Identification and Characterization of SNX15, a Novel Sorting Nexin Involved in Protein Trafficking*

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Sorting nexins are a family of phox homology domain containing proteins that are homologous to yeast proteins involved in protein trafficking. We have identified a novel 342-amino acid residue sorting nexin, SNX15, and a 252-amino acid splice variant, SNX15A. Unlike many sorting nexins, a SNX15 ortholog has not been identified in yeast or Caenorhabditis elegans. By Northern blot analysis, SNX15 mRNA is widely expressed. Although predicted to be a soluble protein, both endogenous and overexpressed SNX15 are found on membranes and in the cytosol. The phox homology domain of SNX15 is required for its membrane association and for association with the platelet-derived growth factor receptor. We did not detect association of SNX15 with receptors for epidermal growth factor or insulin. However, overexpression of SNX15 led to a decrease in the processing of insulin and hepatocyte growth factor receptors to their mature subunits. Immunofluorescence studies showed that SNX15 overexpression resulted in mislocalization of furin, the endoprotease responsible for cleavage of insulin and hepatocyte growth factor receptors. Based on our data and the existing findings with yeast orthologs of other sorting nexins, we propose that overexpression of SNX15 disrupts the normal trafficking of proteins from the plasma membrane to recycling endosomes or the trans-Golgi network.

Intracellular vesicle traffic in both yeast and mammals requires the function of numerous proteins that mediate multiple processes including cargo selection, vesicle budding, and fusion of vesicles with specific targets (1–3). Sorting nexins (SNXs)1 are a family of widely expressed proteins believed to be part of the complex molecular machinery required for protein trafficking (4). Mammalian SNXs are homologous to several yeast proteins (e.g. Vps5p, Mvp1p, and Gdr119p) for which there is strong genetic evidence demonstrating a role in protein trafficking (5–7). SNX1 was identified using the yeast two-hybrid system by virtue of its ability to bind to the cytoplasmic domain of the epidermal growth factor receptor (EGF) receptor (8). Subsequently, SNX1 was shown to also interact with receptors for insulin, platelet-derived growth factor (PDGF), transferrin, and leptin (4). Furthermore, SNX1 is the mammalian ortholog of Vps5p, a yeast protein that is a component of a multimeric complex (termed the “retromer complex”) involved in retrograde transport of proteins from prevacuolar endosomes to the TGN (9). We have previously described three additional sorting nexins: SNX2, SNX3, and SNX4 (4); and 10 additional sorting nexins (SNX5–14) have been deposited in GenBank™. Like SNX1, both SNX2 and SNX4 associate with various receptors. In addition, SNX1, SNX2, and SNX4 assemble into oligomeric structures (4). All 14 SNX molecules contain a phox homology (PX) domain, a conserved sequence of unknown function first identified in the p40phox and p47phox subunits of the NADPH oxidase complex (10). PX domains consist of ~100 amino acid residues, and most contain a proline-rich sequence that may represent an SH3 domain-binding motif (10). PX domains have been identified in >20 proteins, some of which are involved in protein trafficking in yeast (6, 9). Using a PX domain consensus sequence obtained from SNX1–4 to search the NCBI data base, we identified a cDNA encoding a novel SNX protein, SNX15.

EXPERIMENTAL PROCEDURES
Cloning of SNX15 cDNA—We determined a consensus sequence for the phox homology domain of SNX1, 2, 3, and 4 (GenBank™ accession numbers U53225, NP0039001, NP003786, and NP003785, respectively), using clustal W (11). We searched the NCBI data base with this consensus sequence using the BLAST algorithm and identified seven human expressed sequence tags (ESTs) corresponding to a novel sorting nexin, now designated SNX15. Five ESTs (accession numbers T65368, A351071, F11999, R17390, and AA351142) were obtained from Research Genetics (Huntsville, AL), and plasmid DNA was isolated using reagents provided by CLONTECH (Palo Alto, CA). Using appropriate vector- and sequence-specific primers we determined the nucleotide sequences of the ESTs. The sequence data revealed two groups of SNX15 EST clones. The first group of ESTs encoded a long form of the molecule, designated SNX15L. The second group of ESTs encoded a presumed splice-variant of SNX15, designated SNX15A that lacks 270 bp at the 3´ end of the cDNA. Neither group contained a 5´ start site. To obtain additional 5´ sequence including the protein start site, we carried out PCR with a 5´-10 universal cDNA library (CLONTECH) as template. Standard molecular biology techniques were used to construct full-length cDNAs. The existence of each full-length SNX15 isoform was confirmed by sequencing an additional full-length EST (accession number AF001435) and by PCR using tissue-specific cDNA libraries (CLONTECH) and isoform specific primers. All sequencing was performed using the ABI prism dye terminator cycle sequencing kit and an ABI automated sequencer, model 373A (PerkinElmer Life Sciences).

Tissue Distribution of SNX15 mRNA—Multiple tissue Northern blots containing ~2 μg of purified human poly(A) RNA and normalized for equal actin loading were obtained from CLONTECH. To obtain a
probe for Northern blot analysis, SNX15 cDNA was excised from the epitope-tagged pcDNA3.1+ plasmid construct with XhoI and NotI. The resulting fragment was gel purified using reagents supplied by Qiagen (Chatsworth, CA), and labeled with 32P using a random primed DNA labeling kit (Roche Molecular Biochemicals) and diluted in Express Hybridization Solution (CLONTECH). The probe was hybridized to protamine-saline-citrate blotted poly(A) RNA (7) for 16–18 h and washed according to the manufacturer's instructions.

