A large family of endosome-localized proteins related to sorting nexin 1

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Sorting nexin 1 (SNX1), a peripheral membrane protein, has previously been shown to regulate the cell-surface expression of the human epidermal growth factor receptor [Kurten, Cadena and Gill (1996) Science **272**, 1008–1010]. Searches of human expressed sequence tag databases with SNX1 revealed eleven related human cDNA sequences, termed SNX2 to SNX12, eight of them novel. Analysis of SNX1-related sequences in the *Saccharomyces cerevisiae* genome clearly shows a greatly expanded SNX family in humans in comparison with yeast. On the basis of the predicted protein sequences, all members of this family of hydrophilic molecules contain a conserved 70–110-residue Phox homology (PX) domain, referred to as the SNX-PX domain. Within the SNX family, subgroups were identified on the basis of the sequence similarities of the SNX-PX domain and the overall domain structure of each protein. The members of one subgroup, which includes human SNX1, SNX2, SNX4, SNX5 and SNX6 and the yeast Vps5p and YJL036W, all contain coiled-coil regions within their large C-terminal domains and are found distributed in both membrane and cytosolic fractions, typical of hydrophilic peripheral membrane proteins. Localization of the human SNX1 subgroup members in HeLa cells transfected with the full-length cDNA species revealed a similar intracellular distribution that in all cases overlapped substantially with the early endosome marker, early endosome autoantigen 1. The intracellular localization of deletion mutants and fusions with green fluorescent protein showed that the C-terminal regions of SNX1 and SNX5 are responsible for their endosomal localization. On the basis of these results, the functions of these SNX molecules are likely to be unique to endosomes, mediated in part by interactions with SNX-specific C-terminal sequences and membrane-associated determinants.

Key words: membrane transport, protein sorting, receptor trafficking.

INTRODUCTION

The basic organization of the endosomal/lysosomal system of mammalian cells is now fairly well defined: it is a highly dynamic trafficking pathway that includes membrane transport from both the plasma membrane and the late Golgi [1]. The endocytic pathway has functionally and physically distinct compartments, which include early endosomes, recycling endosomes, late endosomes, lysosomes and the late Golgi. A primary function of endosomes is the sorting and segregation of receptors and ligands, a process fundamental for many cellular processes. Integral membrane proteins and macromolecules that enter endosomes, either endocytosed via clathrin-coated pits at the cell surface or transported directly from the *trans*-Golgi network (TGN), have several intracellular fates, including recycling to the cell surface, transport to the lysosomes either to function or to be degraded, or transport to the TGN. Molecular sorting and transport of proteins within the specialized endosomal compartments is a highly regulated process. The internalization of membrane proteins at the cell surface by clathrin-coated vesicles has been well characterized [2]; however, in contrast, the molecular machinery responsible for sorting and trafficking cargo from the early endosome to either the TGN or the late endosome remain largely undefined.

A peripheral membrane protein, sorting nexin 1 (SNX1), regulates the cell-surface expression of the human epidermal growth factor (EGF) receptor [3]. SNX1 was identified, by using the yeast two-hybrid system, as a protein that interacts with cytoplasmic sequences of the EGF receptor that include the tyrosine kinase domain and the adjacent lysosomal targeting signal. Overexpression of SNX1 in transfected cells results in a specific increase in the rate of degradation of the EGF receptor when bound to its ligand [3]. Haft et al. [4] used co-immunoprecipitation experiments to demonstrate a direct interaction between the EGF receptor and SNX1. In addition, these investigators identified three novel SNXs, namely SNX2, SNX3 and SNX4; furthermore, SNX3 and SNX4 interacted with several tyrosine kinase receptors [4]. Recently, SNX1 and SNX2 were found to associate with the human orthologues of Vps26p, Vps29p and Vps35p to form a multimeric complex [5]. Within *Saccharomyces cerevisiae*, the SNX Vps5p associates with Vps17p, Vps26p, Vps29p and Vps35p to form a retromer coat complex on vesicular membranes [6]. This protein coat is proposed to be responsible for the proper retrieval and recycling of the carboxypeptidase Y receptor from the prevacuolar compartment (endosome equivalent) to the late Golgi (TGN). These findings suggest that the emerging family of SNXs might have a role in regulating the intracellular trafficking of numerous membrane receptors.

To gain further insight into the functions of the SNX family, the number of potential members of this family, and their intracellular localization, are required. To determine the potential

Abbreviations used: EEA1, early endosome autoantigen 1; EGF, epidermal growth factor; EST, expressed sequence tag; FLAG, Asp-Tyr-Lys-Asp-Asp-Asp-Asp-Lys; GFP, green fluorescent protein; KLH, keyhole-limpet haemocyanin; RACE, rapid amplification of cDNA ends; SNX, sorting nexin; TGN, *trans*-Golgi network.

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size of this family we analysed various sequence databases and identified additional members of the SNX family in both yeast and humans. In addition, we defined some of the features shared by this protein family; on the basis of these we describe a number of subgroups within the family. Comparison of the intracellular localization of the five SNXs, which form a subgroup that includes SNX1, revealed a similar intracellular distribution that overlaps substantially with early endosomes. The localization of this SNX subgroup supports a role for these molecules in the regulation of cargo trafficking within the endosomal system. This protein family differs from previously characterized protein families implicated in membrane transport, including the mammalian syntaxin [7] and Rab GTPase families [8], in which individual members associate with different intracellular compartments.

EXPERIMENTAL

Cell culture and antibodies

Cells were maintained in a humidified air/CO_2 (9:1) atmosphere at 37 °C as monolayers in DMEM (Dulbecco's modified Eagle's medium) supplemented with 10% (v/v) foetal bovine serum, 2 mM glutamine, 100 i.u./ml penicillin and 0.1% streptomycin (complete DMEM). Transfected cells were maintained in the above medium with 400 μg/ml G418 (Gibco BRL, Rockville, MD, U.S.A.). Asp-Tyr-Lys-Asp-Asp-Asp-Asp-Lys (FLAG) epitope-tagged proteins were detected with monoclonal antibody M2 (Eastman Kodak Company, New Haven, CT, U.S.A.). A rabbit antibody against green fluorescent protein (GFP) was purchased from Molecular Probes (Eugene, OR, U.S.A.). Antibody against early endosome autoantigen 1 (EEA1) was from BD-PharMingen (San Diego, CA, U.S.A.). FITC conjugates were from ICN Pharmaceuticals (Costa Mesa, CA, U.S.A.) and Texas Red conjugates from Jackson Immunodiagnostics (West Grove, PA, U.S.A.). Horseradish peroxidase-conjugated anti-mouse immunoglobulin was obtained from Dako Corporation (Carpinteria, CA, U.S.A.).

Sequence databases and Internet resources

Yeast (*S. cerevisiae*) protein sequence database, non-redundant nucleotide and protein databases, and the public non-redundant expressed sequence tag (EST) cDNA database (dbest [9]) were searched with the National Center for Biotechnology Information (NCBI) BLAST family of programs (http://www.ncbi.nlm.nih.gov/BLAST/). The reported functional properties of yeast open reading frames were retrieved from The *Saccharomyces* Genome Database project (http://genome-www.stanford.edu/saccharomyces/) [10]. DNA sequence trace data for the EST clones derived from the Integrated Molecular Analysis of Genome Expression Consortium (I.M.A.G.E.) was retrieved from the Washington University Genome Sequencing Center (http://genome.wustl.edu/pub/gsc1/est/).

