGA CCA GUU ACA UCA A-3'), hVps26 siRNA (5'-CUC UAU UAA GAU GGA AGU G-3'), Hrs siRNA (5'-CGA CAA GAA CCC ACA CGU C-3'), Tsg101 siRNA (5'-CCU CCA GUC UUC UCU CGU C-3') and a nonspecific (ns) siRNA (5'-GGC UAC GUC CAG GAG CGC ACC-3') was used as a negative control.

Transient Transfections

HeLa cells were plated at 5.0×10^5 cells per well in 6-well dishes or at 1.25×10^5 cells per well in 12-well dishes and grown overnight. Cells were then transiently transfected with a total of 2 μ g of plasmid DNA per well of a 6-well dish or 0.8 μ g plasmid DNA per well of a 12-well dish using LipofectAMINE reagent according to the manufacturer's instructions (Invitrogen). All experiments were performed 48 h after transfection.

Coimmunoprecipitation and Immunoblotting

HeLa cells stably expressing FLAG-tagged PAR1 plated at 5.0×10^5 cells per well in a six-well dish were transiently transfected and grown for 48 h. Cells were lysed and immunoprecipitated with M2 anti-FLAG antibody, as described previously (Wang *et al.*, 2002). Immunoprecipitates were resolved by 9 or 12% SDS-PAGE and transferred, and membranes were then incubated overnight at 4°C with anti-PAR1 rabbit polyclonal antibody. Cell lysates were run in parallel and immunoblotted for SNX1, SNX2, Vps26, Vps35, Hrs, Tsg101, myc or actin proteins. HeLa cells plated at 1.25×10^5 cells per well of 12-well dishes were transiently transfected, lysed, and immunoblotted for endogenous EGFR. Membranes were washed, incubated with species-specific secondary antibodies-conjugated to horseradish peroxidase, and washed again. Immunoblots were then developed using enhanced chemiluminescence (Amersham Biosciences, Piscataway, NJ), imaged by autoradiography, and quantitated using a Fluor-S Multimager (Bio-Rad, Richmond, CA).

Immunofluorescence Confocal Microscopy

HeLa cells plated at 1.5×10^5 on fibronectin-coated glass coverslips in 12-well dishes were transiently transfected and grown for 48 h. Cells were fixed and processed for microscopy as described previously (Trejo *et al.*, 2000). Colocalization of PAR1 with LAMP1 was assessed in cells that were pretreated with 2 mM leupeptin for 1 h. Confocal images were acquired using a Fluoview 300 laser scanning confocal imaging system (Olympus) configured with an IX70 fluorescence microscope fitted with a PlanApo 60 $\times$ oil objective (Olympus). Fluorescent images, X-Y section at 0.28 μ m, were collected sequentially at 800 $\times$ 600 resolution with 2 $\times$ optical zoom. Some images (see Figures 8 and 9) were collected using an Olympus DSU spinning disk confocal microscope configured with a PlanApo 60 $\times$ oil objective and Hamamatsu ORCA-ER digital camera. Fluorescent images of X-Y sections at 0.15 μ m were collected sequentially using Intelligent Imaging Innovations Slidebook 4.1 software. The final composite images were created using Adobe Photoshop CS (Adobe Systems, San Jose, CA).

Internalization Assay

HeLa cells stably expressing PAR1 plated at a density of 1.0×10^5 cells in 24-well dishes were transiently transfected with siRNA and grown for 48 h.

Cells were incubated with or without agonist for various times at 37°C. Cells were fixed with 4% paraformaldehyde for 5 min at 4°C, washed, and then incubated with M1 anti-FLAG antibody diluted in DMEM containing 1 mg/ml BSA, 1 mM CaCl₂, 10 mM HEPES, pH 7.4, for 1 h at 25°C. Cells were washed and then incubated with horseradish peroxidase-conjugated goat anti-mouse secondary antibody for 1 h at 25°C. The amount of bound horseradish peroxidase-goat anti-mouse secondary antibody was determined by incubation with one-step ABTS (2,2'-azino-bis-3-ethylbenz-thiazoline-6-sulfonic acid; Pierce, Rockford, IL) substrate for 10–20 min at 25°C. An aliquot was removed and the optical density was determined at 405 nm using a Molecular Devices SpectraMax Plus microplate reader (Sunnyvale, CA).

RESULTS

SNX1 Is Necessary for Sorting Activated PAR1 to a Degradative Pathway

Genetic and biochemical evidence indicate that SNX1 is part of a "retromer" complex that functions in endosome-to-TGN retrograde trafficking (Horazdovsky *et al.*, 1997; Nothwehr and Hindes, 1997; Seaman *et al.*, 1998; Carlton *et al.*, 2004). SNX1 has also been implicated in endosome-to-lysosome sorting of cell surface receptors in mammalian cells; however, whether SNX1 is required in this process remains to be determined. Toward defining the functional importance of SNX1 in PAR1 trafficking, we used siRNA to deplete HeLa cells of endogenous SNX1 protein. Endogenous SNX1 expression was virtually abolished in cells transfected with SNX1-specific siRNA compared with nonspecific (ns) control siRNA-treated cells (Figure 1, middle panels). To determine whether SNX1 was necessary for agonist-induced PAR1 degradation, HeLa cells stably expressing FLAG-tagged PAR1 were transiently transfected with control or SNX1 siRNA, and PAR1 degradation was assessed. In control siRNA-treated cells, a significant ~60% loss of receptor protein was observed after 90-min exposure to PAR1-specific agonist peptide SFLLRN (Figure 1). These findings are consistent with the extent of agonist-induced PAR1 degradation typically observed in these cells (Trejo *et al.*, 2000). In contrast, activated PAR1 degradation was markedly inhibited in cells lacking endogenous SNX1 (Figure 1); only ~8% of receptors were degraded after agonist treatment. These findings suggest that SNX1 is necessary for agonist-induced PAR1 degradation.