Construction of Expression Vectors—Using PCR catalyzed by Klen-taq (CLONTECH), we introduced c-Myc and influenza hemagglutinin (HA) epitope tags at the 5' end of SNX15 and a C-Myc tag at the 3' end of SNX15A. In addition, using similar methods, we constructed several mutants of SNX15. ΔPX lacks the N terminus and PX domains of SNX15 (amino acids 1–135); ΔC lacks the entire C terminus (amino acids 136–342), whereas the ΔESP domain lacks the entire ESP domain (amino acids 222–308) plus the remaining 34 C-terminal amino acids of SNX15. Epitope-tagged PCR products were ligated into pcDNA3.1 (+) expression vector (Invitrogen, Carlsbad, CA). The sequences of primers used to construct SNX15, SNX15A, and the SNX15 mutant cDNAs are available upon request. pCIS2 expression vector conferring resistance to chloramphenicol (CLONTECH) and IP Labs software (Scanalytics Inc., Fairfax, VA). Adobe Photoshop (Adobe Systems Inc., Mountain View, CA) was used to process the images obtained from the Western blot images.

Immunofluorescence—Transfected cells were fixed in 2% formalin (v/v) in PBS, incubated with the primary antibody in PBS, 1% fetal bovine serum (v/v), and 0.075% saponin (w/v) for 1.5 h, washed, and labeled with secondary antibody (the same buffer) for 40 min (20). Cells were then viewed in a Zeiss Axioshot inverted microscope (Carl Zeiss Inc., Thornwood, NY). Images were captured with a PentaMAX camera (Princeton Instruments Inc., Trenton, NJ) and IP Labs software (Scanalytics Inc., Fairfax, VA).

Coimmunoprecipitation Experiments—Transfected COS7 cells were washed in ice-cold PBS and scraped into 250 μl of cold lysis buffer/well (50 mM Tris-HCl, pH 7.5, 0.5% (v/v) Triton X-100, 0.3 mM NaCl, Plus protease inhibitor tablet) (Roche Molecular Biochemicals). The cells were solubilized on ice for 30 min and centrifuged at 14,000 × g for 20 min at 4 °C to remove insoluble debris. Proteins were detected by standard immunoblotting procedures or by immunoprecipitation of extracts (400 μl) with specific antibodies (1:100 dilute; see “Antibodies”). Immune complexes were sedimented with 30 μl of Ultralink immobilized protein A or protein G (Pierce). The beads were washed and boiled in Laemmli sample buffer. Proteins were separated by SDS-PAGE (7.5 or 12.5%). Proteins were then transferred to nitrocellulose (21), and epitope-tagged proteins were detected by Western blot followed by chemiluminescence using ECL reagents (Amersham Pharmacia Biotech).

Subcellular Distribution of SNX15—COS7 cells (2–3 × 105 cells/well) were transfected with Myc-tagged SNX15 or mutant forms of SNX15 (see above), washed with ice-cold PBS, and lysed in 250 μl of ice-cold homogenization buffer (10 mM HEPES, pH 7.4, 0.25 mM sucrose, 1 mM ethylenediaminetetraacetate, 0.5 mM MgCl2, and protease inhibitor tablet) (Roche Molecular Biochemicals) by 15 passages through a 25-gauge needle. To obtain total membrane and cytosolic fractions, the lysates were centrifuged at 240,000 × g for 45 min at 4 °C in a Beckman TL120.2 rotor (Beckman Instruments, Palo Alto, CA). Following centrifugation, the pellet was resuspended in 250 μl of homogenization buffer using eight strokes with a Teflon pestle and a Potter homogenizer. Aliquots of the total lysates, as well as cytosolic and membrane fractions, were solubilized in Laemmli buffer and separated on a 12% (w/v) polyacrylamide-SDS gel. Epitope-tagged molecules were detected by immunoblotting with anti-Myc antibody and ECL reagents obtained from Amersham Pharmacia Biotech.

Transient Expression of Sorting Nexins and Receptors—COS7 cells (2–3 × 105 cells/well) were transfected with 2 μg of DNA-LipofectAMINE complexes in serum free DMEM and then incubated for the times indicated in figure legends, media were removed, cells were washed twice with ice-cold PBS and fixed on liquid nitrogen. Cells were scraped, and lysates were prepared by solubilization in ice-cold Kahane buffer + octylglucoside (20 mM octylglucoside, 0.5% Triton X-100, 0.3 mM NaCl, 0.025 mM sodium phosphate, pH 7.4, and 0.02% Na2S) plus Complete protease inhibitor (Roche Molecular Biochemicals). Cell lysates were spun at 14,000 rpm for 20 min, and the supernatants were then precleared by incubating with 30 μl of protein A-agarose (Pierce) for 3 h on a rotating wheel at 4 °C. Samples were subsequently centrifuged (14,000 rpm for 1 min), and then the supernatants were incubated overnight at 4 °C with specific antibody (see figure legends). Antibody complexes were sedimented with 30 μl of protein A-Sepharose (Pierce) for 3 h at 4 °C on a rotating wheel. Protein A pellets were washed twice with Kahane buffer + octylglucoside, once with Kahane buffer, and once with Tris-buffered saline. Gel samples were prepared (see above), and the proteins were separated by SDS gel electrophoresis and transferred to nitrocellulose. The blots were directly exposed to film and scanned for intensity using a Phosphorlmager (Molec-ular Dynamics, Sunnyvale, CA).
mCi/ml; PerkinElmer Life Sciences) for 4 h at 4 °C. The media were removed; the cells were washed again and then incubated in fresh binding buffer at 37 °C. After various times at 37 °C, the media were removed, incubated with 12% (w/v) trichloroacetic acid overnight at 4 °C, and then spun at (16,000 g for 15 min) to separate the trichloroacetic acid-insoluble counts (intact 125I-PDGF) from the trichloroacetic acid soluble counts (degraded 125I-PDGF) present in the medium. 125I-PDGF present at the cell surface at each time was determined by treating the cells twice with DMEM plus 25 mM HEPES (pH 3.0) for 5 min at 4 °C to remove the cell surface counts. Preliminary experiments showed that this acid wash procedure removes >95% of the surface bound counts. Lastly, the cells were incubated for 30 min at 4 °C in PBS containing 1% (v/v) Triton X-100 and 1 mg/ml bovine serum albumin to collect the remaining cell associated counts (22), which were then treated with trichloroacetic acid (see above). Each fraction was collected and counted (Autogamma Cobra II, Packard Inc., Downers Grove, IL).