Analysis of protein and nucleotide sequences

Analyses of DNA sequence data and contig assembly were performed with Sequencher™ 3.0 (Gene Codes Corporation, Ann Arbor, MI, U.S.A.). ClustalX was used for multiple alignments of protein sequences [11]. Phylogenetic trees, based on the output from the ClustalX analysis, were constructed with the Growtree and Distances programs from the Genetics Computer Group (GCG) Wisconsin Package (version 8.1) located at Australian National Genomic Information Service (ANGIS). Direct comparison of two protein sequences was performed with BestFit within the GCG package. Coiled-coil predictions were performed with the COILS program (http://www.isrec.isb-sib.ch/software/coils-form.html) [12,13]. Predictions from the COILS program containing stretches of 21 or more amino acids with a probability greater than 0.5 were considered significant. Predictions of secondary structure (PHDsec) [14,15], detection of transmembrane helix location and topology (PHDhtm, PHDtopology) [16] and the detection of functional motifs (PROSITE) [17] were all performed with the PredictProtein software located at EMBL (http://www.embl-heidelberg.de/predictprotein/predictprotein.html) [18]. The detection of protein domains was performed with SMART (v3.1; http://smart.embl-heidelberg.de/) [19] and Pfam (v5.5; http://www.sanger.ac.uk/pfam/) [20].

Confirmation of EST cDNA clones

EST cDNA clones were purchased from Genome Systems (Incyte Genomics, St Louis, MO, U.S.A.) or A.T.C.C. (Manassas, VA, U.S.A.). EST cDNA clones were confirmed by DNA sequencing with fluorescent dye terminators and an ABI 377 sequencer; sequences were compared with the predicted contig generated from partial EST sequences.

Rapid amplification of cDNA ends (RACE)

5′ RACE was performed on G-tailed cDNA prepared from human brain poly(A)$^+$ RNA by two sequential rounds of PCR by using different nested gene-specific primers with an 18 nt poly(C) primer. The resulting PCR products were cloned into the pCR 2.1 vector (TA cloning kit; Invitrogen, San Diego, CA, U.S.A.). The nucleotide sequences of the 5′ RACE products were compared with the original contigs.

Expression of genes encoding SNX in different tissues

Twenty-five tissue and cell-specific human cDNA libraries (Clontech, Palo Alto, CA, U.S.A.) were screened for the expression of the individual genes encoding SNX by using PCR. Initially, PCR reactions were performed on each library with gene-specific primers. PCR products encoding genes for SNX were detected by hybridization with internal ^{32}P-labelled 40-base oligonucleotides. After separation on non-denaturing polyacrylamide gels, the hybridized products were detected by autoradiography.

Production of antibodies

Synthetic peptides encoding the N-terminal or C-terminal residues of the predicted SNX proteins were coupled to keyhole-limpet haemocyanin (KLH) by using glutaraldehyde [21]. New Zealand White rabbits were immunized with dialysed KLH-peptide (50 μg) in Freund's complete adjuvant and boosted three times at intervals of 3 weeks with 50 μg of KLH-peptide in Freund's incomplete adjuvant.

cDNA constructs

Full-length SNX proteins

The mammalian expression vector pCMU [22] was modified to encode three repeats of the FLAG epitope flanked by restriction endonuclease sites for convenient subcloning. The modified vector is referred to as pCMU-FLAG. The full-length open reading frames of SNX4, SNX5, SNX6 and SNX10 were amplified by PCR and the resulting PCR products were cloned

into pCMU-FLAG. The full-length open reading frames of SNX1 and SNX2 were amplified by PCR and the resulting PCR products were cloned into pCR3.1 (Invitrogen).

Truncations of SNX1

The construct, termed FLAG-SNX1$_{77–522}$, encoding residues 77–522 of SNX1 fused to three FLAG epitopes at the N-terminus, was generated by digestion of pCR3.1-SNX1 with *Bam*HI and *Not*I and subcloning the 1.7 kb fragment into the corresponding endonuclease sites in pCMU-FLAG. The FLAG-SNX1$_{77–522}$ plasmid was used to generate FLAG-SNX1$_{77–272}$, encoding residues 77–272 of SNX1, by digestion with *Pml*I to remove a 0.65 kb fragment followed by religation of the vector. This construct had four additional non-SNX1 amino acids (Asp-Glu-Val-Pro) at the C-terminus before the stop codon.

GFP fusions with domains of SNX1 and SNX5

The N-terminal domain (residues 1–183), the SNX-PX domain (residues 184–266) and the C-terminal domain (residues 267–522) of SNX1 and the C-terminal domain (residues 167–404) of SNX5 were amplified by PCR. The resulting PCR products were each cloned into the multiple cloning site at the 3′ end of the GFP cDNA within the plasmid pEGFP–C1 (Clontech).

Molecular cloning techniques were performed by the methods of Sambrook et al. [23] with reagents from New England Biolabs (Beverly, MA, U.S.A.). All constructs were confirmed by DNA sequencing.

Transfection of HeLa cells

HeLa cells were transfected with 20 μg of plasmid DNA by the calcium phosphate method [22] or FuGENE 6 (Boehringer Mannheim, Germany) and stable transfected cells were selected with 800 μg/ml Geneticin (Gibco BRL).

Indirect immunofluorescence

Transfected cell monolayers were fixed in 4 % (v/v) paraformaldehyde and permeabilized in 0.2 % (v/v) saponin [24]. FLAG-tagged proteins were detected by indirect immunofluorescence with the FLAG-epitope-specific mouse monoclonal antibody M2, followed by FITC-conjugated anti-mouse immunoglobulin. Rabbit polyclonal antiserum was detected with FITC-conjugated anti-rabbit immunoglobulin or Texas-Red-conjugated anti-rabbit immunoglobulin. For double-labelling experiments, the early endosomal protein EEA1 was detected with a mouse monoclonal antibody followed by FITC-conjugated anti-mouse immunoglobulin. The monolayers were then treated with 5 % (v/v) normal mouse serum before incubation with biotin-conjugated M2 mouse monoclonal antibody; the biotin-conjugated M2 mouse monoclonal antibody was detected with Cy3-conjugated streptavidin (Amersham Pharmacia Biotech, Uppsala, Sweden). No cross-reactivity between the anti-immunoglobulin conjugates or between the anti-immunoglobulin conjugates and the irrelevant primary antibodies was observed. Stained monolayers were analysed with a Bio-Rad MRC 1021 confocal imaging system and Lasersharp 3.1 software.

Fluid-phase uptake of dextran

Cell monolayers were incubated for 30 min at 37 °C with DMEM containing 5 mg/ml biotinylated dextran (10 kDa; Molecular Probes). The monolayers were washed twice with ice-cold PBS and then incubated with 50 μg/ml avidin on ice for 30 min to quench any cell-surface biotin–dextran. After this treatment, monolayers were fixed, permeabilized and processed for indirect immunofluorescence as described above, in ice-cold solutions. The biotinylated dextran was detected with Cy3-conjugated streptavidin.