We next determined whether expression of a siRNA-resistant SNX1 mutant protein could restore agonist-induced PAR1 degradation in cells lacking endogenous SNX1. PAR1-expressing HeLa cells were cotransfected with control siRNA and pcDNA or with SNX1 siRNA and either pcDNA, wild-type myc-SNX1 or myc-SNX1 siRNA-resistant mutant cDNAs. A significant decrease in endogenous SNX1 and wild-type myc-SNX1 protein was observed in cells transfected with SNX1 siRNA compared with control siRNA-treated cells (Figure 2, lanes 1–6). In contrast, expression of myc-SNX1 siRNA-resistant mutant protein was unaffected in SNX1 siRNA-transfected cells, as expected (Figure 2, lanes 7 and 8). A 90-min exposure to agonist peptide SFLLRN caused a marked ~50% decrease in PAR1 protein in control siRNA-transfected cells (Figure 2, lanes 1 and 2). By contrast, activated PAR1 failed to efficiently degrade in cells cotransfected with SNX1 siRNA and either pcDNA or wild-type myc-SNX1 (Figure 2, lanes 3–6), indicating that SNX1 is required for agonist-induced PAR1 degradation. Strikingly, however, coexpression of SNX1 siRNA-resistant mutant protein together with SNX1 siRNA restored the ability of agonist to induce a significant ~60% degradation of PAR1 protein (Figure 2, lanes 7 and 8). Together these observations strongly suggest that SNX1 is necessary for sorting activated PAR1 to a lysosomal degradative pathway in mammalian cells.

Figure 1. SNX1 is essential for agonist-induced PAR1 degradation. HeLa cells stably expressing FLAG-tagged PAR1 were transiently transfected with nonspecific (ns) or SNX1 siRNA and then incubated in the absence or presence of 100 μ M SFLLRN for 90 min at 37°C. Cells were lysed and immunoprecipitated with M2 anti-FLAG antibody, and the amount of PAR1 remaining was detected by immunoblot analysis using an anti-PAR1 polyclonal antibody (top panel). Cell lysates were immunoblotted for expression of endogenous SNX1 or actin to control for equal loading (bottom panels). The data (mean $\pm$ SE) in the bar graph are expressed as a percentage of PAR1 remaining compared with untreated control cells. Data were determined for each transfection condition and represent the average of three separate experiments.

To determine whether SNX1 siRNA blocked PAR1 degradation by inhibiting receptor internalization, we assessed agonist-induced loss of cell surface PAR1 quantitatively by ELISA. PAR1-expressing HeLa cells transfected with control or SNX1 siRNA were incubated with agonist for various times, and the amount of PAR1 remaining on the cell surface was then measured. In control siRNA-treated cells, agonist induced rapid PAR1 internalization from the cell surface within 10 min (Figure 3A), and receptor continued to slowly internalize to ~50% loss of cell surface PAR1 after 30 min of agonist exposure. The addition of agonist caused a similar decrease in PAR1 internalization at various times in SNX1 siRNA-treated cells (Figure 3A), which were depleted of endogenous SNX1 protein as assessed by immunoblot and immunofluorescence microscopy (Figure 3C). Studies of stable PAR1-expressing HeLa cells using immunofluorescence microscopy were consistent with a SNX1-independent regulation of receptor internalization. In control siRNA-treated cells, 10-min incubation with agonist caused PAR1 to redistribute from the cell surface into endocytic vesicles (Figure 3B, a' and b'). Similar results were observed in cells transfected with SNX1 siRNA after 10-min agonist exposure (Figure 3B, d' and e'). We then examined whether SNX1 was necessary for delivery of PAR1 from an endosomal to a lysosomal compartment by examining PAR1 endosomal accumulation after prolonged agonist exposure. After 60 min of agonist incubation, PAR1-positive endosomes were no

Figure 2. Ectopic expression of SNX1 siRNA-resistant mutant restores agonist-induced PAR1 degradation in cells lacking endogenous SNX1. HeLa cells stably expressing FLAG-tagged PAR1 were transiently cotransfected with either nonspecific (ns)-siRNA and pcDNA (lanes 1 and 2) or with SNX1 siRNA and pcDNA, myc-SNX1, or myc-SNX1 siRNA-resistant mutant (siRNA-mut) cDNAs (lanes 3–8). Cells were then incubated in the presence or absence of 100 μ M SFLLRN for 90 min at 37°C, lysed, and immunoprecipitated with M2 anti-FLAG antibody, and the amount of PAR1 remaining was assessed by immunoblot with anti-PAR1 polyclonal antibody. Cell lysates were immunoblotted with anti-SNX1 antibody to detect both endogenous and ectopically expressed myc-SNX1 (middle panels) or actin expression to control for equal loading (bottom panels). The results (mean $\pm$ SE) shown are expressed as a percentage of PAR1 remaining compared with untreated control and are the average of three independent experiments.

longer apparent in control siRNA-transfected cells (Figure 3Bc'), consistent with activated PAR1 lysosomal sorting and degradation. By contrast, in SNX1 siRNA-transfected cells, PAR1-containing endosomes were apparent and easily detected after 60 min of agonist exposure (Figure 3Bf'). Thus, activated PAR1 accumulates in endosomes and fails to efficiently sort to a degradative pathway in cells depleted of endogenous SNX1, suggesting a critical function for SNX1 in endosome-to-lysosome trafficking of PAR1.