**Phylogenetic Analysis of the Sorting Nexins—** PX domain sequences were identified by an eight-round PSI-BLAST (23) search with the PX domain of SNX4 as the seed, and an initial stringency of 1e^-26, keeping known PX domain containing proteins and high scoring unknowns in each round. The PX-containing regions of most available human and Saccharomyces cerevisiae proteins were aligned initially by clustal W (11), followed by manual editing, removal of fragmentary and poorly alignable sequences, and trimming of the poorly conserved ends of the alignment. The resulting curated alignment of the ~100-amino acid residue core of the PX domain sequences contained about 80 potentially informative positions for phylogenetic analysis. This manually curated multiple alignment of PX domain core region sequences was used for phylogenetic analysis, using a variety of methods, including maximum parsimony (24), neighbor joining with both PAM-based (25) and Poisson-corrected distance measures in the PHYLO_WIN program (26) and both the Fitch-Margoliash (27) and neighbor joining in the Phyllip program (28). Clustering based on sequence similarity rather than phylogenetics was performed by the unweighted pair group method using arithmetic averages algorithm.2

RESULTS

**Identification and Tissue Distribution of SNX15—** In an effort to identify additional members of the sorting nexin family, we compared the predicted amino acid sequences of SNX1–4...
The PX domain was the only region of homology shared by all four molecules. A consensus sequence for the PX domain was created using the clustal W algorithm (11) and then used to search the dbEST data base of the NCBI. We identified multiple ESTs encoding portions of a new sorting nexin, SNX15. We determined the complete sequence of two ESTs, both of which lacked the 5'-start site. Clone 1 (accession number T65368) was 1779 bp, and clone 2 (accession number AA351071) was 1543 bp. Where the two clones overlapped, their sequences were identical except that clone 2 had an in-frame deletion of 258 bp. To obtain the 5' start site, we performed nested PCR on brain, lung, and liver cDNA libraries. The longest amplified cDNA fragment contained 358 bp of additional 5' sequence. We then searched the nonredundant data base of NCBI with full-length SNX15 and identified an independent clone (AF001435) with identical sequence at the 5' end. This clone (AF001435) had been identified previously as an expressed gene on chromosome 11q13 during the course of positional cloning of the gene for multiple endocrine neoplasia type 1 (29). The coding sequence for the long form of SNX15 is 2061 bp, and the mRNA appears to contain at least 250 bp upstream from the putative start site and 780 bp of 3'-untranslated region including a poly(A) tail. The protein is predicted to contain 342 amino acid residues and to have a pI of 4.95 (Fig. 1A). SNX15 has 12 amino acid residues at its N terminus containing poly(A) and normalized for equal actin loading were hybridized with a [32P]-labeled human SNX15 cDNA probe. The filters were washed and exposed to film as described under “Experimental Procedures.”

Both isoforms of SNX15 lack predicted transmembrane domains or leader sequences and are expected to be soluble proteins. However, when we examined the subcellular distribution of recombinant Myc-SNX15 and Myc-SNX15A in COS7 cells, we found both isoforms in particulate, as well as cytosolic fractions (Fig. 2A). Likewise, fractions prepared from nontransfected COS7 cells and probed with a polyclonal antibody to SNX15, detected SNX15 (~51 kDa) in both particulate and cytosolic fractions (Fig. 2B), whereas the short isoform, SNX15A (~37 kDa), was not detected in either fraction.

Northern blots of numerous human tissue libraries probed with a SNX15 cDNA probe detected a broad band of ~2 kilobases. The highest levels of SNX15 mRNA expression were detected in skeletal muscle, heart, brain, kidney, spleen, thymus, and small intestine (Fig. 3). Because the conditions used for northern analyses did not allow us to discriminate messages that differed by only a few hundred nucleotides, we screened eight cDNA tissue libraries by PCR using sequence-specific primers to determine the expression pattern of SNX15 and SNX15A. Amplicons representing the long isoform of SNX15 were observed in lung, liver, skeletal muscle, prostate, pancreas, and adult and fetal brain. The short isoform, SNX15A, was present in adult brain, liver, and placenta (data not shown).