Membrane fractionation

Subconfluent monolayers of HeLa cells were detached by being scraped into PBS and washed in chilled hypotonic sucrose buffer [250 mM sucrose/10 mM Hepes (pH 7.4) containing Complete Protease Inhibitors (Boehringer Mannheim, Germany)]. Cells were resuspended in 500 μl of hypotonic sucrose buffer and passaged 20 times through a 26-gauge needle. Intact cells and nuclei were removed by centrifugation at 2700 ***g*** for 10 min and the resulting supernatant was centrifuged at 100000 ***g*** and 2 °C for 60 min. The resulting microsome pellet was resuspended in hypotonic buffer. Equivalent proportions of each fraction were analysed by SDS/PAGE and immunoblotting.

Immunoblotting

Immunoblotting with enhanced chemiluminescence (NEN, Boston, MA, U.S.A.) was performed as described previously [25].

RESULTS

S. cerevisiae proteins related to SNX1

The sequences of SNX1 and SNX1-related proteins were used to search the NCBI yeast protein database, which contains all the predicted protein sequences of the *S. cerevisiae* genome. Six yeast sequences were identified that showed significant similarity to the SNXs (Figure 1). Three of these protein sequences, namely Vps5p [26,27], Mvp1p [28] and Grd19p [29], have been identified as peripheral membrane proteins that function within the late Golgi or in vacuolar membrane trafficking in yeast and have previously been considered to be members of the SNX family [29]. The other three sequences are the open reading frames YHR105W, YJL036W (also termed SNX4 [4]) and YDL113C. The similarities of the full-length predicted yeast proteins to SNX1, on the basis of pairwise BLASTp alignments, were as follows: Mvp1p (43 %), Vps5p (47 %), Grd19p (49 %), YHR105W (51 %), YJL036W (44 %) and YDL113C (45 %).

SNX1-related proteins within the human EST libraries

Having identified a number of proteins related to SNX1 in yeast, we next analysed various databases for human sequences related to SNX1 and the SNX1-related proteins. At the start of this study no other human proteins related to this group were found in sequence databases, such as GenBank®, that contain experimentally annotated information. The SNX1 and SNX1-related *S. cerevisiae* sequences were then used to search the EST database obtained from the NCBI. The overlapping EST contigs identified were challenged by comparing the putative sorting nexin-related proteins with the NCBI non-redundant protein database. These extended cDNA species were considered SNX family members only if the most significant relative within this database remained a protein within the SNX-related group. The results from this analysis identified eleven human sequences related to SNX1, termed SNX2 to SNX12 (Figure 1). Three of these sequences, namely SNX2, SNX3 and SNX4, were reported during this study [4] and consequently we have adopted this terminology. Independently, Otsuki et al. [30] identified human SNX5 as a component that interacts with the Fanconi Anemia

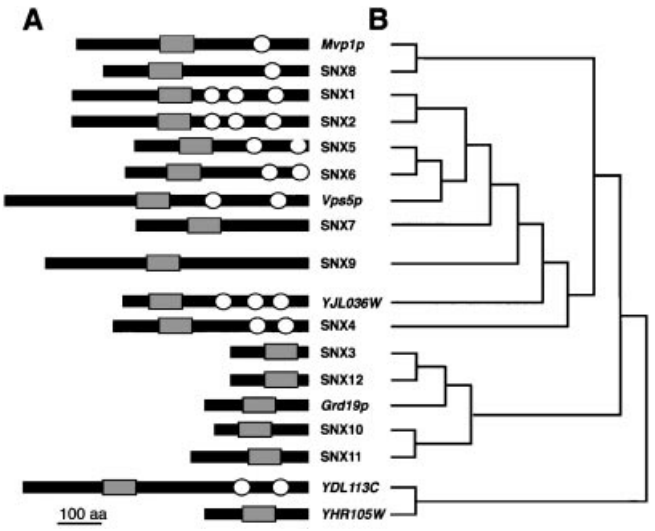

Figure 1 Phylogenic relationships between members of the SNX protein family

(**A**) Yeast (*S. cerevisiae*) protein sequence database, non-redundant nucleotide and protein databases, and the public non-redundant EST cDNA database were searched with the NCBI BLAST family of programs (http://www.ncbi.nlm.nih.gov/BLAST/). Overlapping nucleotide sequences contiguous with the partial putative SNX cDNA species identified were retrieved from the EST databases. The extended cDNA sequences were challenged by comparing the putative sorting nexin-related proteins with the NCBI non-redundant protein database. These extended cDNA species were considered SNX family members only if the most significant relative within the database remained a protein within the SNX-related group. The results from this analysis identified eleven human sequences related to SNX1, termed SNX2 to SNX12, and six yeast sequences (italics). The relative locations of the conserved SNX-PX domain (rectangles) and protein sequences predicted to form coiled-coils (ellipses) are indicated. Coiled-coil predictions were performed with the COILS program [13]. (**B**) The phylogenic tree for the SNX-PX domain was constructed with the multiple sequence alignment shown in Figure 2. The pairwise evolutionary distances were calculated by using the Jukes–Cantor distance correction method; the neighbour-joining tree construction method was used to plot this cladogram. GenBank accession numbers are as follows: Vps5p, AAB62976; Mvp1p, AAA67884; Grd19p, AAC15913; YHR105W, AAB68857; YJL036W, CAA89327; YJL113C, CAA98681; SNX1, AAA98672; SNX2, AAC17181; SNX3, AAC16040; SNX4, AAC83149; SNX5, AAD27828; SNX6, AAD27829; SNX7, AAD27830; SNX8, AAD27831; SNX9, AAD27832; SNX10, AAD27833; SNX11, AAD27834; SNX12, AAD48491.

Complementation Group A protein (FANCA) with the use of a two-hybrid system.

Identification of full-length cDNA clones encoding SNX family members

EST clones containing the most 5′ sequence of each SNX were purchased and sequenced. The sequences of the longest clones, which also included a 3′ stop codon, were compared with the original contigs for verification. To assess whether the cDNA species encoding the novel SNX1-related proteins were full length, the 5′ sequences were analysed; if a stop codon was present in the putative 5′ untranslated sequence then the cDNA was considered full length. Alternatively, 5′ RACE with human brain mRNA was performed to detect any additional 5′ sequences not encoded by the longest EST clone. When several independent 5′ RACE clones contained the same sequence, the cDNA was considered complete. Thus it is likely that the cDNA species of SNX2–SNX12 were all full length. The cDNA sequences for SNX2, SNX3 and SNX4, generated in this study, matched the previously published sequences [4]. The GenBank accession numbers for the novel SNX cDNA species are AF121855 (SNX5), AF121856 (SNX6), AF121857 (SNX7), AF121858 (SNX8), AF121859 (SNX9), AF121860 (SNX10), AF121861 (SNX11) and AF171229 (SNX12).