Lysosomal Degradation of PAR1 Is Independent of Retromer, Hrs, and Tsg101 Activity

Recent studies indicate that SNX1 functions as part of the mammalian retromer complex that mediates endosome-to-TGN retrograde trafficking of CI-MPR (Carlton *et al.*, 2004). To determine whether SNX1 regulation of PAR1 trafficking involves retromer activity, we used siRNA to deplete cells of endogenous Vps26, a protein subunit important for maintaining retromer activity. PAR1-expressing HeLa cells were transfected with control or Vps26 siRNA, and the effects on PAR1 degradation were assessed. In Vps26 siRNA-treated cells, the amounts of Vps26 and Vps35, a core retromer subunit protein, were substantially reduced compared with control siRNA-treated cells (Figure 4A), whereas endogenous SNX1 and SNX2 expression were unaffected (Figure 4C, bottom panels). In both control and Vps26 siRNA-treated cells, agonist induced a similar ~50–60% degradation of PAR1 protein (Figure 4A), suggesting that Vps26 is not essential for activated PAR1 degradation. To confirm that PAR1 degradation is due to lysosomal sorting in Vps26 knockdown cells, we examined activated PAR1 colocalization with the lysosomal-associated membrane protein-1 (LAMP1) in the presence of leupeptin, a classic inhibitor of lysosomal proteases. Activated PAR1 accumulated in vesicles in the presence of leupeptin after 60 min of agonist exposure and extensively colocalized with LAMP1 in both control and Vps26 siRNA-treated cells (Figure 4B), indicat-

ing that PAR1 is targeted to lysosomes under these conditions. In the absence of leupeptin, LAMP1-positive vesicles containing PAR1 were not apparent, consistent with lysosomal sorting and degradation of activated receptor as we previously reported (Trejo and Coughlin, 1999). To ensure that depletion of Vps26 by siRNA disrupted retromer function in retrograde trafficking, we examined the stability of CI-MPR using conditions that we, and others, have recently reported (Arighi *et al.*, 2004; Griffin *et al.*, 2005). HeLa cells transfected with control or Vps26 siRNA were treated with or without cycloheximide, an inhibitor of protein synthesis, and the amount of CI-MPR protein remaining was then determined using immunoblot analysis. In Vps26 siRNA-treated cells, the amount of CI-MPR protein was reduced by ~50% compared with control siRNA-treated cells (Figure 4C). These findings suggest that in Vps26-depleted cells retromer fails to retrieve CI-MPR from endosomes resulting in lysosomal sorting and degradation of CI-MPR, consistent with recently reported studies (Arighi *et al.*, 2004; Seaman, 2004). Together, these studies suggest that SNX1 functions independent of retromer to mediate lysosomal sorting and degradation of activated PAR1.

Hrs associates with Tsg101 and regulates endosome-to-lysosome sorting of EGFR (Bishop *et al.*, 2002; Lu *et al.*, 2003). Hrs has also been shown to directly interact with SNX1 (Chin *et al.*, 2001), raising the possibility that Hrs and Tsg101 might function in lysosomal sorting of PAR1. To assess the function of these proteins in PAR1 trafficking, we depleted HeLa cells of endogenous Hrs and Tsg101 proteins using siRNA and assessed PAR1 degradation. The expression of endogenous Hrs and Tsg101 proteins was virtually abolished after incubation with their specific siRNAs compared with control siRNA-treated cells (Figure 5A, middle panels). However, Tsg101 siRNA also caused partial degradation of Hrs protein, although the mechanism by which this occurs is unknown. In cells lacking either Hrs or Tsg101 proteins, agonist induced a significant ~60–70% degradation of PAR1

Figure 3. SNX1 is not essential for agonist-induced PAR1 internalization. (A) PAR1-expressing HeLa cells were transiently transfected with nonspecific (ns) or SNX1 siRNA and then incubated in the absence or presence of 100 μ M SFLLRN for various times at 37°C. Cells were then fixed, and the amount of receptor remaining on the cell surface was measured by ELISA. The initial level of PAR1 expressed on the cell surface before incubation at 37°C (0 min) was similar for each transfection condition. These results (mean $\pm$ SD) are expressed as a fraction of M1 anti-FLAG antibody (antibody) bound compared with total antibody bound to untreated control cells and are representative of three independent experiments. (B) PAR1-expressing HeLa cells were either left untreated (Ctrl) or incubated with 100 μ M SFLLRN for 10 min or 60 min at 37°C. Cells were fixed and immunostained for PAR1 and imaged by confocal microscopy. The imaged cells are representative of many cells examined in at least three independent experiments. Scale bar, 10 μ m. (C) Immunoblot analysis of lysates transfected with nonspecific (ns) or SNX1 siRNA confirms the loss of endogenous SNX1 expression (left). Immunofluorescence microscopy of HeLa cells transfected with nonspecific (ns) or SNX1 siRNA is consistent with the loss of endogenous SNX1 (right). The imaged cells are representative of many cells examined in separate experiments. Scale bar, 10 μ m.