Association of SNX15 with Other Sorting Nexins—Previously, we showed that SNX1, SNX2, and SNX4 form homo- and hetero-oligomeric complexes (4). To determine whether SNX15 could associate with itself or other SNX molecules, COS7 cells were transiently transfected with HA-tagged SNX15 alone or in combination with Myc-tagged SNX1, SNX2, SNX3, SNX4, or SNX15. Total cell lysates were immunoblotted with anti-Myc antibody. A similar experiment was performed using cells transiently transfected with Myc-SNX15 and HA-SNX4. A, lanes 1–6, total cell lysates were immunoprecipitated with anti-HA antibody, separated by SDS-PAGE, and analyzed by Western blotting with an anti-Myc antibody to detect communoprecipitated SNX molecules. B, lanes 1–6, The same blot was reprobed with anti-HA antibody to determine the amount of HA-SNX15 (~51 kDa) expressed and immunoprecipitated in each sample. C, lanes 1–6, total cell lysates were analyzed by immunoblotting with an anti-Myc antibody to determine the expression levels of each Myc-tagged SNX. Because the SNX proteins vary in molecular mass, the blots were cut and the Myc-positive bands are shown. A similar experiment was performed using cells transiently transfected with Myc-SNX15 and HA-SNX4. A, lane 7, same extract immunoblotted with an anti-HA antibody. B, lane 7, same extract immunoblotted with an anti-HA antibody. C, lane 7, result of reprobing of the blot in A with an anti-Myc antibody. These experiments were repeated three times with similar results.
antibody to show the expression level of the various recombinant Myc-tagged SNXs (Fig. 4C). Cell extracts were then immunoprecipitated with anti-HA antibody and immunoblotted with an anti-Myc antibody. Similar amounts of HA-SNX15 were expressed and immunoprecipitated in each sample (Fig. 4B). Reprobing the same precipitates with an anti-Myc antibody showed that HA-SNX15 associates with itself (Fig. 4A, lane 5), and with Myc-SNX1 (lane 1), Myc-SNX2 (lane 2), and small amounts of Myc-SNX4 (lane 4). In contrast, HA-SNX15 did not associate detectably with Myc-SNX3 (Fig. 4A, lane 3), although Myc-SNX3 was readily expressed (Fig. 4C, lane 3). Because only small amounts of Myc-SNX4 were coimmunoprecipitated with HA-SNX15, we designed confirmatory experiments using a similar approach but switching the epitope tags (Fig. 4, A–C, lane 7). HA-SNX4 was coexpressed with Myc-SNX15, cell lysates were immunoprecipitated with anti-Myc antibody, and the immune complexes were immunoblotted with an anti-HA antibody. A strong band corresponding to HA-SNX4 was coimmunoprecipitated with Myc-SNX15 (Fig. 4A, lane 7).

To determine whether the PX or ESP domains of SNX15 are required for assembly into oligomers, we coexpressed several SNX15 mutants in combination with SNX15. SNX15A is a naturally occurring splice variant lacking the majority of the ESP domain (amino acids 222–308). ΔPX lacks the N terminus and PX domain of SNX15 (amino acids 1–135); ΔESP lacks the entire ESP domain plus 34 remaining C-terminal amino acids (222–342), whereas ΔC lacks the entire C-terminal region of the molecule (amino acid 136–342). SNX15A, ΔESP, and ΔC SNX15 were well expressed (Fig. 5A, middle panel) and associated with SNX15 (Fig. 5A, top panel, lanes 2–4). In contrast, ΔPX did not associate with SNX15 (top panel, lane 5) or with SNX1, SNX2, or SNX4 (data not shown). Furthermore, ΔPX was found almost exclusively in the cytosolic fraction (Fig. 5B), whereas ΔESP and ΔC SNX15 were distributed to both particulate and cytosolic fractions like SNX15 and SNX15A (see Fig. 2).

**Fig. 5.** The PX domain of SNX15 is required for self-association. A, COS7 cells were transiently cotransfected with cDNAs encoding HA-SNX15 and various forms of Myc-SNX15: empty vector (lane 1); Myc-SNX15A (lane 2); ΔESP, Myc-tagged SNX15 lacking amino acids 222–342 (lane 3); ΔC, Myc-tagged SNX15 lacking amino acids 136–342 (lane 4); and ΔPX, Myc-tagged SNX15 lacking amino acids 1–135 (lane 5). Total cell lysates were immunoprecipitated (IP) with anti-Myc antibody followed by immunoblotting with anti-HA antibody to detect coimmunoprecipitated HA-SNX15 (upper panel). The levels of expression of Myc-SNX15A and the various Myc-SNX15 mutants are show in the bottom panel. SNX15A is seen as a doublet. It is presently unclear whether this is due to post-translational processing or proteolysis of the molecule. This experiment was repeated three times with similar results. B, COS7 cells were transfected with the indicated SNX15 mutant cDNAs and 24–30 h later total cell lysates (T), particulate (P), and cytosolic fractions (C) were prepared as described under “Experimental Procedures.” The distribution of the various Myc-tagged mutants of SNX15 was determined in each fraction by immunoblotting with an anti-Myc antibody.

