Evidence for a Role of SNX16 in Regulating Traffic between the Early and Later Endosomal Compartments*

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Sorting nexins (SNXs) are a growing family of proteins characterized by the presence of a PX domain. The PX domain mediates membrane association by interaction with phosphoinositides. The SNXs are generally believed to participate in membrane trafficking, but information regarding the function of individual proteins is limited. In this report, we describe the major characteristics of one member, SNX16. SNX16 is a novel 343-amino acid protein consisting of a central PX domain followed by a potential coiled-coil domain and a C-terminal region. Like other sorting nexins, SNX16 associates with the membrane via the PX domain which interacts with the phospholipid phosphatidylinositol 3-phosphate. We show via biochemical and cellular studies that SNX16 is distributed in both early and late endosome/lysosome structures. The coiled-coil domain is necessary for localization to the later endosomal structures, as mutant SNX16 lacking this domain was found only in early endosomes. Trafficking of internalized epidermal growth factor was also delayed by this SNX16 mutant, as these cells showed a delay in the segregation of epidermal growth factor in the early endosome for its delivery to later compartments. In addition, the coiled-coil domain is shown here to be important for homo-oligomerization of SNX16. Taken together, these results suggest that SNX16 is a sorting nexin that may function in the trafficking of proteins between the early and late endosomal compartments.

The cellular response to a myriad of external signals is known to be elicited by ligand binding to cognate cell surface receptors which in turn become activated and set in motion a variety of signaling cascades. Regulation of signaling pathways is of vital importance and is achieved at multiple levels. One chief mechanism is by the down-regulation of the activated receptors. This may be achieved by rapid internalization of the activated receptor into the endosomal system. For many of the receptors of nutritional macromolecules, entry into the endosomal system is brief and generally results in their recycling back to the cell surface for re-employment in another round of ligand binding (1). On the other hand, receptors of hormones and growth factors are often retained in the endosomal system and shuttled through various intracellular membrane compartments on their way to the lysosome for degradation (2). Trafficking through the endosomal system involves the use of membrane-bound intermediates such as vesicles, hence sorting of the correct cargo into the correct vesicle is critical. Many of the proteins involved in this sorting process are hydrophilic peripheral membrane proteins, and their interaction with the membrane is mediated by direct binding to lipids. This is a key event that has recently gained much support. Extensive research has shown that phosphoinositides, particularly the phosphorylated form of phosphatidylinositol (PI) (1), play a role in multiple aspects of membrane trafficking (for reviews see Refs. 3 and 4), and an increasing number of PI-binding motifs with specific lipid binding affinities have been identified. These include the pleckstrin homology domain (5), the FYVE domain (6), the epsin N-terminal homologue domain (7), and the PX domain (8).

In terms of endosomal transport and, in particular, receptor trafficking, the most interesting phosphoinositide is phosphatidylinositol 3-phosphate (PI3-P). Existing predominantly in endosomal membranes, PI3-P is produced by the action of a family of phosphatidylinositol 3-kinases (PI 3-kinase). Three classes of PI 3-kinase are known. Class I PI 3-kinases are regulated by activated receptor tyrosine kinases and G-protein-coupled receptors and can produce PI3-P, PI3,4-P2, and PI3,4,5-P3 (9). Class II PI 3-kinases contain PX and C2 domains in their C-terminal regions and can generate PI3-P and PI3,4-P2 (9). Finally, class III PI 3-kinases consisting of Vps34 and p150 produce mainly PI3-P and are responsible for providing the bulk of the endosomal PI3-P as both of these proteins are located in the endosomes (10, 11). Involvement of the FYVE domain, which binds PI3-P, in membrane trafficking is well established (for review see Ref. 12), with proteins containing the domain, such as EEA1 (13), Rabip4 (14), and Rabenosyn-5 (15), being involved in endosome docking and fusion. Recently, studies with the PX domain proteins SNX3 (16), Vam7 (17), p40phox, and p47phox (18, 19) have found the PX domain to have a preference for PI3-P as well as PI3,4-P2. The PX domain is present in a large number of proteins, many of which exist in the GenBank™ data base, and contain either the PX domain alone or with C-terminal coiled-coil motifs (for review see Ref. 20). Collectively, they are now known as the sorting nexins (SNXs), in accordance with the naming of the prototypic protein, SNX1 (21).

SNX1 was first identified in a yeast two-hybrid screen using the kinase domain of the epidermal growth factor (EGF) receptor as bait. The C-terminal region of SNX1 following the PX

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1 The abbreviations used are: PI, phosphatidylinositol; SNXs, sorting nexins; EGF, epidermal growth factor; EGF-R, EGF receptor; PI3-P, phosphatidylinositol 3-phosphate; E3Ts, expressed sequence tags; HA, hemagglutinin; PBS, phosphate-buffered saline; FITC, fluorescein isothiocyanate; GST, glutathione S-transferase; BSA, bovine serum albumin; EGF-rh, rhodamine-labeled EGF; SNARe, soluble N-ethylmaleimide-sensitive factor attachment protein receptor.
domain contains three coiled-coil domains, the last of which is responsible for binding to the lysosomal targeting information of the EGF receptor (21). Overexpression of SNX1 has been shown to enhance the degradation of the EGF receptor (21). In addition, SNX1 and the related proteins SNX2 and SNX4 can co-immunoprecipitate not only the EGF receptor but also other receptor tyrosine kinases, such as receptors for insulin- and platelet-derived growth factor and the receptor for transferrin (22). With the characterization of SNX6, a sorting nexin identified as platelet-derived growth factor and the receptor for transferrin (Stratagene), pDMyc neo has an insert encoding two Myc epitope sequences inserted 5' to the multiple cloning site (16, 28). All of the deletion mutants were generated by PCR, and the Y145A point mutant was made by direct mutation of the codon using PCR. Full-length SNX16 was also cloned into pDHAneo, which has the two Myc epitope tags of pMyc neo replaced with two hemagglutinin (HA) sequence tags, as mentioned above. For expression of full-length SNX16 and selected mutants in bacteria as GST fusion proteins, the cDNA was excised from pDMyc neo and cloned into the corresponding sites of pGEX4T-1 (Amersham Biosciences). All constructs were confirmed by sequencing. 