protein that was similar to that observed in control siRNA-treated cells (Figure 5A), indicating that neither Hrs nor Tsg101 is essential for degradation of PAR1. To exclude the possibility of aberrant PAR1 degradation by proteases in a nonlysosomal compartment formed in Hrs and Tsg101 knockdown cells, we examined PAR1 colocalization with LAMP1 in the presence of leupeptin. In all cases, activated PAR1 accumulated in vesicles and showed marked colocalization with LAMP1 in the presence of leupeptin, whereas in the absence of protease inhibitor, receptor-containing vesicles were no longer detectable (Figure 5B). These results suggest that activated PAR1 is targeted to lysosomes and degraded in cells lacking endogenous Hrs and Tsg101. In

contrast to PAR1, however, activated EGFR degradation was significantly inhibited in the same cells depleted of Hrs and Tsg101 proteins (Figure 5C), consistent with previous studies showing a requirement for Hrs and Tsg101 in EGFR lysosomal sorting and degradation (Lu *et al.*, 2003). These data strongly suggest that SNX1 mediates sorting of PAR1 to a lysosomal degradative pathway that is independent of Hrs and Tsg101 proteins.

SNX2 Regulates Lysosomal Sorting of PAR1, But Is Not Essential for This Process

SNX1 and SNX2 are highly homologous and display functional redundancy in certain cellular processes (Schwarz *et al.*, 2002), suggesting that SNX2 might be involved in endosome-to-lysosome sorting of PAR1. Toward determining the function of SNX2 in PAR1 trafficking, we initially assessed the effect of SNX2 overexpression on agonist-induced PAR1 degradation. HeLa cells transiently cotransfected with FLAG-tagged PAR1 and either SNX1 or pcDNA showed a significant $\sim$ 50% degradation of PAR1 protein after exposure to agonist (Figure 6A), consistent with our previously published studies (Wang *et al.*, 2002). In striking contrast, however, agonist-induced degradation of PAR1 was markedly inhibited in cells coexpressing SNX2 (Figure 6A, lanes 3–4), only 7% of receptors were degraded after 90-min incubation with agonist. To determine whether SNX2 was necessary for activated PAR1 degradation, we used siRNA to deplete cells of endogenous SNX2 protein. In cells lacking endogenous SNX2, agonist induced a significant $\sim$ 60% decrease in PAR1 protein that was comparable to that observed in control siRNA-treated cells (Figure 6B), indicating that SNX2 is not essential for sorting activated PAR1 to a degradative pathway. These findings suggest that SNX2 is capable of regulating PAR1 trafficking, but that it is not required for this process.

To identify the domain(s) that specify the distinct functions of SNX1 and SNX2 in regulation of PAR1 trafficking, we generated chimeras in which the N- or C-terminal domains of these proteins were exchanged (Figure 7A). HeLa cells transiently cotransfected with FLAG-tagged PAR1 and SNX1, SNX21 or SNX1/2 chimeras were incubated in the absence or presence of agonist, and the effect on receptor degradation was then assessed by immunoblot. Agonist induced a comparable $\sim$ 60–70% decrease in PAR1 protein in cells cotransfected with either wild-type SNX1, S1N-S2PXC1, or S1NPX-S2C chimeras (Figure 7B, lanes 3 and 4, and 9–12), suggesting that neither the SNX2 PX domain nor C-terminus is sufficient to block PAR1 degradation. By contrast, activated PAR1 degradation was significantly inhibited in cells coexpressing S2N-S1PXC or S2NPX-S1C chimeras containing either the SNX2 N-terminus or N-terminal/PX domain (Figure 7B, lanes 5–8), similar to that observed with wild-type SNX2 (Figure 7B, lanes 1 and 2). Together, these findings suggest that the amino-terminal domain of SNX2 specifies its ability to inhibit activated PAR1 sorting to a lysosomal degradative pathway.

Regulation of PAR1 Lysosomal Degradation by SNX2 Involves Disruption of Endogenous SNX1 Endosomal Localization

SNX1 and SNX2 are capable of forming heterodimeric complexes *in vitro* and *in vivo* (Haft *et al.*, 1998; Wang *et al.*, 2002; Zhong *et al.*, 2002), raising the possibility that SNX2 might regulate activated PAR1 sorting via interaction with endogenous SNX1. We therefore examined whether ectopic expression of SNX2 affected endogenous SNX1 subcellular localization using confocal microscopy. In untransfected