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**Fig. 6.** SNX15 association with receptor tyrosine kinases. COS7 cells were transiently transfected with mammalian expression vectors for the receptors for PDGF (PDGFR, lanes 1 and 2), insulin (IR, lanes 3 and 4), or EGF (EGFR, lanes 5 and 6) in the absence or presence of recombinant Myc-SNX15. Total cell lysates were analyzed by immunoblotting to detect expression of transfected cDNAs (bottom and middle panels). In addition, total cell lysates were immunoprecipitated (IP) with the appropriate anti-receptor antibodies, separated by SDS-PAGE, and analyzed by Western blotting using an anti-Myc antibody to detect coimmunoprecipitated Myc-SNX15 (top panel). The insulin receptor precursors (~210 and ~190 kDa) are indicated by two and one asterisks, respectively. The mature receptor β-subunit is found at ~95 kDa. This experiment was repeated three times with similar results.

Associations of SNX15 with Receptor Tyrosine Kinases—SNX1, SNX2, and SNX4 bind to receptor tyrosine kinases (4, 8). To investigate whether SNX15 can also bind to growth factor receptors, Myc-SNX15 was coexpressed in COS7 cells together with expression vectors encoding receptors for insulin, EGF, or PDGF. Total cell lysates were immunoblotted to show expression of the recombinant receptor proteins (Fig. 6, bottom panel) and Myc-SNX15 (Fig. 6, middle panel, even lanes). Although SNX15 was coimmunoprecipitated by antibody to the PDGF receptor (Fig. 6, top panel, lanes 2), we did not detect association with receptors for EGF and insulin (Fig. 6, top panel, lanes 4 and 6). We next sought to determine whether the tyrosine kinase activity of the PDGF receptor was required for its association with SNX15. COS7 cells were transiently cotransfected with Myc-SNX15 together with various forms of the PDGF receptor: wild type, kinase inactive (K684R), or the F5 mutant receptor in which five tyrosine phosphorylation sites were mutated to phenylalanine (Y740F, Y751F, Y771F, Y1009F, and Y1021F) (15, 16). Immunoblotting of cell extracts showed that...
Myc-SNX15 and the various PDGF receptor proteins were all well expressed (Fig. 7, middle and bottom panels). Cell extracts were also immunoprecipitated with antibody directed against the PDGF receptor, and the immune complexes were analyzed by immunoblotting with anti-Myc antibody (Fig. 7, top panel). Small amounts of Myc-SNX15 associated with endogenous PDGF receptors, despite their low levels of expression in COS7 cells (Fig. 7, top panel, lane 1). When PDGF receptors were overexpressed, increased levels Myc-SNX15 were detected in immunoprecipitates of the wild type PDGF receptor (Fig. 7, lane 2). In addition, Myc-SNX15 was communoprecipitated with both the kinase inactive and the F5 mutant PDGF receptor (Fig. 7, lanes 3 and 4). Addition of PDGF to the incubation medium did not affect the association of SNX15 with the PDGF receptor (data not shown). Taken together, these data suggest that SNX15 association with the PDGF receptor is unchanged by activation of the PDGF receptor.

The PX Domain of SNX15 Is Required for Association with the PDGF—

To determine the region of SNX15 that is re-
required for its association with the PDGFR, we coexpressed our Myc-tagged SNX15 mutants with the PDGF receptor. Cell lysates were immunoprecipitated with antibody directed against the PDGF receptor followed by immunoblotting with anti-Myc (Fig. 8A). Deletion of the PX domain of SNX15 abolished association with the PDGF receptor (Fig. 8A, lane 6). However, removal of some or the entire ESP domain (lanes 3 and 4) or the entire C-terminal region of SNX15 (lane 5) had no effect on the association of SNX15 with the PDGF receptor.

Overexpression of SNX15 Slows the Internalization and Degradation of 125I-PDGF—To investigate whether association of SNX15 with the PDGFR affects the trafficking of the receptor; we transiently transfected COS7 cells with recombinant PDGF receptors in the absence or presence of coexpressed Myc-SNX15. After allowing the cells to bind 125I-PDGF for 4 h at 4 °C, the cells were washed to remove unbound ligand and warmed to 37 °C for the indicated times. At the end of the 37 °C incubation, the cells were placed on ice, and the medium was collected. The medium was treated with trichloroacetic acid to determine the amount of 125I-PDGF degraded (trichloroacetic acid-soluble radioactivity) and the amount of intact 125I-PDGF dissociated and/or recycled (trichloroacetic acid-precipitable radioactivity). The cells were then acid-washed to collect the 125I-PDGF bound to the cell surface, and the remaining cell-associated counts were collected. In cells expressing only PDGF receptors, 125I-PDGF was lost from the cell surface during the warm-up period (Fig. 9A, open symbols) and continued to increase for the remainder of the time course. During the last hour at 37 °C, no change was seen in the amount of intracellular 125I-PDGF, indicating that a new steady state had been reached (i.e. for each molecule internalized, a molecule was

Fig. 10. Overexpression of SNX15 slows processing of the insulin receptor. A, the insulin receptor is synthesized as a 190-kDa precursor. Its high mannose N-linked oligosaccharides undergo processing to yield complex oligosaccharides leading to an increase in the molecular mass of the precursor to 210 kDa. Subsequently, the 210-kDa precursor is cleaved by furin to yield mature α- and β-subunits. B, pulse-chase labeling studies. COS7 cells were transfected transiently with an expression vector for the insulin receptor in the presence (filled squares) or absence (open squares) of expression vector for Myc-SNX15. Cells were pulse labeled for 20 min with [35S]cysteine and [35S]methionine and then chased for 0–20 h in complete medium containing excess cysteine and methionine. Cell lysates were immunoprecipitated with anti-insulin receptor antibody. Immune complexes were analyzed by SDS-PAGE and transferred to nitrocellulose. A PhosphorImager was used to quantify the radioactivity in the bands corresponding to the 190-kDa pro-receptor, the 210-kDa precursor, and the 95-kDa β-subunit. These data, expressed in arbitrary units, are plotted as a function of time.