Antibodies—SNX16 was produced as a GST fusion protein in bacteria and then cleaved with thrombin. The released SNX16 was used to immunize rabbits with Freund's adjuvant (Inovotrace). To affinity-purify antibodies against SNX16, serum from the immunized rabbits was incubated with the antigen, GST-SNX16, chemically coupled to cyanogen bromide-Sepharose (Amersham Biosciences). Bound antigen was eluted with Immuno-Pure IgG elution buffer (Pierce), neutralized with phosphate-buffered saline (PBS), pH 7.4, and then dialyzed against the same solution. The goat antibody against GST was obtained from Amersham Biosciences. Monoclonal antibodies against EEA1, an early endosome marker, from Transduction Laboratories, and the mouse anti-LAMP1 monoclonal antibody (HA3), developed by J. T. August and J.E.K. Hildreth, was obtained from the Developmental Studies Hybridoma Bank developed under the auspices of the NICHD and maintained by the University of Iowa, Department of Biological Sciences, Iowa City, IA 52242. Polyclonal anti-Myc antibodies were from Upstate Biotechnology, Inc., and monoclonal antibody against HA was purchased from Roche Applied Science. The secondary fluorescein isothiocyanate (FITC)-, Alexa Fluor 555-, or Alexa Fluor 647-conjugated anti-mouse or anti-rabbit antibodies were from Jackson Immunoresearch and Molecular Probes, respectively.

Tissue Distribution of SNX16 mRNA—Specific primers representing the start and end of the human SNX16 coding region were used for PCR using first-strand cDNA generated from the indicated human tissues obtained from Clontech. PCR conditions were as instructed by the manufacturer, and primers for human glyceraldehyde-3-phosphate dehydrogenase were used as control. PCR products were analyzed on 1.2% TAE-agarose gels.

Protein-Lipid Overlay Assay—Protein-lipid overlay assays were based on the procedure described by Dowler et al. (27). Nitrocellulose membranes spotted with 100 pmol of the indicated ligands were obtained from Echelon Research Laboratories and blocked with 3% bovine serum albumin (BSA) in 10 mM Tris/HCl, pH 8.0, 150 mM NaCl, and 0.1% (v/v) Tween 20 (Tris-buffered saline) for 4 h. Membranes were incubated overnight at 4 °C with 1 µg/ml of the relevant GST fusion protein in the blocking solution, after which the membranes were washed 12 times for 5 min with Tris-buffered saline. For detection of bound protein, the membranes were incubated with goat anti-GST polyclonal antibody, washed 6 times for 5 min with Tris-buffered saline, then incubated with rabbit anti-goat antibody conjugated with horseradish peroxidase, and finally washed again prior to being visualized using SuperSignal West Pico chemiluminescence substrate (Pierce).

EXPERIMENTAL PROCEDURES

Cloning of SNX16 cDNA—By using the protein sequence of the PX domain of SNX1, we searched the NCBI database using the BLAST algorithm (25) and identified two homologous human expressed sequence tags (ESTs). The two ESTs (GenBank accession numbers AA280333 and AA284866) were purchased from Research Genetics (Huntsville, AL), and plasmid DNA was isolated using miniprep DNA isolation products from Clontech (Palo Alto, CA). The full sequence of the two ESTs was determined, and the 5' start site was not found. PCR was employed to amplify further the 5' sequence from a 5'-rapid amplification of cDNA ends human liver cDNA library, and sequencing of the amplified products identified the start codon. Standard molecular biology techniques were used to construct full-length cDNA. The identity of the full-length clone was confirmed by sequencing, and it contains a 1035-bp coding region, encoding a 343-amino acid residue sorting nexin, now designated SNX16.

Expression Constructs—PCR using VENT polymerase (New England Biolabs) was used to introduce a 5'-XhoI site and a 3'-XmaI site at the beginning and ending of the SNX16 coding region. The full-length cDNA as well as truncated cDNAs were cloned via these restriction enzyme sites into pDMyneo, which is a modified version of the pCIneo vector (Stratagene). pDMyneo has an insert encoding two Myc epitope sequences inserted 5' to the multiple cloning site (16, 28). All of the deletion mutants were generated by PCR, and the Y145A point mutant was made by direct mutation of the codon using PCR. Full-length SNX16 was also cloned into pDHAneo, which has the two Myc epitope tags of pMyc neo replaced with two hemagglutinin (HA) sequence tags, as mentioned above. For expression of full-length SNX16 and selected mutants in bacteria as GST fusion proteins, the cDNA was excised from pDMyneo and cloned into the corresponding sites of pGEX4T-1 (Amersham Biosciences). All constructs were confirmed by sequencing.