Figure 4. Agonist-induced lysosomal degradation of PAR1 in cells depleted of retromer. (A) PAR1-expressing HeLa cells were transiently transfected with nonspecific (ns) or Vps26 siRNA and then incubated with or without 100 μ M SFLLRN agonist peptide for 90 min at 37°C. Cell lysates were immunoprecipitated with M2 anti-FLAG antibody, and the amount of PAR1 remaining was detected by immunoblot. To confirm depletion of Vps26 and Vps35 subunits by siRNA, cell lysates were immunoblotted with anti-Vps26 and anti-Vps35 antibodies, respectively. Membranes were stripped and reprobed with an anti-actin antibody to control for equal loading. The data (mean $\pm$ SE) in the bar graph are expressed as a percentage of PAR1 remaining compared with untreated control cells. Data were determined for each transfection condition and represent the average of three separate experiments. (B) PAR1-expressing HeLa cells transiently transfected with nonspecific (ns) or Vps26 siRNA were preincubated in the absence or presence of 2 mM leupeptin for 1 h and then stimulated with 100 μ M SFLLRN for 60 min at 37°C. Cells were fixed and coimmunostained for PAR1 (green) and LAMP1 (red) and imaged by confocal microscopy. Colocalization of PAR1 and LAMP1 is revealed by the yellow color in the merged image (arrowheads). The insets are magnifications of boxed areas. Scale bar, 10 μ m. The imaged cells are representative of many cells examined in three different experiments. (C) PAR1-expressing HeLa cells transfected with either nonspecific (ns) or Vps26 siRNA were incubated in the absence or presence of 10 μ M cycloheximide diluted in serum-free DMEM for 18 h and lysed, and the amount of CI-MPR remaining was determined by immunoblot analysis. Membranes were stripped and reprobed for actin expression to control for equal loading. The expression of SNX1, SNX2, and Vps26 was assessed in the same cell lysates by immunoblot analysis. The data (mean $\pm$ SE) shown in the bar graph represent the percentage of CI-MPR remaining compared with noncycloheximide-treated cells for each transfection condition after normalization to total actin expression. The data represent the average of three separate experiments.

HeLa cells, endogenous SNX1 localized primarily to endosomal vesicles, whereas in cells overexpressing wild-type myc-SNX2 endosomal localization of endogenous SNX1 was severely disrupted (Figure 8A, arrow). Immunoblot analysis demonstrates a similar amount of endogenous SNX1 in myc-SNX2 and vector-transfected cells (Figure 8B), suggesting that endogenous SNX1 is likely mislocalized, but not degraded in cells overexpressing SNX2. The expression of chimeras containing the SNX2 N-terminal domain or N-terminal/PX domain also displaced endogenous SNX1, albeit less effectively than wild-type SNX2 (Figure 8C, a'–d'). In contrast, overexpression of wild-type SNX2 failed to affect endosomal location of endogenous SNX2 (Figure 8C, e' and f'). These findings suggest that SNX2 might indirectly regulate PAR1 lysosomal sorting by disrupting endosomal localization of endogenous SNX1.

To determine whether targeting of SNX2 to endosomal vesicles is required for inhibiting PAR1 degradation and for disruption of endogenous SNX1 localization, we examined

the effect of a previously described SNX2 Δ RRF mutant that fails to localize to early endosomes (Gullapalli *et al.*, 2004). The SNX2 PX domain contains highly conserved R¹⁸²RF¹⁸⁴ residues that are critical for PtdIns(3)P binding and endosomal localization (Worby and Dixon, 2002; Zhong *et al.*, 2002). Consistent with our findings described above, agonist-induced PAR1 degradation was markedly inhibited in cells overexpressing wild-type SNX2 compared with vector-transfected cells (Figure 9A, lanes 1–4). In contrast, coexpression of SNX2 Δ RRF mutant failed to inhibit activated PAR1 degradation (Figure 9A, lanes 5 and 6). Moreover, overexpression of SNX2 Δ RRF mutant failed to disrupt endosomal localization of endogenous SNX1 compared with wild-type SNX2 (Figure 9B, a'–f'), suggesting that targeting of SNX2 to endosomal membranes is critical for disrupting localization of endogenous SNX1. Taken together these findings provide further evidence that endosomal localization of endogenous SNX1 is important for sorting activated PAR1 to a lysosomal degradative pathway.

Figure 5. Hrs and Tsg101 are not required for lysosomal degradation of PAR1. (A) PAR1-expressing HeLa cells transiently transfected with nonspecific (ns), Hrs-, or Tsg101-specific siRNAs were incubated in the absence or presence of 100 μ M SFLLRN for 90 min at 37°C, and the amount of PAR1 protein degradation was then assessed as described above. Cell lysates were immunoblotted with anti-Hrs or -Tsg101 antibodies to confirm loss of these proteins in siRNA-transfected cells or for actin expression to control for equal loading. The results (mean $\pm$ SE) shown in the graph are represented as a percentage of PAR1 remaining compared with untreated control cells for each transfection condition and are an average of at least three experiments. (B) PAR1-expressing HeLa cells were treated with or without 2 mM leupeptin and then incubated with 100 μ M SFLLRN as described above. Cells were fixed and coimmunostained for PAR1 (green) and LAMP1 (red) and imaged by confocal microscopy. Colocalization of PAR1 and LAMP1 is revealed by the yellow color in the merged image (arrowheads). The insets are magnifications of boxed areas. Scale bar, 10 μ m. (C) Serum-starved HeLa cells transfected with Tsg101, Hrs, or nonspecific (ns) siRNAs were incubated in the absence or presence of 100 ng/ml EGF ligand for 30 min at 37°C, and the amount of receptor protein remaining was then determined by immunoblot analysis using anti-EGFR antibodies. Cell lysates run in parallel were immunoblotted for Hrs and Tsg101 to confirm loss of protein in siRNA-transfected cells or for actin expression to control for equal loading. Similar results were observed in three separate experiments.