Properties of the family of SNX1-related proteins

The presence of conserved regions within the SNX protein family was assessed by multiple sequence alignment generated with the ClustalX program. From this analysis it was observed that one region of 70–110 residues was conserved between all members of the family. The sequences of this region are depicted in Figure 2. This region is related to the previously described Phox homology (PX) domain identified by Ponting [31]. The PX domain is found in a large group of proteins with very diverse functions. Indeed, the original definition of the PX domain included some partial protein sequences identified here as Mvp1p, Vps5p, Grd19p, YHR105W, YJL036W, YDL113C, SNX1, SNX3, SNX4, SNX8, SNX9 and SNX10. However, we propose that the PX domain of

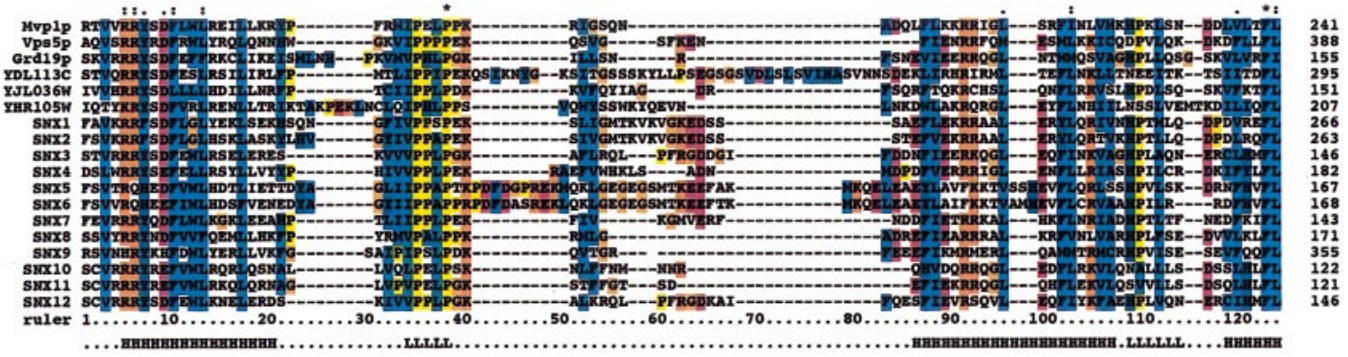

Figure 2 Sequence alignment of the SNX-PX domain

Sequences encoding the SNX-PX domain of each family member were aligned by using ClustalX. The symbols above indicate strongly conserved positions: asterisks, positions with a single, fully conserved residue; colons and full points, fully conserved residue groups as defined in [11]. Proline and glycine residues are highlighted individually. Additional residues are highlighted where more than 80% conservation occurred (blue, hydrophobic residues; red, acid residues; orange, basic residues). The location of the last residue of the SNX-PX domain within the full-length sequence of the protein is indicated at the right. Predictions of secondary structure (bottom line) (PHDsec) were performed with the PredictProtein software located at EMBL (http://www.embl-heidelberg.de/predictprotein/predictprotein.html) [18]: H, helix; L, loop.

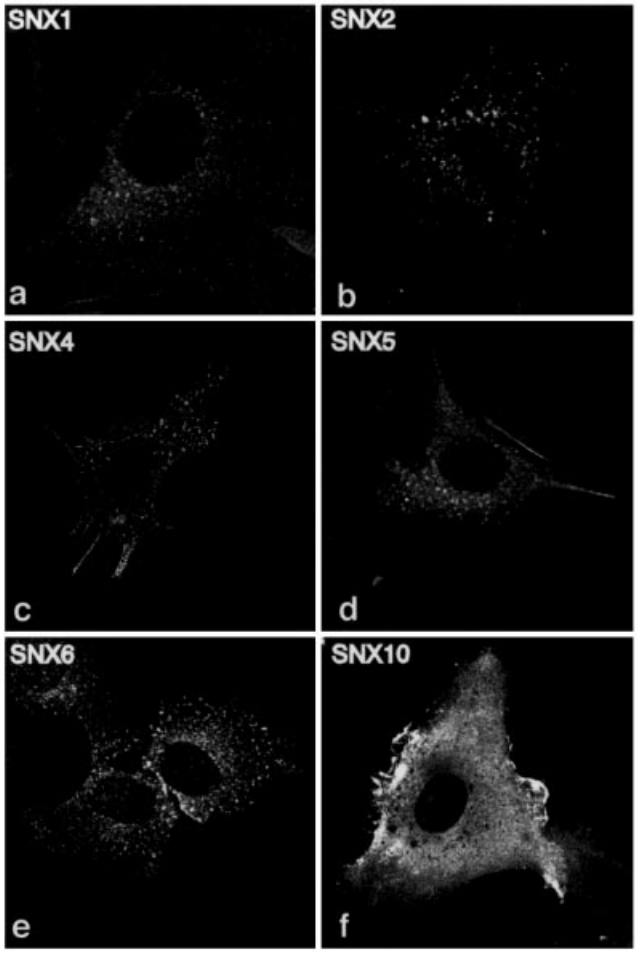

Figure 3 Intracellular distribution of human SNX proteins in transfected HeLa cells

Transiently transfected HeLa cells expressing pCR3.1-SNX1 (**a**) and pCR3.1-SNX2 (**b**), or HeLa cells stably expressing the constructs pCMU-FLAG-SNX4 (**c**), pCMU-FLAG-SNX5 (**d**), pCMU-FLAG-SNX6 (**e**) and pCMU-FLAG-SNX10 (**f**) were fixed with paraformaldehyde. Indirect immunofluorescence was performed in the presence of saponin by using the M2 monoclonal antibody against the FLAG epitope followed by FITC-conjugated anti-mouse immunoglobulin (**c**–**f**). Alternatively, indirect immunofluorescence was performed with rabbit anti-peptide antisera for SNX1 (**a**) and SNX2 (**b**) and FITC-conjugated anti-rabbit immunoglobulin. The peptides used in the generation of the rabbit antisera to SNX1 and SNX2 were ASGGGGCSASERLPPP and KYWEAFLPEAKAIA (single-letter amino acid codes) respectively; the preimmune sera from these immunized rabbits showed no reactivity. The mouse anti-FLAG M2 monoclonal antibody displayed no reactivity with untransfected HeLa cells.

SNX molecules represents a very distinct subgroup of the whole family of PX domains. Reciprocal detection within the non-redundant protein database using any of the PX domains from the SNX molecules failed to detect any of the other PX-containing sequences (Blastp cutoff value $E < 0.2$). In contrast, the SNX family members shown in Figure 1 all have more than 50% similarity between their PX domain and the corresponding region of the SNX1 protein. This level of similarity is a criterion for membership to the SNX family of proteins, which excludes many of the previously identified proteins containing the PX domain. In addition, the generation of phylogenetic trees including both non-SNX PX domains (obtained from Pfam) and PX domains from SNXs revealed that the SNXs clustered into distinct branches (results not shown). Therefore the PX domain of these SNX molecules clearly represents a subgroup of the original definition of the PX domain. For these reasons we have termed the PX domain of the SNX family members the SNX-PX domain.