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Cell Culture and Transient Transfection—A431 cells were maintained in Dulbecco’s Modified Eagle’s medium supplemented with 10% fetal calf serum (v/v, Hyclone Laboratories) and antibiotic/antimycotic (Invitrogen) and grown in a 5% CO2 incubator at 37 °C. Transfection was carried out using EFFECTENE (Qiagen) for ~5 h and processed for immunofluorescence, Western slot analysis, or immunoprecipitation 24 h after transfection.

Density Gradient—A431 cells from four 10-cm plates were harvested by scraping into 500 µl of 20 mM Hepes, pH 7.3, 1 mM EDTA, and 8% (w/v) sucrose and lysed by 20 passes through a 26-gauge needle. Post-nuclear supernatant was obtained by centrifugation of the lysate at 1000 × g for 10 min, and 360 µl of the post-nuclear supernatant was
loaded on the top of a tube containing 360-μl aliquots of 40, 37, 34, 31, 28, 25, 22, 19, 16, 13, and 10% (w/v) sucrose in 20 mM Hepes, pH 7.3, 1 mM EDTA, sequentially overlaid. Following centrifugation at 55,000 rpm in an SW60TI rotor, 250 μl were collected from the bottom of the tube, resolved by SDS-PAGE, transferred to nitrocellulose, and then probed with the indicated antibodies as described below.

Cell Imaging—A431 cells were cultured onto 18 × 18-mm glass coverslips and transiently transfected with the indicated plasmids. All cells were washed twice with PBS/CM (PBS containing 1 mM CaCl2 and 1 mM MgCl2) and fixed with 3% paraformaldehyde in PBS/CM for 30 min at 4°C, followed by two rinses with 50 mM NH4Cl in PBS/CM. Cells were permeabilized with 0.1% (w/v) saponin in PBS/CM containing 5% BSA for 30 min at room temperature prior to incubation with the indicated primary antibodies. All primary and secondary antibody incubations were performed in fluorescence dilution buffer (FDB: 5% fetal calf serum, 5% goat serum, and 2% BSA in PBS/CM). After mounting the cells were viewed using a laser scanning confocal microscope (Zeiss).

Internalization of EGF—A431 cells were transfected with the indicated plasmids as described above and plated at 1.5 dilution on coverslips the following day. Cells were then serum-starved (Dulbecco’s modified Eagle’s medium supplemented with 20 mM Hepes, pH 7.3, and 0.2% BSA) 24 h after plating for 3 h. To label cell surface EGF receptors, cells were incubated with 1.6 μg/ml EGF conjugated with rhodamine (EGF-rh) for 1 h at 4°C followed by several washes with starvation media to remove unbound EGF-rh. Surface-bound EGF-rh was internalized by incubation with normal growth media at 37°C for the respective periods followed by fixation. Following permeabilization for 5 min with 0.1% (w/v) Triton X-100, cells were processed for triple labeling and analyzed by confocal microscopy as described above.

Immunoprecipitation and Western Blot—A431 cells on 100-mm dishes were transiently transfected with the indicated plasmids and then lysed in 500 μl of lysis buffer (25 mM Tris/HCl, pH 7.4, 150 mM NaCl, 1% Triton X-100, and Complete EDTA-free protease inhibitor mixture (Roche Diagnostics)). After dilution to 0.5% Triton X-100, immunoprecipitation was performed at 4°C with 25 μl of anti-Myc cross-linked to protein A-Sepharose for 4 h. The Sepharose was then washed three times with lysis buffer containing 0.5% Triton X-100, and bound proteins were eluted with SDS-PAGE sample buffer. Immunoprecipitates were resolved by SDS-PAGE and transferred to nitrocellulose for immunodetection. The blots were blocked for at least 1 h with 5% skimmed milk and 20% fetal calf serum in PBST (PBS containing 0.05% Tween 20) and incubated with primary antibody overnight at 4°C. Following several washes with PBST, the blots were incubated with the appropriate secondary antibody conjugated with hors eradish peroxidase (Jackson ImmunoResearch) and washed again with PBST prior to detection of antibody complexes with SuperSignal West Pico Chemiluminescence Substrate.

RESULTS

Identification and Tissue Distribution of SNX16—To identify additional members of the sorting nexin family, we performed a Blast search of the NCBI human EST data base for proteins homologous/identical to the protein sequence of the SNX1 PX domain. A large number of ESTs were identified, two of which were homologous to each other and represent a partial clone of a novel SNX. A rapid amplification of cDNA ends library was used to PCR-amplify the missing 5′ region. Assembling the additional 5′ nucleotide sequence with the EST sequence results in the identification of a complete open reading frame of 1035 bp which we have deposited into GenBank™ as SNX16 (GenBank™ accession number AF305780). SNX16 is a 343-residue hydrophilic protein, which contains a central PX domain (residue 101–213) followed by a single putative coiled-coil domain (residue 230–278) and non-structural C-terminal region (residue 279–343) (Fig. 1A). Comparison of the human, mouse (GenBank™ accession number NM_029068), and rat (GenBank™ accession number AF305780) SNX16 protein sequences (Fig. 1B) showed that whereas SNX16 is highly conserved in the middle region of the protein, containing both the PX domain and the coiled-coil domain, both the N-terminal and especially the C-terminal regions show substantial difference, with many of the acidic residues present in the C-terminal region of the protein absent in the rodent counterparts. Analysis of the human genome has shown 3 transcript variants of SNX16, variant 1 (GenBank™ accession number NM_022133) and 2 (GenBank™ accession number NM_152836) differ in their 5′-untranslated region and gives rise to isoform A, simply referred to as SNX16 here, whereas variant 3 (GenBank™ accession number NM_152837) results in a 29-residue deletion of part of the PX domain producing isoform B (Fig. 1B). SNX16 showed little homology to other proteins in regions outside the PX domain. The PX domain showed the highest homology (Fig. 1C) to the PX domains of the cytokine-independent survival kinase (CISK: GenBank™ accession number AF312007 (28)), the uncharacterized PXK protein which contains a kinase-like domain in addition to the PX domain (GenBank™ accession number AF399753), SNX1 (GenBank™ accession number U53225 (21)), and the yeast sorting nexin Mvp1p (GenBank™ accession number U16137 (29)).