DISCUSSION

In the present study, we have defined for the first time an essential role for endogenous SNX1 in endosome-to-lysosome sorting of a cell surface receptor in mammalian cells. We specifically show that SNX1 is necessary for sorting activated PAR1 to a lysosomal degradative pathway in HeLa cells. Interestingly, PAR1 lysosomal degradation does not require retromer activity, suggesting that SNX1 has distinct functions in endosome-to-lysosome sorting and retrograde trafficking. Hrs and Tsg101 are also not essential for activated PAR1 degradation, indicating that multiple distinct pathways exist for lysosomal sorting of cell surface receptors in mammalian cells. In contrast to SNX1, however, SNX2 is not required for PAR1 degradation, but can regulate PAR1 lysosomal sorting through its ability to disrupt endosomal localization of endogenous SNX1. Analysis of chimeric SNX1/2 proteins suggest that regulation of PAR1 sorting by SNX2 is specified by the amino-terminal domain, the least conserved region of these proteins with only ~26% identity at the amino acid level. Together these studies strongly suggest an essential function for endogenous SNX1 in sorting PAR1 to a distinct lysosomal degradative pathway that is independent of retromer, Hrs, and Tsg101 functions.

A recent study using RNAi and HeLa cells suggests that SNX1 has retained a conserved function in endosome-to-TGN retrograde trafficking in yeast and mammals (Carlton *et al.*, 2004). We also recently demonstrated that endogenous SNX1 is not essential for lysosomal sorting of EGFR in HeLa cells (Gullapalli *et al.*, 2004). These studies suggested that SNX1 might not function in endosome-to-lysosome sorting of cell surface receptors in mammalian cells. However, we report here that SNX1 is essential for sorting PAR1, a GPCR, to a lysosomal degradative pathway. Depletion of endogenous SNX1 by siRNA markedly inhibited activated PAR1 degradation, whereas receptor internalization was unaffected. Moreover, expression of a SNX1 siRNA-resistant mutant protein restored the ability of agonist to promote PAR1 degradation in cells lacking endogenous SNX1, strongly suggesting that SNX1 is required for lysosomal sorting of activated PAR1. We previously showed that EGFR degradation occurs normally in these same siRNA-transfected cells depleted of endogenous SNX1 (Gullapalli *et al.*, 2004), excluding the possibility of global defects in lysosomal degradation. Moreover, SNX1-dependent lysosomal sorting of PAR1 is consistent with our previous work in which we showed that SNX1 associates with PAR1 and that a SNX1

Figure 6. SNX2 can regulate activated PAR1 degradation, but is not required for this process. (A) HeLa cells transiently cotransfected with FLAG-tagged PAR1 and either pcDNA, myc-SNX2 or myc-SNX1 were incubated in the absence or presence of 100 μ M SFLLRN for 90 min at 37°C. Cells were lysed and immunoprecipitated, and the amount of PAR1 remaining was determined by immunoblot. Cell lysates run in parallel were immunoblotted with anti-myc antibody to confirm expression of SNX1 and SNX2 (bottom panels). The data (mean $\pm$ SE) shown in the bar graph are represented as a percentage of PAR1 remaining compared with untreated control cells and are an average of three separate experiments. (B) HeLa cells stably expressing PAR1 were transfected with nonspecific (ns) or SNX2 siRNA and treated with 100 μ M SFLLRN for 90 min at 37°C, and the amount of PAR1 remaining was determined as described above. Cell lysates were immunoblotted with SNX2-specific antibodies to confirm loss of protein or anti-actin antibodies to control for equal loading. The results (mean $\pm$ SE) shown in the bar graph were derived as described above.

deletion mutant blocked degradation of activated PAR1 (Wang *et al.*, 2002). These data strongly support a role for SNX1 in endosome-to-lysosome sorting of a cell surface GPCR in mammalian cells. Interestingly, SNX1 has recently been shown to directly interact with the cytoplasmic carboxyl tail of at least 10 other distinct GPCRs in vitro (Heydorn *et al.*, 2004). However, whether SNX1 regulates intracellular trafficking of these receptors remains to be determined.

These studies also indicate that SNX1 is capable of regulating distinct intracellular trafficking processes in mammalian cells. Several recent studies showed that retromer (Vps26 and Vps35 subunits) and SNX1 function in retrograde trafficking of CI-MPR in HeLa cells (Arighi *et al.*, 2004;

Carlton *et al.*, 2004; Seaman, 2004), analogous to regulation of Vps10p receptor by the yeast retromer complex (Horazdovsky *et al.*, 1997; Nothwehr and Hindes, 1997; Seaman *et al.*, 1998). In the studies reported here, we demonstrate that activated PAR1 is efficiently degraded in cells lacking Vps26 and Vps35 proteins, but not in cells depleted of SNX1 protein, indicating that SNX1, and not retromer, is important for PAR1 degradation. CI-MPR trafficking was perturbed in cells depleted of Vps26 and Vps35, indicating that retromer activity was indeed disrupted in these cells. The ability of SNX1 and SNX2 to dimerize and the survival of *Snx1*^{-/-} and *Snx2*^{-/-} null mice, but not the doubly deficient *Snx1*^{-/-};*Snx2*^{-/-} mice (Schwarz *et al.*, 2002), suggests that the proteins may act together as a heterodimeric complex or separately as a homodimer. Our studies indicate that SNX2 is not essential for lysosomal sorting of PAR1, but it can regulate PAR1 degradation by disrupting endosomal localization of endogenous SNX1. The effect of SNX2 on endogenous SNX1 localization is unlikely to involve generalized disruption of the endocytic sorting machinery because overexpression of SNX2 does not induce extensive tubulation or affect EGFR degradation (Gullapalli *et al.*, 2004; Carlton *et al.*, 2005). Thus, lysosomal sorting of PAR1 appears to be regulated primarily by a homodimeric SNX1:SNX1 complex, whereas SNX1:SNX2 heterodimeric complexes may have other important functions in mammalian cells. Indeed, our recent work with mice provides the first genetic evidence for a mammalian retromer complex containing SNX1 and SNX2, which has an essential role in embryonic development that does not involve regulation of CI-MPR trafficking (Griffin *et al.*, 2005). In addition, the mammalian retromer Vps35-Vps29-Vps26 subcomplex has been shown to regulate transcytosis of the pIgR-pIgA receptor in polarized epithelial cells independent of SNX1 and SNX2 (Verges *et al.*, 2004). Together these studies indicate that SNX1 and retromer have evolved considerably from yeast and have acquired a variety of distinct functions in regulation of intracellular trafficking in mammalian cells.