Although few individual amino acids are conserved within the SNX-PX domain of the entire family, overall there is significant sequence similarity among family members throughout this domain (Figure 2). In addition, analysis of the secondary structures, using ProteinPredict software, predicts that all the SNX-PX domains form similar secondary structures. The prediction for secondary structure of the SNX-PX domain is indicated in Figure 2. The N-terminus of the SNX domain is predicted to form an α-helix and within this helix an Arg-Arg-Tyr-Xaa-Asp(Glu) motif is highly conserved throughout the family. In addition, the conserved aliphatic amino acids are predicted to form one face of this α-helix. The most striking feature of the SNX-PX domain that is conserved throughout the family is a proline-rich motif, Pro-Pro-Xaa-Pro-Xaa-Lys, located within the first predicted loop. Similar proline-rich sequences have been shown to function directly in protein–protein interactions [32]. Numerous residues within the C-terminal region of the SNX-PX domain show similarity throughout the family. In particular, the Phe-Leu residues located at the C-terminus are present in all except one member of the family and a His-Pro motif is present in most SNX proteins. The SNX-PX domain shows variability in length because some SNX members have additional sequences inserted within the domain, for example SNX5 and SNX6. In addition, regions flanking the SNX-PX domain displayed in Figure 2 show a low level of similarity [31] and are likely to be structural sequences required for the folding of the SNX-PX domain. So far, no biochemical functions have been directly associated with the PX or SNX-PX domains.

No sequences outside this SNX-PX domain were identified as being conserved in all family members. In addition, a direct comparison of the C-terminal domains of the family, which for SNX1 is proposed to bind directly to the EGF receptor, revealed no conserved residues. Furthermore, scanning the individual members of the SNX family for protein patterns or protein domains associated with biological functions did not reveal any additional shared protein features within this family. This is in contrast with many other proteins with PX domains outside the SNX-PX group that contain an associated domain termed PXA (R. D. Teasdale and P. A. Gleeson, unpublished work; see SMART database) [19]. SNX9 was predicted to encode an additional domain to the SNX-PX domain, a SH3 domain near its N-terminus (see also [33]). Although the only domain conserved throughout the entire SNX family is the SNX-PX domain, many of the SNX proteins nevertheless show a strong relationship with one other family member. For example, SNX5 and SNX6 are 81% similar over their full-length protein sequences.

The SNX family was also analysed for conserved secondary structure properties. First, from hydropathy analysis, none of the members contain either putative transmembrane domains or signal peptides. Secondly, the members of the SNX family were analysed for the presence of coiled-coil domains by using the COILS prediction method. Coiled-coil domains, potential sites for protein–protein interactions, are crucial to the function of other membrane transport proteins such as syntaxins [34]. Protein sequences within the SNX family members predicted to form coiled-coils are indicated in Figure 1(A). Many, but not all, of the SNX proteins have between one and three stretches of 21 or more amino acids within their C-terminal sequences that have the potential to form coiled-coil structures.

The phylogenetic relationship between the members of the SNX family was assessed. Comparison of the SNX-PX domain between the members clearly indicates that distinct groups exist

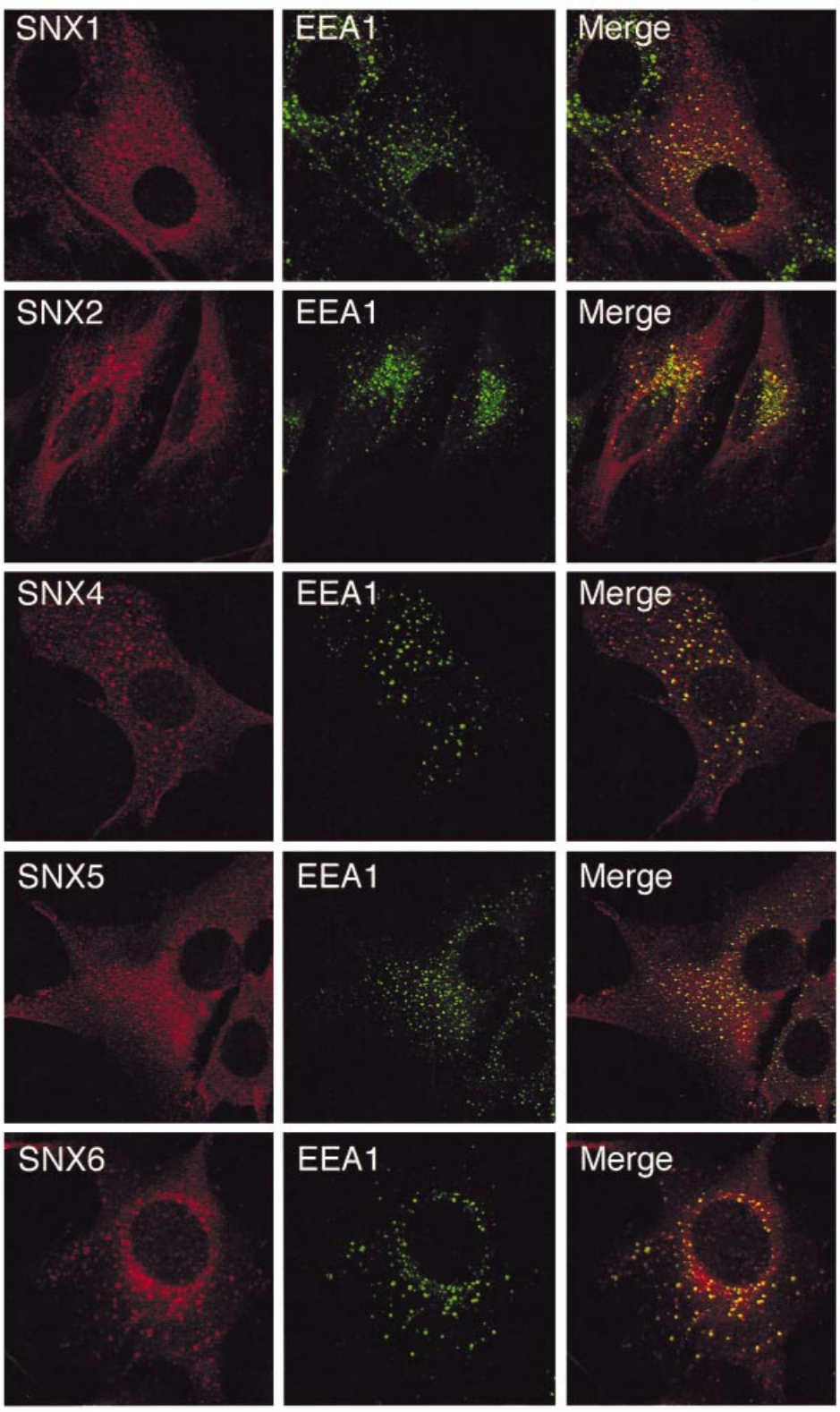

Figure 4 Human SNX proteins are localized to the early endosome in transfected HeLa cells

Transiently transfected HeLa cells expressing pCR3.1-SNX1 and pCR3.1-SNX2, or HeLa cells stably expressing the constructs pCMU-FLAG-SNX4, pCMU-FLAG-SNX5 and pCMU-FLAG-SNX6, as indicated, were immunolabelled for the early endosomal protein EEA1 with a mouse monoclonal antibody followed by FITC-conjugated anti-mouse immunoglobulin. The monolayers were then treated with 5% (v/v) normal mouse serum before incubation with a biotinylated mouse anti-FLAG M2 monoclonal antibody and Cy3-conjugated streptavidin. No cross-reactivity between the anti-immunoglobulin conjugate and the irrelevant primary antibody was observed. Superimposed images are shown (merge).