To obtain the full-length SNX16 cDNA, we performed high fidelity PCR on a human liver cDNA library. We obtained a PCR product ~1050 bp in length, in agreement with the expected size of the SNX16 coding region. The identity of the PCR product was confirmed by sequencing. This 1050-bp fragment is present in diverse tissues, as first strand cDNA generated from eight human tissues subjected to PCR using the same primers employed for the initial cloning of SNX16, and revealed the presence of an ~1050-bp band present in most of the tissues tested (Fig. 2A). The highest level of SNX16 was detected in pancreas, lung, liver, placenta, and heart, with little SNX16 detected in the brain and virtually no SNX16 present in skeletal muscle or kidney under these experimental conditions.

In order to study endogenous SNX16, we prepared specific antibodies by immunizing rabbits with recombinant full-length human SNX16 produced in Escherichia coli. The antibodies were affinity-purified and used to probe an immunoblot of cell lysates from A431 cells or A431 cells expressing Myc-SNX16. The antibodies identified a single protein species migrating at ~49 kDa in A431 cells (Fig. 2B, lane 3) as well as the Myc-tagged SNX16 protein which migrates at ~53 kDa (data not shown). SNX16 has a calculated molecular mass of ~39 kDa, and the apparent larger size can be explained by the high number of acidic residues present in the C-terminal region of human SNX16. Pre-treatment of the affinity-purified antibody with SNX16 covalently attached to Sepharose completely abolished the detection of this ~49-kDa protein and Myc-SNX16 (Data not shown), indicating that the affinity-purified antibodies are specific for SNX16.

As mentioned previously, the protein sequence of rodent SNX16 is substantially different to that of the human protein in the N-terminal and C-terminal regions, and this is reflected in the apparent molecular size of the protein from different species. When the in vitro translated protein products of both the human and rat clones were illuminated by the SNX16 antibodies (Fig. 2B, lanes 1 and 2), human SNX16 migrated at the size observed for SNX16 in A431 cells (49 kDa), whereas rat SNX16 migrated closer to its calculated molecular mass at 40 kDa, which is expected given the absence of acidic residues in the C-terminal region as compared with human protein (Fig. 1B). To verify the authenticity of the size difference between the human and rodent proteins, an immunoblot of lysates from various tissue culture cells was prepared and probed with the SNX16 antibodies (Fig. 2B, lanes 3–9). As can be seen lysates from cells of human A431, HeLa, and 293T, or monkey COS7 origin yielded a protein of ~49 kDa, whereas those of rodent origin AtT20 (mouse), NRK, and L2 (rat) were characterized by the presence of the ~40-kDa protein confirming the results seen with the in vitro translated proteins. Immunoblot analysis of various rat tissue samples revealed a restricted distribution...
of SNX16 (Fig. 2B, lanes 10–17). Although high levels of protein were detected in skeletal muscle, substantial amounts of SNX16 were also identified in the heart and brain lysates. Interestingly, liver, kidney, and spleen lysates did not show a protein the size of SNX16 but rather revealed the presence of a lower molecular weight band (~36 kDa), also present in skeletal muscle, which may correspond to isoform B mentioned previously.

SNX16 Binds to PI3-P in Protein-Lipid Overlay Assays—

Given the recent findings by our laboratory and others (16–19)
Fig. 2. SNX16 is present in multiple tissues. A, tissue distribution of human SNX16 transcripts as revealed by PCR (upper panel) using its specific oligonucleotides and first strand cDNA synthesized from the indicated tissues, a product of 1035 bp is expected. PCR of glyceraldehyde-3-phosphate dehydrogenase (G3PDH) (lower panel) was used as a control. B, antibodies produced against human SNX16 in rabbits were affinity-purified and used at 0.1 μg/ml to probe immunoblots containing human (lane 1) and rat (lane 2) SNX16 produced by in vitro translation (IVT); 70 μg of lysates prepared from the indicated tissue cultured cells of human (lanes 3, 4, and 6), monkey (lane 7), mouse (lane 5), and rat (lanes 8 and 9) origin; and 70 μg of lysates prepared from the indicated rat tissues (lanes 10–17). The antibodies detect a band of ~49 kDa in samples of human origin and 40 kDa in samples of rodent origin, which is expected given the difference in acidic residues highlighted in the C-terminal region of SNX16 (Fig. 2). The lower (~35 kDa) band observed in lanes 12–15 may represent SNX16 isoform B, which contains a deletion in the PX domain. The immunoblots were also probed with an antibody against β-tubulin as a loading control.