Fig. 11. Expression of SNX15 impairs post-translational processing of the insulin receptor and the HGF receptor. A, COS7 cells were transiently transfected with a cDNA encoding the insulin receptor in the absence or presence of recombinant Myc-SNX15. Cell lysates were analyzed by SDS-PAGE, followed by immunoblotting with an anti-insulin receptor antibody to detect the insulin receptor precursors (pre-, 210 and 190 kDa), and the mature β-subunit (95 kDa). An anti-Myc antibody was used to detect recombinant Myc-SNX15. B, COS7 cells were transiently transfected with a cDNA encoding the HGF receptor in the absence or presence of recombinant Myc-SNX15. Cell lysates were analyzed by SDS-PAGE, followed by immunoblotting with an anti-HGF receptor antibody to detect the receptor precursor (pre, 170 kDa), and the mature β-subunit (145 kDa). An anti-Myc antibody was used to detect recombinant Myc-SNX15.
SNX15 and Protein Trafficking

Fig. 12. Localization of tac-furin and endogenous SNX15. COS7 cells were transfected with tac-furin and a Golgi marker, EYFP-GT. After 20 h the cells were fixed, permeabilized, and stained. A, cells were stained for endogenous SNX15 with a rabbit anti-SNX15 primary antibody and visualized with a Rhodamine Red-conjugated donkey anti-rabbit IgG secondary antibody (red). B, cells were also stained for tac-furin with mouse anti-tac primary antibody and visualized with an Alexa 350 conjugated goat anti-mouse IgG secondary antibody (blue). C, cells expressing EYFP-GT, a medial and trans-Golgi marker, are shown (green). Endogenous SNX15 is seen in the cytoplasm and in numerous small puncta (A, red). Recombinant tac-furin is located predominantly in perinuclear structures, and at higher levels of expression, the chimera is also found in small puncta, presumably endosomes, through which it normally cycles (B, blue). As expected, EYFP-GT is seen in brightly stained perinuclear Golgi structures (C, green). D, in the merged image, the TGN is stained aqua, indicating colocalization of tac-furin and EYFP-GT. In addition, the cell expressing high levels of tac-furin shows some colocalization of tac-furin with SNX15 in small puncta (violet).

degraded). In contrast, in cells coexpressing PDGF receptors and Myc-SNX15, there was a marked decrease in the internalization of 125I-PDGF from the cell surface (Fig. 9, A and B, solid symbols) and a 3-fold decrease in the amount of 125I-PDGF degraded (Fig. 9C, solid symbols).

Overexpression of SNX15 Impairs the Post-translational Processing of the Insulin Receptor Precursor—In the course of studying whether SNX15 associated with receptor tyrosine kinases other than the PDGF receptor, we consistently found that overexpression of SNX15 led to a decrease in the amount of insulin receptor β-subunit present in COS7 cells (Fig. 6A, bottom panel). The insulin receptor is synthesized as a 190-kDa pro-receptor containing N-linked high mannos oligosaccharides (35). The N-linked oligosaccharides undergo processing to yield complex oligosaccharides and an increase in molecular mass to 210 kDa (36). Cleavage of the 210-kDa precursor protein by the endoprotease furin results in mature α- (135 kDa) and β-subunits (95 kDa) (Fig. 10A) (36–38). To investigate whether the decrease in mature insulin receptor β-subunit seen in cells overexpressing SNX15 might be due to impairment in the post-translational processing of the receptor precursor, we performed pulse-chase studies in COS7 cells. After labeling for 20 min with [35S]cysteine plus [35S]methionine, transfected cells were chased for 0–20 h in complete medium containing excess unlabeled cysteine and methionine. Cell lysates were then immunoprecipitated with anti-insulin receptor antibody and analyzed by SDS-PAGE followed by autoradiography (Fig. 10B). In cells expressing only recombinant insulin receptors (Fig. 10B, open boxes), the 190-kDa pro-receptor accumulates, attaining a peak level ~4 h into the chase. Moreover, the 210-kDa form of the receptor is present only at low levels, suggesting that the pro-receptor undergoes efficient proteolytic processing by furin to the mature mature α- and β-subunits. In contrast, when SNX15 was coexpressed, this led to an increase in the levels of both the 190-kDa precursor and the 210-kDa species (Fig. 10B, solid boxes, left and middle panels, respectively) with a corresponding decrease in the level of mature β-subunit (Fig. 10B, right panel, solid boxes). Similar delays in pro-receptor processing were seen by Western blotting of total cell extracts from cells overexpressing both SNX15 and the insulin receptor (Fig. 11A, lanes 1 and 2). Interestingly, overexpression of SNX15 also delayed the post-translational processing of the HGF receptor, another substrate of the endoprotease furin (Fig. 11B). When COS7 cells were transfected with HGF receptors in the presence of recombinant Myc-SNX15, there was a 2-fold increase in the immunodetectable 170-kDa HGF pro-receptor and a corresponding decrease in the 145-kDa mature β-subunit as compared with cells expressing the HGF receptor alone (Fig. 11B, lanes 1 and 2). In contrast, when COS7 cells were transiently transfected with a cDNA encoding the PDGF receptor (a receptor that does not undergo furin-dependent processing) in the presence or absence of Myc-SNX15, there was no change in the immunodetectable precursor or mature form of the PDGF receptor (Fig. 6, bottom panel). These findings suggest that SNX15 overexpression interferes with the activity and/or localization of furin.