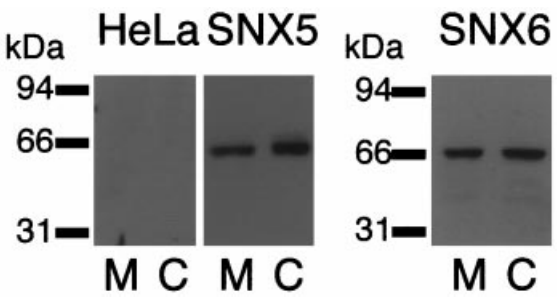

Figure 5 Human SNX5 and SNX6 are distributed in both cytosolic and membrane fractions

Sub-confluent monolayers of HeLa cell lines stably transfected with the constructs pCMU-FLAG-SNX5 and pCMU-FLAG-SNX6 were detached by scraping into hypotonic buffer and fractionated by passage through a 26-gauge needle and syringe. Cell debris and nuclei were removed by low-speed centrifugation and membranes were collected by ultracentrifugation. Samples (equivalent loads in relation to the total homogenate) were analysed by immunoblotting with monoclonal antibody M2 against the FLAG epitope, followed by peroxidase-conjugated anti-mouse immunoglobulin. Bound antibodies were detected by chemiluminescence. Abbreviations: M, membrane fraction; C, cytosolic fraction. The positions of molecular mass markers are indicated at the left.

(Figure 1B). On the basis of the similarity of the SNX-PX domain and the domain structures outside the SNX-PX domain (Figures 1A and 1B), the SNX family can be divided into three subgroups as follows: (1) the SNX8 subgroup consisting of SNX8 and Mvp1p, which both contain large C-terminal domains with a single coiled-coil region; (2) the SNX1 subgroup consisting of SNX1, SNX2, SNX4, SNX5, SNX6, Vps5p and YJL036W, which all contain large C-terminal domains with multiple coiled-coil domains; and (3) the SNX10 subgroup consisting of SNX3, SNX10, SNX11, SNX12 and Grd19p, which are smaller proteins than other SNX family members and lack coiled-coil regions. SNX7 and SNX9, although closely related to the SNX1 subgroup on the basis of the SNX-PX domain (Figure 2), are excluded from this subgroup owing to the lack of coiled-coil domains. In addition, the SNX-PX domains of the two additional yeast members, YDL113C and YHR105W, do not show obvious close relationships to any of these subgroups.

Tissue distribution of SNX molecules

SNX1, SNX2, SNX3, SNX7 and SNX9 were analysed for their tissue distribution by using a PCR-based analysis of cDNA libraries derived from 25 different human tissues and cell lines. Each of the SNX mRNA species were detected in all libraries. Other groups have also shown that SNX1, SNX2, SNX3, SNX4 and SNX5 have a broad tissue distribution by using Northern hybridization [4,30]. These SNX family members are therefore expressed in a wide range of tissues.

Mammalian SNX1 subgroup molecules are localized to early endosomes

Overexpression of SNX1 results in ligand-induced degradation of the EGF receptor [3]. Knowledge of the intracellular location of SNX1 is clearly important to an understanding of the mechanism regulating the turnover of this cell-surface receptor. We therefore analysed the steady-state distribution of SNX1 and the other human members of the SNX1 subgroup, namely SNX2, SNX4, SNX5 and SNX6. Rabbit antibodies against synthetic peptides encoding either the C-terminal or N-terminal sequences of SNX1 to SNX6 were generated. Of the anti-peptide antisera raised, only antibodies against SNX1 and SNX2 proved suitable for intracellular localization studies. However, these peptide antisera could not detect the endogenous molecule in HeLa cells. However, HeLa cells transfected with the SNX1 and SNX2 cDNA species showed positive immunofluorescence with these antisera, with a punctate staining pattern seen throughout the cytoplasm (Figure 3). To determine the intracellular localization of the other full-length SNX molecules, cDNA species encoding SNX4, SNX5 and SNX6 were engineered to include a triple FLAG epitope at their N-terminus. These epitope-tagged forms of the SNX1-related proteins were stably expressed in human HeLa cells and the expressed products were detected by indirect immunofluorescence with a monoclonal antibody against the FLAG epitope (Figures 3c–3e). The staining patterns of the tagged SNX molecules were all very similar, with punctate cytoplasmic distributions similar to those of SNX1 and SNX2. In addition, co-staining experiments with transiently expressed SNX1 and SNX2 in the stable HeLa cell lines expressing the FLAG-tagged SNX4, SNX5 and SNX6 showed high levels of co-localization of the different SNX proteins on the same punctate structures (results not shown).

To determine whether the SNX proteins were localized within the endosomal pathway, co-staining experiments were performed with an endocytic fluid-phase marker. Biotinylated dextran 10000 was incubated with the stable HeLa cell lines expressing the different SNX molecules for 30 min at 37 °C to allow for fluid-phase uptake in multiple endocytic compartments. The cell monolayers were then fixed and surface-blocked with biotin conjugated with avidin before membrane permeabilization. The intracellular biotinylated dextran was detected with steptavidin-Cy3 and the SNX proteins were detected with either the anti-peptide antisera or the anti-FLAG M2 monoclonal antibody. All members of the SNX1 subgroup showed extensive co-localization with the endosomal compartments defined by the fluid-phase marker (results not shown).

To determine whether the SNX1 subgroup molecules were localized to the endosome compartment, dual staining was performed with the defined early endosome marker EEA1 [35]. Figure 4 shows the dual staining of SNX1, SNX2, SNX4, SNX5 and SNX6 with EEA1. Significantly, SNX1, SNX2, SNX4, SNX5 and SNX6 all showed co-localization with EEA1, indicating that these SNX molecules were associated with early endosomes. In addition, in each case a proportion of the SNX molecules were also detected on similar-sized intracellular structures that were EEA1-negative.

As discussed above, the different SNX subgroups show differences both in the relationships of their SNX-PX domain and in their overall domain structures. For example, SNX10 is a member of the subgroup that also includes SNX3, SNX11, SNX12 and Grd19p (Figures 1 and 2). The SNX10 subgroup have short C-terminal domains that lack coiled-coil regions. To ascertain whether there was a difference between the intracellular localizations of the SNX molecules in the SNX1 and SNX10 subgroups, FLAG-tagged SNX10 was stably expressed in HeLa cells and the epitope-tagged product detected by indirect immunofluorescence. In contrast with the members of the SNX1 subgroup, SNX10 was found diffusely distributed throughout the cytoplasm, with no evidence of an association with a membrane compartment (Figure 3f).