that the PX domain is capable of binding to phosphatidylinositides (PI), we examined whether SNX16 was capable of binding to any of these phosphorylated lipids. The protein lipid overlay assay (27) involves probing strips of nitrocellulose spotted with various lipids with potential target molecules. This method has become increasingly popular for assessing the lipid binding capability of proteins (30–33) due to the ease of the method and the availability of commercial strips. As SNX3 has been shown previously to bind PI3-P (16), we first tested for the ability of this protein to bind to PI3-P strips. As shown in Fig. 3, recombinant SNX3, which is structurally little more than a PX domain, bound exclusively to PI3-P in agreement with our previous findings using the plate binding assay, thereby validating the accuracy of this assay method. We next tested for the ability of SNX16 to bind to the phospholipids. We found that full-length recombinant SNX16 specifically recognized PI3-P. No detectable binding was seen for PI, for PIs phosphorylated at other positions (PI4-P and PI5-P), for the bis-phosphorylated PIs (PI3,4-P2, PI3,5-P2, and PI4,5-P2) or for the tris-phosphorylated PI (PI3,4,5-P3). Similarly no binding was found for the other lipids tested: lysophosphatidic acid, lysophosphatidylycholine, phosphatidylethanolamine, phosphatidylcholine, sphingosine 1-phosphate, phosphatidic acid, and phosphatidylerine.

Mutation of a conserved tyrosine residue at position 145 to alanine, shown previously (16) to be responsible for the lipid binding capability of the PX domain of SNX3, abolished the lipid binding of SNX16 confirming that the PX domain was responsible for the lipid binding activity (data not shown). Interestingly, when the PX domain alone of SNX16 was used in this assay, beside the binding to PI3-P, a small amount of binding was also seen for PI3,4-P2 (Fig. 3B). Deletion of the putative coiled-coil domain does not affect PI3-P binding (Fig. 3B).

SNX16 Localizes to Early Endosomes and the Late Endosome/Lysosome—The SNX16 antibodies discussed earlier were used for immunofluorescence but failed to show any staining, indicating that the epitopes recognized by the antibody are not accessible under the conditions of this assay. Therefore, to study the cellular distribution of endogenous SNX16, we fractionated cells according to compartment density on a 10–40% sucrose gradient (Fig. 4), a strategy used by Stockinger and co-workers (34) to examine the cellular distribution of SNX17. Early endosomal EEA1, SNX1, and SNX2 as well as late endosomal/lysosomal LAMP1 were used as markers of the respective compartments. SNX16 was found mainly in fractions of lower density (fractions 4–7), which is similar to the profile of the early endosome marker EEA1 (fractions 5–7), and the early endosome localized sorting nexins SNX1 (fractions 5–8) and SNX2 (fractions 4–8). The marker for the late endocytic compartment, LAMP1, eluted out in heavier fractions with a profile largely distinct from EEA1. Some SNX16 was also found in these heavy fractions (see below), as was a portion of SNX2. These results suggest that the majority of SNX16 resides in fractions that have similar density endosomal membranes marked by EEA1. In addition, SNX16 may also be present in late endosomes/lysosomes as suggested by its presence in the heavier fractions marked by LAMP1.

As mentioned above, immunofluorescence studies of SNX16 could not be performed; therefore, to examine the morphologi-
above using the sucrose gradient confirmed these findings as SNX1 and SNX2 show similar elution profiles. As SNX16 showed a similar elution profile to SNX2 on the sucrose gradient, we next sought to determine whether they are co-localizing in endocytic vesicles by immunofluorescence. As can be seen in Fig. 7 (a–d), SNX16 and SNX2 did show some overlap in the perinuclear region. However, similar to that observed with EEA1, the majority of the SNX16 peripheral puncta were distinct from those labeled by SNX2, suggesting that significant amounts of these two sorting nexins reside in distinct compartments.

As indicated above, the sucrose density gradient study, SNX16 was found to elute in fractions that also contained LAMP1; therefore, we proceeded to examine the possibility that SNX16 may be present in the late endosome/lysosome compartment. A431 cells expressing Myc-SNX16 were processed for immunofluorescence using a monoclonal antibody against LAMP1 as a marker for late endosome/lysosome. As shown in Fig. 5B, a good co-localization between SNX16 and LAMP1. Not only is this evident in the perinuclear region, but the majority of peripheral puncta observed for SNX16 (Fig. 5B, a) overlapped very well with the peripheral puncta (late endosome/lysosomes) marked by LAMP1 (d). Even where the level of Myc-SNX16 was high enough to aggregate the endosome and SNX16 in the perinuclear region, many of the remaining peripheral structures overlapped with LAMP1 (Fig. 5B, e–h). These results collectively suggest that SNX16, at least in steady state, resides in both the early and late endosome/lysosome system.