Our studies further indicate that SNX1 mediates sorting of activated PAR1 to a distinct lysosomal sorting pathway that is independent of Hrs and Tsg101. The best-characterized route from endosomes to lysosomes involves the formation of early endosomal tubular extensions that mature into multivesicular bodies/late endosomes that then fuse with lysosomes. Sorting of EGFR through this pathway involves ubiquitin-dependent interaction with Hrs/clathrin and Tsg101 (Raiborg *et al.*, 2001; Bishop *et al.*, 2002; Lu *et al.*, 2003). Our findings indicate that neither Hrs nor Tsg101 is essential for activated PAR1 lysosomal degradation, whereas in the same cells depleted of Hrs or Tsg101, degradation of EGFR is significantly inhibited. Moreover, SNX1 is required for lysosomal degradation of PAR1, but is not involved in EGFR degradation. In addition to PAR1, the delta opioid G protein-coupled receptor (DOR) does not require Tsg101 for agonist-induced lysosomal degradation (Hislop *et al.*, 2004). This study also reported that lysosomal sorting of DOR is dependent on Hrs. However, in this case, overexpression or RNAi-mediated knockdown of Hrs appears to only partially inhibit DOR degradation. Moreover, Hrs is involved in lysosomal sorting of ubiquitinated membrane proteins, and agonist-induced lysosomal degradation of DOR occurs independent of ubiquitin modification (Tanowitz and Von Zastrow, 2002). Thus, DOR may follow a lysosomal sorting pathway similar to PAR1. SNX1 has been shown to directly bind to the DOR cytoplasmic tail in vitro (Heydorn *et al.*, 2004); however, whether SNX1 is required for lysosomal degradation of DOR remains to be determined. Together,

Figure 7. Expression of SNX2 amino-terminal domain chimeras inhibit activated PAR1 degradation. (A) Chimeras of SNX1 and SNX2 were generated by exchanging either the N-terminal or C-terminal domain. SNX1 bearing the N-terminus of SNX2 is designated "S2N-S1PXC", and SNX2 containing the SNX1 N-terminal domain is designated "S1N-S2PXC", whereas SNX1 containing the SNX2 C-terminus is termed "S1NPX-S2C", and SNX2 with the SNX1 C-terminus is designated "S2NPX-S1C". (B) HeLa cells were transiently cotransfected with FLAG-tagged PAR1 and cDNAs encoding either SNX1, SNX2 or various SNX1/2 chimeras and then incubated in the absence or presence of 100 μ M SFLLRN for 90 min at 37°C. Cell lysates were immunoprecipitated with M2 anti-FLAG antibody, and remaining PAR1 was detected with anti-PAR1 polyclonal antibody. Cell lysates run in parallel were immunoblotted with anti-myc antibody to confirm expression of various sorting nexins (lower panel). The data (mean $\pm$ SE) shown in the graph are represented as a percentage of PAR1 remaining compared with untreated control cells determined for each transfection condition and are the average of at least three independent experiments.

these findings suggest that SNX1 regulates a distinct lysosomal sorting pathway that targets at least certain GPCRs for degradation independent of Hrs and Tsg101.

The mechanism by which SNX1 regulates endosome-to-lysosome sorting of PAR1 probably involves its localization to and pinching off of PAR1-positive endosomal tubules. Recent work indicates that SNX1 localization to early endosomes is mediated by two membrane binding domains, a PX domain that interacts with PtdInsP and a BAR (Bin/Amphiphysin/Rvs) domain that allows SNX1 to dimerize and

to sense membrane curvature (Carlton *et al.*, 2004). SNX1 binds to the tubular portion of early endosomes and forms oligomers that may facilitate pinching off of endosomal tubules. This process probably involves SNX1 interaction with other proteins, because tubulation induced by SNX1 *in vitro* is rather weak compared with *Drosophila* amphiphysin. In addition, the localization of SNX1 to endosomal membranes is not directly responsible for recruitment of PAR1, because we, and others, have failed to detect a direct interaction between PAR1 cytoplasmic domains and SNX1 (Heydorn *et*