SNX5 and SNX6 are peripheral membrane proteins

In addition to the association with endosomal membranes, immunofluorescence experiments detected a diffuse cytosolic

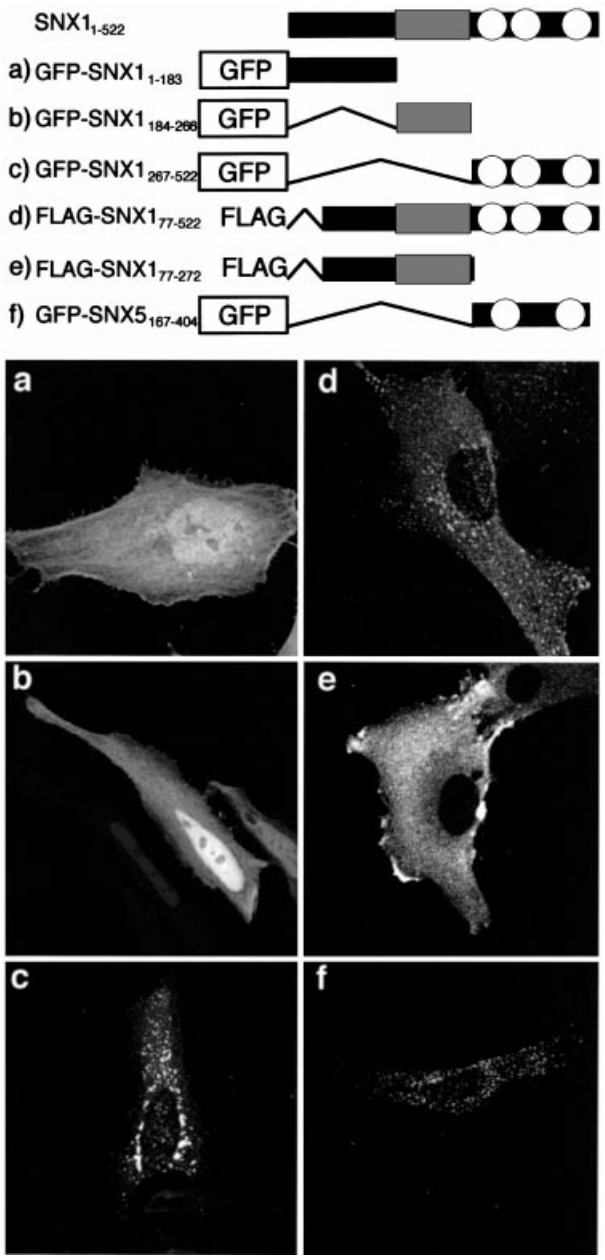

Figure 6 Endosomal localization of SNX1 and SNX5 is dependent on the C-terminal domains

HeLa cells transiently expressing the constructs pEGFP–$SNX1_{1–183}$ (**a**), pEGFP–$SNX1_{184–266}$ (**b**), pEGFP–$SNX1_{267–522}$ (**c**), pCMU-FLAG-$SNX1_{77–522}$ (**d**), pCMU-FLAG-$SNX1_{77–272}$ (**e**) and pEGFP–$SNX5_{167–404}$ (**f**) were fixed with formaldehyde. Monolayers were either observed directly for GFP fluorescence (**a**–**c**, **f**) or by indirect immunofluorescence in the presence of saponin with the M2 monoclonal antibody against the FLAG epitope, followed by FITC-labelled anti-mouse immunoglobulin (**d**, **e**).

staining for each of the SNX1 subgroup proteins (for example, see Figure 4). Immunoblotting analysis of cell extracts from the transfected cell lines, with the use of either the rabbit antisera raised against SNX1 or SNX2 or the anti-FLAG M2 monoclonal antibody against SNX4, SNX5 and SNX6, detected only one product of the expected molecular mass (results not shown). The cytosolic staining was therefore unlikely to be due to proteolytic processing of the SNX molecules. We therefore examined further the distribution of SNX5 and SNX6 molecules between the cytosol and intracellular membranes. Stably transfected HeLa cell lines expressing the FLAG-tagged SNX5 and SNX6 were homogenized in hypotonic buffer and cytosolic and membrane fractions were prepared. Immunoblotting of the fractions revealed that SNX5 and SNX6, detected as 62 and 63 kDa components respectively, were present in both the membrane and cytosolic fractions in approximately equal proportions (Figure 5). In addition, treatment of the membrane fraction with 1 M NaCl resulted in the dissociation of the FLAG-tagged SNX5 and SNX6 molecules from the membranes, as revealed by immunoblotting (results not shown). The behaviour of SNX5 and SNX6 is therefore consistent with these molecules' being peripheral membrane proteins.

C-terminal domains of SNX1 and SNX5 are required for endosomal localization

We next explored the basis for the recruitment of the SNX1 subfamily of SNX proteins to the membranes of the endosomes. The roles of the N-terminal domain, the SNX-PX domain and the C-terminal domain of SNX1 in intracellular targeting were examined by fusing each of these three domains individually to GFP. HeLa cells were transiently transfected with these three constructs and immunoblotting experiments with anti-GFP antibodies demonstrated in each case that the GFP fusion proteins were of the expected size (results not shown). The localization of the products in transfected HeLa cells was assessed by examining the fluorescence of GFP (Figure 6). Both the GFP fusion with the N-terminal SNX1 domain (GFP–$SNX1_{1–183}$) and the GFP–SNX-PX domain (GFP–$SNX1_{184–266}$) fusion proteins were distributed throughout the cytoplasm and nucleus of transfected HeLa cells (Figures 6a and 6b). These fusion proteins showed no apparent association with intracellular membrane structures. In contrast, the GFP fusion with the C-terminal domain of SNX1 (GFP–$SNX1_{267–522}$) showed an additional punctate fluorescence pattern (Figure 6c). Furthermore, GFP–$SNX1_{267–522}$ co-localized with the corresponding full-length SNX1 (results not shown), suggesting that the C-terminal domain of SNX1 is responsible for endosomal localization.

To examine further the role of the C-terminal domain of SNX1 in targeting, a deletion mutant of SNX1 was generated. To be able to distinguish the SNX1 deletion mutant from the full-length SNX1 we generated a FLAG-tagged chimaera (FLAG-$SNX1_{77–522}$) that lacked the N-terminal 77 residues, a sequence that includes the epitope detected by our rabbit polyclonal antibodies. HeLa cells were transiently transfected with the FLAG-$SNX1_{77–522}$ construct and the product was localized by indirect imunofluorescence with the FLAG-specific monoclonal antibody. The punctate pattern observed for FLAG-$SNX1_{77–522}$ was similar to the endosome staining pattern observed for SNX1 (Figure 6d) and, moreover, the FLAG-$SNX1_{77–522}$ product co-localized with the full-length SNX1 (results not shown). The deletion of the N-terminal 77 residues therefore did not affect the association of SNX1 with endosomes, a conclusion consistent with the behaviour of the GFP fusion proteins. Next we investigated the effect of deleting the C-terminal domain of FLAG-$SNX1_{77–522}$ on endosomal localization. In contrast with full-length SNX1, a construct that lacked the entire C-terminal domain of SNX1, FLAG-$SNX1_{77–272}$, was distributed throughout the cytoplasm of transfected HeLa cells (Figure 6e).

To ascertain whether the C-terminal domains of other members of the same SNX subgroup were also important in endosomal localization, a GFP fusion protein was constructed

with the C-terminal domain of SNX5, namely GFP–SNX5$_{167–404}$. The C-terminal domain of SNX5 shows no sequence similarity to the C-terminus of SNX1. HeLa cells were transiently transfected with GFP–SNX5$_{167–404}$ and an endosome-like staining pattern was observed (Figure 6f). We therefore conclude from these results that the C-terminal domains of both SNX1 and SNX5 are essential for their endosomal association.