Deletion of the Coiled-coil Domain of SNX16 Abrogates Its Late Endosome/Lysosome Localization—Because the majority of sorting nexins described show an early endosomal localization and SNX16 appears to exhibit a substantial localization to the late endosome/lysosome, we decided to express a number of deletion mutants of SNX16 to define the structural/molecular basis for its distribution in the late endosome/lysosome. As expected for some but not all sorting nexins, deletion of the entire PX domain of SNX16 totally abolished its association with membrane structures, resulting in distribution to the cytosol (data not shown). Deletion of the N-terminal region immediately preceding the PX domain did not have an effect on the residence of SNX16 in either the early or late endosome/lysosome, as the pattern of staining observed for SNX16ΔNT by immunofluorescence was indistinguishable from that of the full-length protein (data not shown). However, when the putative coiled-coil domain of SNX16 was removed, the late endosome localization seen with wild type SNX16 was no longer present. As can be seen in Fig. 6, expression of SNX16 lacking the coiled-coil domain (SNX16ΔCT1) (Fig. 3A) resulted in somewhat enlarged punctate structures (Fig. 6, a and e) in comparison to those observed for wild type SNX16. SNX16ΔCT1 also differed from the wild type SNX16 as it co-localized nearly completely with EEA1 in both the perinuclear and peripheral regions (Fig. 6, c and d). In fact, expression of SNX16ΔCT1 altered the labeling pattern of EEA1 compared with that of non-transfected cells with EEA1 showing fewer but enlarged punctate structures (Fig. 6b). Similarly, SNX2, which was distinct from the peripheral puncta observed for wild type SNX16, now showed partial overlap with the majority of the peripheral structures observed for SNX16ΔCT1 (Fig. 7, e–h). When double labeling was performed with LAMP1, in direct contrast to the results obtained with wild type SNX16, the peripheral lysosomes illuminated by the LAMP1 antibody showed no significant overlap with those containing SNX16ΔCT1 (Fig. 6, e–h). To ensure that the difference in localization observed for SNX16ΔCT1 was not due to alteration of the lipid binding characteristics of the protein, the mutant was expressed as a fusion
with GST in bacteria and was used in the protein lipid overlay assay described previously. As expected GST-SNX16ΔCT1 bound only PI3-P (Fig. 3B). Taken together, these results suggest that deletion of the coiled-coil domain of SNX16 somehow inhibits its localization to the late endosome/lysosome, resulting in retention of the mutant in the early endosome thereby causing alteration of its structure.

Expression of the SNX16ΔCT1 Mutant Delays Early to Late Endosome Trafficking—The prototypic sorting nexin, SNX1 was first identified for its ability to interact with the lysosomal targeting signal of epidermal growth factor receptor (EGFR) (21). SNX1 interacts with EGFR, and its overexpression was shown to enhance the degradation of the receptor, as measured by following the internalization and subsequent degradation of fluorescently labeled ligand EGF. Since this first discovery, the ability of a sorting nexin to either associate with EGFR (22, 23) or to affect the degradation of EGFR or its ligand (16, 35) has provided a useful system to study sorting nexin function. We have therefore examined the effect SNX16 and its mutants on trafficking of rhodamine-labeled EGF (EGF-rh). In this triple labeling immunofluorescence assay, EGF-rh bound to surface-expressed EGFR at 4 °C was internalized with incubation at 37 °C, followed by fixation of cells at specified times. Fig. 8 (a–d) shows that after 10 min at 37 °C, EGF has been internalized to the early endosome. While at the 60-min time point segregation of EGF from the early endosome marked by EEA1 was observed (Fig. 8, i–l). At the 180-min time point decrease of the majority of internalized of EGF-rh was observed, probably due to degradation in the lysosome (Fig. 8, q–t), in cells expressing wild type SNX16. Importantly, SNX16 together with EGF was seen to segregate away from the EEA1-marked early endosomes (Fig. 8, j), an effect that is most obvious after a 45–60-min chase. The observed rate of EGF-rh trafficking after internalization and subsequent degradation in cells expressing wild type SNX16 was indistinguishable from the results obtained from non-transfected cells. However, expression of the coiled-coil domain mutant had a marked effect on the trafficking of EGF-rh. After a 10-min chase, cells expressing SNX16ΔCT1 showed normal internalization to the early endosome as EGF-rh co-localized with both EEA1 (Fig. 8, g and h) and Snx16ΔCT1 (f and h). At 60 min, however, the presence of SNX16ΔCT1 delayed the segregation of EGF-rh from the early endosome, as the majority of EGF-rh was still present in a compartment marked by both EEA1 (Fig. 8, o and p) and the Myc-tagged protein (n and p). The delay of EGF-rh segregation was still observable after 180 min (Fig. 8, w), although some EGF-rh was beginning to segregate away from the early endosome (u–x). The efficient degradation of internalized EGF-rh seen in SNX16-expressing (Fig. 8, q–t) and non-transfected cells was somehow compromised in cells expressing SNX16ΔCT1. In order to quantify the inhibition observed with overexpression of Myc-SNX16ΔCT1, a similar experiment to that described above was performed. In this case, cells expressing either SNX16 or SNX16ΔCT1 were fixed after 30, 90, and 180 min of internalization. By using confocal microscopy the difference in EGF-rh intensity between overexpressing and neighboring control cells was determined for 100 cells at any given time point. Fig. 9 shows the plot of these intensity differences. As can be seen, cells expressing SNX16 show little difference in EGF-rh intensity to that observed for control cells across all time points. However, although the EGF-rh intensity difference from cells overexpressing SNX16ΔCT1 shows little difference to those overexpressing SNX16 at 30 min, the latter time points, where EGF-rh is transported through later compartments of the endosome to the lysosome for degradation, show a substantially higher level of EGF-rh as quantified by the increase in intensity difference for SNX16ΔCT1-expressing cells, indicating that EGF-rh is not being efficiently transported to the lysosome.