Figure 8. Expression of SNX2 amino-terminal domain chimeras disrupt endogenous SNX1 endosomal localization. (A) HeLa cells transiently transfected with myc-tagged SNX2 wild-type or pcDNA were fixed and immunostained for endogenous SNX1 (left panel) and myc-tagged SNX2 (right panel). The arrow indicates the localization of endogenous SNX1 in myc-SNX2-transfected cells. Scale bar, 10 μ m. (B) Cell lysates prepared from pcDNA or myc-SNX2-transfected cells were immunoblotted for endogenous SNX1 using anti-SNX1-specific antibodies or for myc-SNX2 expression using anti-myc antibodies. (C) Cells transfected with myc-tagged S2N-S1PXC or S2NPX-S1C chimera were fixed and immunostained for endogenous SNX1 (a' and c') and myc-tagged SNX2 chimeric proteins (b' and d'), and imaged by confocal microscopy. HeLa cells transiently transfected with myc-SNX1 were fixed, immunostained for endogenous SNX2 or myc-SNX1 (e' and f', respectively). These images are representative of many cells examined in three separate experiments. Scale bar, 10 μ m.

Figure 9. A SNX2 Δ RRF mutant defective in lipid-binding fails to disrupt activated PAR1 degradation and endogenous SNX1 endosomal localization. (A) HeLa cells transiently cotransfected with FLAG-tagged PAR1 and either myc-SNX2, myc-SNX2 Δ RRF mutant, or pcDNA were incubated in the absence or presence of 100 μ M SFLLRN for 90 min at 37°C. Cell lysates were immunoprecipitated with M2 anti-FLAG antibody, and the remaining PAR1 was detected by immunoblotting with anti-PAR1 polyclonal antibody (top panels). The expression of myc-SNX2 wild-type or mutant in total cell lysates was detected with anti-myc antibody (lower panel). The results (mean $\pm$ SE) in the bar graph shown are expressed as a percentage of PAR1 remaining compared with untreated control cells determined for each transfection condition and are the average of three independent experiments. (B) HeLa cells transiently transfected with myc-SNX2 or myc-SNX2 Δ RRF mutant were fixed and coimmunostained for endogenous SNX1 or myc-tagged SNX2 and imaged by confocal microscopy. Endogenous SNX1 and myc-SNX2 wild-type or mutant localization are shown together in the merged image. These images are representative of many cells examined in at least three different experiments. Scale bar, 10 μ m.

al., 2004). Thus, other proteins associated with SNX1 tubules probably select cargo such as PAR1 for sorting from endosomes to lysosomes. One potential candidate that can mediate transport of cargo from tubular sorting endosomes to lysosomes is adaptor protein complex-3 (AP3) (Ihrke *et al.*, 2004; Peden *et al.*, 2004). AP3-dependent lysosomal sorting from tubular sorting endosomes is distinct from Hrs/Tsg101-mediated trafficking via multivesicular bodies that form by inward budding of the limiting membrane. The μ 3 subunit of AP3 binds directly to di-leucine- or tyrosine-based sorting signals within the cytoplasmic regions of transmembrane proteins. The cytoplasmic tail of PAR1 contains two tyrosine-based motifs (Yxx ϕ) and we recently demonstrated that the μ 2 subunit of AP2 binds directly to the distal tyrosine-based motif to mediate PAR1 constitutive internalization, an important process for cellular recovery of thrombin signaling (Paing *et al.*, 2006). However, whether AP3 can also bind directly to one or both of these tyrosine-based motifs to promote lysosomal sorting of PAR1 remains to be determined.

Our data suggest that SNX1 function is critical for lysosomal sorting of PAR1 in HeLa cells, a human epithelial-like cell line isolated from an adenocarcinoma. However, SNX1 lysosomal function is not solely responsible for the lethality reported for *Par1*^{-/-} null mice, because *Snx1*^{-/-} mice are normal and viable (Connolly *et al.*, 1996; Schwarz *et al.*, 2002). By contrast, mice with targeted deletions for *Par1* are 50% lethal at midgestation due to loss of PAR1 function in endothelial cells, resulting in defective blood vessel formation and subsequent bleeding that occurs independent of platelets (Connolly *et al.*, 1996; Griffin *et al.*, 2001). Platelets are not present at midgestation and PAR1 is not expressed in murine platelets. To date no mice have been generated with defects in PAR1 lysosomal sorting and degradation. Thus, perhaps *Snx1*^{-/-} mice do not share a similar phenotype of embryonic lethality with *Par1*^{-/-} mice because PAR1 lysosomal degradation is not essential during embryonic development. Alternatively, other compensatory mechanisms for PAR1 lysosomal sorting and degradation could exist in the mouse. Clearly, further analysis of PAR1 trafficking in en-

dothelial cells lacking SNX1 would help integrate our findings in HeLa cells with the existing genetic data reported for *Par1*^{-/-} and *Snx1*^{-/-} null mice.

In summary, our studies demonstrate for the first time an essential role for endogenous SNX1 in endosome-to-lysosome sorting of a cell surface receptor in mammalian cells. Moreover, endogenous SNX1 is necessary for sorting PAR1 to a distinct lysosomal degradative pathway that is independent of retromer, Hrs and Tsg101, whether PAR1 sorting to lysosomes involves transit through multivesicular bodies is not known. These findings bring important insight into how PAR1, and perhaps other GPCRs, are sorted from endosomes to lysosomes and degraded. The challenge now becomes to elucidate the mechanism by which PAR1, and perhaps other GPCRS, are recruited to the distinct SNX1-dependent lysosomal sorting degradative pathway.

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