DISCUSSION

Here we have identified a family of related proteins with the use of one previously characterized member, namely SNX1. Overall, the shared characteristics of the human SNX family include the presence of the 70–110-residue SNX-PX domain and the absence of a signal sequence or a putative transmembrane domain. Sequence comparisons of the SNX-PX domain from each family member showed more than 50% sequence similarity to the corresponding domain of SNX1. Within the large SNX family, we identified three subgroups on the basis of the sequence similarity of the SNX-PX domain and the overall domain structure. Significantly, members of each of the three subgroups have been implicated in cargo trafficking through the endosomal system of either mammals or yeast [26–29]. Additional cDNA sequences that encode SNX-PX domains were identified during the searches of the EST databases but have not been discussed owing to the absence of full-length sequences [deposited in GenBank with accession numbers AF121862 (SNX13) and AF121863 (SNX14)]. An additional SNX, SNX15, has recently been described [36] that affects endosome morphology and also influences trafficking through the endocytic pathway [37]. However, SNX15 is not a member of the subgroups defined in this paper and is therefore an additional PX-domain-containing protein involved in endosomal trafficking.

The SNX1 subgroup, consisting of SNX1, SNX2, SNX4, SNX5, SNX6, Vps5 and YJ036W, all contain large C-terminal domains with multiple coiled-coil regions. Membrane fractionation studies showed that SNX5 and SNX6 are associated with both the membrane and cytosolic fractions, which is indicative of peripheral membrane proteins. A similar membrane and cytosolic distribution has previously been reported for the other human SNX1 subgroup members, namely SNX1, SNX2 and SNX4 [4], and also the yeast SNX protein Vps5p [26,27]. In addition to the shared structural features of this SNX subgroup we have shown (1) that all human SNX1 subgroup proteins are localized to an endosome compartment, predominantly the early endosome, and (2) that membrane association for both SNX1 and SNX5 is dependent on the unique sequences found at the C-terminal domain of these molecules.

Various members of the human SNX1 subgroup, namely SNX1, SNX2 and SNX4, have been shown to interact with tyrosine kinase membrane receptors. For example, SNX1 recognizes sequences within the cytoplasmic tail of the EGF receptor that include the lysosomal targeting signal [3]. The interaction of SNX1 with the EGF receptor is dependent on the activation of the cytoplasmic tyrosine kinase domain, induced by the extracellular binding of its ligand, EGF. Recently, two other novel human SNX1 subgroup molecules, SNX2 and SNX4, have also been shown to associate with tyrosine kinase membrane receptors [4]. Vps5p, a yeast SNX1 subgroup member, seems to be necessary for the recycling of numerous membrane proteins from the prevacuolar compartment to the late Golgi. Significantly, SNX1 subgroup molecules, namely SNX1, SNX2 and Vps5p, have been identified as being part of a membrane-associated complex, termed a retromer, that contains Vps26p, Vps29p and Vps35p [5,6]. The retromer complex functions in the formation of the transport vesicles required for the retrieval of Vps10p from endosome to Golgi in *S. cerevisiae*. It remains unclear whether in mammalian cells the retromer complex functions in the same transport step as yeast, namely from endosome to TGN, but it is likely to be involved in one of the transport pathways from the endosome. It is still unresolved whether these SNX-related proteins function in the sorting of cargo into the transport vesicle or as a coat protein required for the formation of the transport vesicle.

Knowledge of the intracellular location of the SNX molecules is critical in defining their function. An early study demonstrated that SNX1 is localized to perinuclear vesicles [3]. Here we have examined the intracellular location of the SNX1 subgroup and shown that all human members of this subgroup are partly localized to early endosome membranes. Localization of these SNX proteins, with the exception of SNX1 and SNX2, was based on transfection experiments with epitope-tagged proteins. Although it is possible that the epitope tag might have resulted in aberrant localization, the localization of SNX1 and SNX2 to the early endosomes of HeLa cells with the use of anti-peptide antisera argues that the results obtained from epitope-tagged SNX molecules are valid. Our finding that SNX1, in part, accumulates on early endosomes suggests that it might function by regulating the transport of the activated EGF receptor from the early endosome to the late endosome/lysosome, where the receptor is degraded.

We also examined the basis for the specific localization of the SNX1 subgroup members to endosomal membranes. If there is a common mechanism of membrane recruitment for the SNX1 subgroup members, then a similar endosomal targeting sequence is likely to be present in all the SNX1-related molecules. If, in contrast, the specific recruitment of each of the SNX proteins to the membrane is due to interactions with different sets of molecules, then the endosomal targeting sequence is likely to differ between SNX molecules. Indeed, our results showed that the C-terminal domains of both SNX1 and SNX5 are necessary and sufficient for endosomal localization; it seems that the endosomal targeting sequence differs between SNX molecules. A role for the C-terminal domain in membrane targeting is also supported by the finding that SNX10, which has only a small C-terminal domain, was not recruited to intracellular membranes but was instead located diffusely throughout the cytoplasm of transfected HeLa cells. In addition, SNX3, another member of the same subgroup as SNX10, was also found predominantly in the cytosol of transfected NIH 3T3 cells [4]. Thus members of the SNX10 subgroup that have been implicated in membrane cargo sorting in yeast, for example Grd19p [29], might not interact with membranes directly but could nevertheless be recruited by interactions via other cytosolic proteins. The finding that the C-terminal domain of SNX1 is crucial for endosome recruitment *in vivo* is of particular interest in view of the earlier report [3] that the C-terminal 58 residues of SNX1 interacted with the cytoplasmic domain of the EGF receptor in a yeast two-hybrid assay. It therefore seems likely that membrane recruitment of the SNX1 subgroup molecules might be due to an interaction with cargo and also possibly interactions with other members of retromer protein complexes.

The similar intracellular localization of the five human proteins from the SNX1 subgroup to predominantly one intracellular organelle, the early endosome, distinguishes these molecules from other protein families involved in membrane trafficking, such as Rab GTPases, which perform similar functions on distinct intracellular organelles. This location of the SNX1 subgroup indicates that these SNX proteins are unlikely to be involved in a mechanism common to all transport steps, such as the formation

or the docking of transport vesicles. It is more likely that the function of the SNX family is unique to endosomes. As mentioned, a number of the SNX molecules have now been shown to interact directly with cargo and might therefore have a direct role in cargo trafficking. Thus different SNX proteins might sort different sets of cargo within the early endosome for subsequent transport to various locations. This suggestion is supported by (1) the lack of sequence similarity of the 60-residue C-terminal domain of SNX1, responsible for binding to the EGF receptor, to the C-terminal domains of other members of the SNX family and (2) defects in the yeast SNX gene products that show cargo selective phenotypes [29]. The challenge now is to define in more detail the molecular basis of cargo specificity and the transport steps promoted by the different SNX molecules.

We thank Jackson Wan and Steve Franks for support in searching the EST databases; Arne Huvar and Ranelle Salunga for assistance with the 5′ RACE and tissue distribution experiments respectively; Rene Huvar, Kathy Whitmeyer and all members of the DNACore, Vivarium and Protein Chemistry facilities at R. W. Johnson Pharmaceutical Research Institute for their technical support; Dr. Michael Jackson, Dr Mark Erlander and Dr Per Peterson for helpful discussions; Professor Ban Hock Toh for the antiserum against EEA1; and Dr Melvena Teasdale for critical reading of the manuscript. R.D.T. was supported by NHMRC Howard Florey Centenary Fellowship (no. 987210).

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Received 13 March 2001/21 May 2001; accepted 13 June 2001