The Coiled-coil Domain Is Required for Homo-oligomerization of SNX16—The formation of homo- and hetero-oligomers is a common feature of many of the sorting nexins studied to date. SNX1 can associate with itself (36) as well as with SNX2 and SNX4 (22). Similarly, SNX6 can associate with itself and with SNX1, SNX2, and SNX4 (23); and SNX15 can also associate with itself, SNX1, and SNX2 and weakly with SNX4 (37). Interestingly, SNX3 has not been found to associate with any other sorting nexins (22, 23, 36, 37). To determine whether SNX16 was able to form homo- and hetero-oligomers, we co-transfected cells with HA-tagged SNX16 and Myc-SNX1, Myc-SNX3, Myc-tagged SNX16, or each of its various deletion mutants (Fig. 10A). Cell lysates were immunoprecipitated with anti-Myc antibodies. The immunoprecipitates were analyzed by immunoblot analysis with anti-Myc antibodies to determine the efficiency of the immunoprecipitation as well as anti-HA antibodies to detect the amounts of co-immunoprecipitated HA-tagged SNX16. SNX16 was found to associate with itself (Fig. 10, lane 1). This interaction is not dependent on its ability to bind to the membrane, as mutation in a key residue of the PX domain responsible for PI3-P-binding (RRA) had no effect on its interaction with HA-SNX16 (lane 2). The SNX16 N-terminal region plays no role in the self-association, as its deletion had no effect and the N-terminal region alone could not interact with HA-SNX16 (Fig. 10, lanes 3 and 7, respectively). Deletion of the entire C-terminal region following the PX domain abolished self-association (Fig. 10, lane 4). The C-terminal fragment alone was able to associate with SNX16 (Fig. 10, lane 8). Because this region seemed important for self-association, more truncation mutants were made to delineate the C-terminal region critical for this function. Deletion of the region corresponding to the very extreme C-terminal amino acid residues 295–343 did not effect homo-oligomer formation at all, as
the amount of HA-SNX16 immunoprecipitated by this mutant was similar to that observed with wild type Myc-SNX16 (Fig. 10, lane 6). However, when the region corresponding to the coiled-coil domain (amino acid residues 213–295) was deleted, homo-oligomer formation was abolished in a similar manner to that observed for the entire C-terminal deletion (Fig. 10, lane 5). This result indicates that the coiled-coil domain is a critical determinant for the self-association of SNX16. Furthermore, SNX16 was unable to hetero-oligomerize with either SNX1 or SNX3 (Fig. 10, lanes 9 and 10), suggesting that unlike the other sorting nexins mentioned above, SNX16 is not part of the SNX1 sorting nexin complex.

**DISCUSSION**

The sorting nexins are a family of proteins identified by the presence of a PX domain and are implicated in regulating
protein trafficking and associated signaling pathways. In the present study, we describe the biochemical, cell biological, and functional characteristics of SNX16, a 343-residue hydrophilic protein containing a central PX domain and a C-terminal flanking coiled-coil domain. Examination of SNX16 from different mammalian species has found that although high conservation is observed throughout the region containing both the PX domain and coiled-coil domain, substantial difference in the N-
Expression of the SNX16/H9004CT1 mutant delays early to late endosome trafficking by preventing segregation of EGF from the EEA1 compartment. A431 cells expressing either Myc-SNX16 (a–d, i–l, and q–t) or Myc-SNX16ΔCT1 (e–h, m–p, and u–x) were incubated with EGF-rhodamine on ice. Surface-bound EGF was then internalized at 37 °C for the indicated times. Cells were fixed and processed for triple labeling.
A Role of SNX16 in Endosomal Traffic

The PX domain is a region of PI3-P and that of p47^phox to PI3,4-P_2. This view is further supported with studies of other PX domain proteins that show the PX domain of SNX2 (33) binds PI3-P, the PX domain of RGS-PX1 binds strongly to PI5-P and PI3-P, and weakly to PI3,5-P_2 (35), and the PX domain of CISK binds PI3,5-P_2, PI3,4,5-P_3, and to some extent PI4,5-P_2 (30). The lipid binding specificity of SNX1 is slightly complicated as the lipid binding specificity varied depending on the type of assay used. However, consensus shows a preference for PI3,5-P_2 and PI3,3-P (33, 38). We have shown here that SNX16 preferentially binds PI3-P. Interestingly, when only the PX domain of SNX16 was tested, some binding to PI3,4-P_2 was also observed. This may be due to increased flexibility in the structure of the isolated PX domain, allowing entry of the bis-phosphorylated lipid into the binding site and may explain some of the weak binding seen with some of the other PX proteins, as isolated PX domains were used in these assays.

Localization studies of SNX16 by both biochemical and cellular means have suggested a novel distribution for it in several endosomal compartments. When we separated membrane structures according to density on a sucrose gradient, we found that SNX16 was present in fractions marked by the early endosomal marker EE1 and also to those characterized by the late endosome/lysosome marker LAMP1. By using indirect immunofluorescence, we found that Myc-tagged SNX16 resides in discrete punctate structures present in both perinuclear and peripheral regions of the cell. In accordance with the gradient result, perinuclear structures of Myc-SNX16 overlapped quite well with EE1, whereas the majority of the more peripheral puncta exhibited co-localization or partial overlap with LAMP1.