To investigate whether SNX15 overexpression affected the localization of furin, we transiently transfected COS7 cells with a tac-furin chimera. The chimera consisted of the extracellular domain of the α subunit of the interleukin-2 receptor fused to the transmembrane and cytoplasmic domains of furin (19). It has previously been shown that both native furin and the tac-furin chimera are localized predominantly in the TGN. However, both molecules continually cycle from the TGN to the
Chimera is now colocalized in SNX15-positive aberrant structures (blue, C overexpression, green EYFP-GT. After 20 h the cells were fixed, permeabilized, and stained. B, cells were also stained for tac-furin with mouse anti-tac primary antibody and visualized with an Alexa 350 conjugated goat anti-mouse IgG secondary antibody (blue). C, cells expressing EYFP-GT are shown (green). Recombinant Myc-SNX15 is seen in rings, fused rings, and larger membrane-limited structures rather than in small puncta (A, red). Although some recombinant tac-furin remains in perinuclear structures, in cells overexpressing SNX15, the majority of the chimera is now colocalized in SNX15-positive aberrant structures (B, blue). In contrast, EYFP-GT staining is unchanged by Myc-SNX15 overexpression (C, green). D, the merged images show that some tac-furin remains localized to the Golgi with EYFP-GT (indicated by arrowheads and aqua color). However, the majority of the tac-furin staining is now colocalized in rings, fused rings, and larger SNX15-containing aggregates (violet).

**DISCUSSION**

Structure of SNX15—In this study, we have identified and characterized a novel sorting nexin, SNX15. Like other sorting nexins, SNX15 contains a PX domain. We show here that the PX domain of SNX15 is required for its self-association, association with other sorting nexins, and its association with the PDGF receptor. Deletion of the PX domain from SNX15 not only blocks its associations with other molecules but also prevents association of SNX15 with intracellular membranes. We have also identified a new homology domain in the C terminus of SNX15 (amino acids 265–337), designated the ESP domain. The ESP domain is found in two known proteins and three as-yet uncharacterized genes from *Drosophila* and human. Fungal PalB and its yeast ortholog, Rim1p, are involved in a conserved signal transduction cascade mediating adaptive responses to changes in pH (32, 33). Yeast End13p/Vps4p and its mammalian ortholog Skd-1 (31) are members of the AAA ATPase family of proteins important in movement of proteins from the prevacuolar endosome (30, 40, 41). The function of the ESP domain is presently unknown. However, the N-terminal portion of the domain shares many characteristics with the tetra-tripeptide repeat homology (TPR) consensus sequence. A region of the ESP domain is 32% identical and 53% similar to the first TPR of protein phosphatase 5, for which the crystal structure is known. Each TPR forms a structural element with two anti-parallel α-helices but in a different structural context. The ESP domain forms a structural element with two anti-parallel α-helices joined by a turn. Tandem TPR domains stack into structures that may be involved in protein–protein interaction. The ESP domain may contain a similar pair of anti-parallel α-helices but in a different structural context (42).

The PX domain is the only structural feature shared by all sorting nexins. This domain of ~100 amino acid residues was first identified in the p40phox and p47phox subunits of NADPH oxidase. To gain insight into the evolution of sorting nexins, we

**FIG. 13.** SNX15 overexpression results in mislocalization of tac-furin. COS7 cells were transfected with Myc-SNX15, tac-furin, and EYFP-GT. After 20 h the cells were fixed, permeabilized, and stained. A, cells were stained for Myc-SNX15 with a rabbit anti-Myc primary antibody and visualized with a Rhodamine Red-conjugated donkey anti-rabbit IgG secondary antibody (puncta, A red EYFP-GT are shown (green). Recombinant Myc-SNX15 is seen in rings, fused rings, and larger membrane-limited structures rather than in small puncta (A, red). Although some recombinant tac-furin remains in perinuclear structures, in cells overexpressing SNX15, the majority of the chimera is now colocalized in SNX15-positive aberrant structures (B, blue). In contrast, EYFP-GT staining is unchanged by Myc-SNX15 overexpression (C, green). D, the merged images show that some tac-furin remains localized to the Golgi with EYFP-GT (indicated by arrowheads and aqua color). However, the majority of the tac-furin staining is now colocalized in rings, fused rings, and larger SNX15-containing aggregates (violet).
constructed a dendrogram based upon amino acid sequence similarities among PX domains contained in mammalian and yeast proteins (Fig. 14). Among the PX domains of known sorting nexins, the SNX15 PX domain is most closely related to that of SNX1 and SNX2. However, whereas the homologies between SNX1 and SNX2 extend along the entire length of the molecules, the homology with SNX15 is limited to the PX domain. Interestingly, another human protein, BAA06542, also has a PX domain closely related to the PX domain of SNX15. As is the case with SNX1 and SNX2, the homology between SNX15 and BAA06542 is restricted to the PX domains. However, based upon the sequence of its PX domain, BAA06542 belongs to the family of sorting nexins and might be designated SNX16.