The majority of PX domain proteins studied so far are believed to be located only in the early endosome. Although the majority of SNX1 and SNX2 structures exhibited similar density with EE1-containing compartment in the sucrose gradient experiment, SNX1 resides in discrete punctate structures identified as early endosome, which overlap only partially with EE1 and are for the most part distinct from LAMP1 (33, 36). These structures have also been found to contain SNX2 (33), SNX4, SNX5, and SNX6 (23, 39), which is not surprising because with the exception of SNX5, these proteins have been found to interact with each other (22, 23). SNX3 also resides in punctate structures, although they co-localize more with the transferrin receptor and EE1 and are identified as structures involved in trafficking from early endosomes to recycling endosomes (16). Recently, SNX17 was identified and similar to the other sorting nexins has an early endosome localization (34). Until now, the only PX domain proteins exhibiting late endosome/lysosome localization are the yeast target SNARE Vam7, which localizes to vacuole membranes (17, 40), and SNX15, which shows partial overlap with LAMP1 (41). However, the overlap observed between SNX15 and LAMP1 is only evident when SNX15 is expressed to very high levels and may represent an aggregation of the endosomal system rather than residence in the late endosome/lysosome per se, as these structures also contained markers for the early endosome and trans-Golgi network (41). Vam7 consists of a PX domain and a coiled-coil SNARE domain homologous to that of SNAP-25. Mutation of either of these regions destroyed the vacuolar localization of

![Figure 9. SNX16 ACT1 inhibits EGF degradation. Degradation of rhodamine-conjugated EGF in the lysosome was measured by a loss of fluorescence using Zeiss confocal microscope (LSM510 software provided by the supplier). To quantify the effect of SNX16 ACT1 on this process, the difference of the average intensity of EGF-rh between cells expressing either SNX16 or SNX16 ACT1 and neighboring control (non-transfected) cells was determined at the given time points following release of surface-bound EGF-rh as described in Fig. 8 (100 pairs of cells were compared at each time point). Plotting the differences reveals a block in the degradation of EGF-rh in cells expressing SNX16 ACT1 at time points indicative of trafficking through later endosomal compartments. The width of the lines indicates the number of times the intensity difference was observed with 1 cm indicating 13 calculations. The height of the bar indicates the average of 100 calculations.](image-url)
Vam7, suggesting that it is the combined action of both the PX domain and the SNARE domain that is important for vacuolar localization (40).

Examination of the distribution of SNX1 and SNX2 on the sucrose density gradient revealed a surprising result. Whereas SNX1 showed a profile where most of the protein was present at densities corresponding to the early endosome, SNX2 showed a distribution similar to SNX1 in the early endosome region but had a substantial amount of the protein present at densities corresponding to the late endosome/lysosome similar to SNX16 and LAMP1. Localization of SNX2 to these structures has not been reported previously. However, when we used SNX2 in double labeling immunofluorescence studies with SNX16, co-localization between the two proteins showed few of the peripheral puncta of SNX16 overlapping with SNX2. Although this result supports the findings that SNX2 resides in the early endosome (33), it does not rule out the possibility that some SNX2 is also present in other late endosomal structures, which may not be the same as the SNX16 containing late endosomes.

The molecular and biochemical basis underlying the distribution of SNX16 between the early endosome and the late endosome/lysosome were then addressed. By using deletion mutants we have shown that the coiled-coil domain of SNX16 is necessary. Similar to the full-length protein, the SNX16 coiled-coil deletion mutant bound exclusively to PI3-P. However, immunofluorescence localization showed that the usual punctate structure was somewhat enlarged as compared with wild type SNX16. The most interesting aspect of the coiled-coil deletion mutant punctate structures is that they are almost completely localized to the early endosome, evident by the near complete overlap with EEA1 and SNX2 and the lack of overlap with LAMP1. Therefore, the consequence of removal of the coiled-coil domain of SNX16 was the inability of the protein to move out of the early endosome to the late endosome/lysosome. Overexpression of this mutant affected the trafficking of proteins destined for degradation in the lysosome.

SNX1 was identified by its interaction with the lysosomal targeting signal of the EGF receptor, and overexpression of the protein enhanced the degradation of the receptor (21). Based on these studies we examined the effect of overexpression of SNX16 on the trafficking of the EGF receptor as traced by its
ligand. Overexpression of SNX16 generally did not have an effect on the internalization and subsequent degradation of the EGF, with SNX16-positive cells showing normal rates of EGF degradation. When the coiled-coil deletion mutant was expressed, trafficking of internalized EGF was delayed at the level of the early endosome. The internalization of EGF to the early endosome was not affected by expression of the mutant as these cells showed normal levels of EGF in the early endosome after 10 min of internalization. However, the segregation of EGF away from the EEA1 marked early endosome was delayed. Indeed EGF was only beginning to move out of early endosomes in the mutant-expressing cells when normal cells had almost completely degraded the internalized EGF.

Our results suggested that the coiled-coil domain of SNX16 is pivotal to its function, as its absence not only altered the distribution of SNX16, but also delayed the trafficking of internalized EGF. Examination of the coiled-coils of a variety of proteins suggests that this domain may represent one of the principal determinants in oligomerization of proteins (42) and/or interaction with other proteins. Like some other sorting nexins, SNX16 was revealed to form homo-oligomers. By using various deletion mutants we found that the coiled-coil domain of SNX16 is both necessary and sufficient for this homo-oligomerization based on two observations. First, its removal abolished its ability to self-associate, and all of the SNX16 mutants without the coiled-coil region did not show any association with full-length SNX16. Second, the domain on its own could associated with the full-length protein. It is clear from our studies that the PX domain does not contribute to the oligomer formation of SNX16.

With the exception of SNX3 and SNX8 (Mvp1p in yeast), most of the sort nexins characterized so far contain two or three coiled-coil domains following their PX domains (39), and the role these domains play in the function of the sorting nexins is characterized by recruitment other trafficking proteins to the early endosomes for trafficking to the later structures of the endocytic pathway. This could be achieved either by causing the formation of the vesicle itself, where the assembly of SNX16 oligomers may cause deformation of the membrane or by recruiting other trafficking proteins to the early endosomes which result in vesicle formation.

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