Previously, we had concluded that the PX domains of SNX1, SNX2, SNX3, and SNX4 were more closely related to one another than to PX domains in other proteins. Indeed, this contributed to the rationale for defining sorting nexins as a subgroup of PX domain-containing proteins. We have now extended this conclusion to a larger group of 31 PX domain-containing proteins in yeast and mammals. With the exception of SNX14, the dendrogram suggests that there is a close evolutionary relationship among the PX domains of all the sorting nexins in the data base. In this analysis, SNX14 does not segregate in the group of proteins containing other sorting nexins or their yeast orthologs. Rather, SNX14 lies outside the group of sorting nexins together with a number of other proteins including the p40phox and p47phox subunits of NADPH oxidase, class II phosphatidylinositol 3-kinase, and phospholipase D2. This raises questions about the appropriateness of the designation SNX14.

In yeast, several PX domain-containing proteins are involved in protein trafficking. For example, Vps5p, the S. cerevisiae ortholog of SNX1, is a subunit of a multimeric complex termed “the retromer complex” that is involved in recycling of the carboxypeptidase Y receptor from endosomes to the TGN (5, 9, 43). Grd19, the yeast ortholog of SNX3, is required to maintain the steady state localization of two late-Golgi enzymes (dipeptidyl amino peptidase A and Kex2) by retrieving mislocalized molecules from prevacuolar endosomes (7). In addition, Mvp1p, the yeast ortholog of SNX8, is thought to function in the formation of transport vesicles that facilitate vacuolar protein targeting (6). Here we show that overexpression of SNX15 affects at least two membrane trafficking events. Excess SNX15 decreases the internalization and degradation of the PDGF receptor and also delays the post-translational processing of the pro-receptors for insulin and HGF into their mature subunits. Because direct binding of SNX15 to the insulin receptor (Fig. 6) or tac-furin3 was not demonstrated, it is unlikely that the above noted effects result from a direct interaction of the molecules. Rather, our findings are most likely due to the effects of SNX15 overexpression on endosome structure and function. Our immuno-fluorescence studies also show that overexpression of SNX15 leads to mislocalization of furin and inefficient processing of furin substrates. This SNX15-induced defect in processing of the insulin receptor precursor can be partially corrected by overexpressing additional furin.3 These findings, as well as studies in yeast, suggest a possible mechanism for the observed defect in insulin receptor processing. Yeast Kex2p is a furin-like protease. Retention of Kex2p in its normal location in the Golgi is dependent upon Grd19p, the yeast ortholog of SNX3. It is possible that overexpression of SNX15 disrupts a similar sorting nexin-dependent pathway required to retain furin in the TGN, thereby disrupting proteolytic processing of the insulin receptor. Recent findings from our laboratory support this idea. In addition to disrupting the normal trafficking of furin, we have shown that SNX15 overexpression also disrupts the endocytosis and normal trafficking of transferrin and of TGN38, another protein that cycles between the TGN, plasma membrane, and endosomes. SNX15 overexpression very dramatically affects the morphology of the endocytic pathway leading to the formation of membrane-limited structures containing markers for early endosomes, late endosomes and lysosomes but not markers of the secretory pathway (44). Taken together these findings clearly demonstrate that SNX15 is involved in protein trafficking. At present, we do not know whether SNX15 interacts with TIP47 (45) or PACS-1 (46) that are also involved in retrograde trafficking in mammals. SNX15 and the many other members of the sorting nexin family of proteins share in common the ability to bind to receptor tyrosine kinases, to self-associate, to associate with each other, and to associate with intracellular membranes (4). Further studies are necessary to define the exact functions that each of these molecules has in directing protein trafficking in mammalian cells.

Fig. 14. Phylogenetic tree of PX-containing proteins. A phylogenetic tree of PX-containing proteins based on a multiple alignment of the PX domains. Columns of the alignment containing gaps in a least one sequence were ignored, leaving 80 columns. The PAM (25) model of mutational distance was used for the neighbor-joining algorithm of tree construction by PHYLO_WIN (26). The branch that contains p40phox, p47phox, and phosphoD2, among others, was selected as an outgroup, which roots the tree. SNX15 can be seen to descend from the same node as the last common ancestral node of SNX1, Vps5p, Grd19p, and other sorting molecules. The GenBank™ protein accession numbers for yeast proteins are as follows: Vam7p (AAC4949), Vps5 (AAB62976), Vps17p (NP 014775), Grd19p (NP015002), Mdm1p (NP013603), Mvp1p (NP013717), YDL13c (CAA88681), YDR425w (NP 010713), YJL066w (CAAS9327), and YBR200w (CAAS5163). The GenBank™ protein accession numbers for human proteins are as follows: SNX1 (AAC17182), SNX2 (AAC17181), SNX3 (NP003786), SNX4 (AAC83149), SNX5 (AAD27828), SNX6 (AAD27829), SNX7 (AAD27830), SNX8 (AAD27831), SNX9 (AAD27832), SNX10 (AAD27833), SNX11 (AAD27834), SNX12 (AAD34941), SNX14 (AAD27836), SNX15 (AF175267), p40phox (BAA06542), and p47phox (BAA06542), phosphatidylinositol 3-kinase (JC5500), I-1 receptor candidate protein (AAC33104), BAA06542, and AAD32686. The SNX13 PX domain fragment is not included in the tree.

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