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Association of Mouse Sorting Nexin 1 with Early Endosomes[1]

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Sorting nexin 1 (SNX1) is a protein that binds to the cytoplasmic domain of plasma membrane receptors. We found that mouse sorting nexin 1 (SNX1) (521 amino acid residues) could partially rescue a yeast *vam3* mutant defective in docking/fusion of vacuolar membranes. In mammalian cells, SNX1 is peripherally associated with membrane structures and localized immunochemically with EEA1, a marker protein of early endosomes. These results suggest that SNX1 regulates endocytic trafficking of plasma membrane proteins in early endosomes. Gel filtration of cell lysates and the purified recombinant protein, together with two-hybrid analysis, indicated that SNX1 self-assembles into a complex of ~300 kDa.

Key words: early endosome, membrane trafficking, self-assembly, sorting nexin 1.

Endosomes are involved in the traffic between the plasma membrane and lysosomes, and between the *trans*-Golgi network (TGN) and lysosomes (*1–3*). They are classified into at least two morphologically and functionally distinct compartments, early and late endosomes. In receptor-mediated endocytosis, ligand/receptor complexes are first transported to early endosomes before targeting to their destination. EGF (epidermal growth factor) and its receptor are then delivered to lysosomes for degradation (*4*), whereas transferrin and low-density lipoprotein receptors are recycled back to the plasma membrane (*5*).

Previous studies revealed that sorting nexin 1 (SNX1) is a protein that binds to the cytoplasmic domain of the EGF receptor (*6*). SNX1 is able to associate with a variety of receptors, including those for transferrin, insulin, platelet-derived growth factor, and leptin (*7*). Many human SNX1-related proteins have been identified based on the sequence similarities (*7–10*). All the proteins contain an approximately 100-amino-acid region, termed the phox homology (PX) domain (*11*). This domain is also found in five yeast proteins, Vps5p, Vps17p, Mvp1p, Grd19p, and Vam7p, involved in post-Golgi membrane trafficking (*12–16*). SNX1 shows the highest sequence similarity to Vps5p, which is required for the sorting and retrograde transport at prevacuolar compartment (*14, 17*), suggesting that SNX1 has a similar function in mammalian cells.

The biogenesis of yeast vacuoles, a lysosome-like compartment, has been studied by combined biochemical and genetic approaches (*18*). Vam3p (*19*) and Pep12p (*20*) are syntaxin-related proteins acting on vacuolar and late endosomal compartments, respectively. Mutants of these genes have contributed to the identification of *Ardbidopsis thaliana* vacuolar/prevacuolar syntaxins, AtVam3p and AtPep12p, respectively (*21, 22*). Furthermore, mouse syntaxin 7 can complement the *vam3* mutant, and its functional site was shown to be late endosomes (*23*). In this study, we found that the mouse SNX1 could partially rescue yeast *vam3* mutant. We demonstrated that SNX1 forms a homooligomer within murine cells. Immunofluorescence microscopy revealed that SNX1 is associated with early endosomes. These results suggest that SNX1 plays important roles in membrane trafficking through early endosomes.

EXPERIMENTAL PROCEDURES

Antibodies and Other Materials—The monoclonal antibodies for lamp-2 (rat clone GL2A7) and EEA1 (mouse clone 14) were purchased from the Developmental Studies Hybridoma Bank (University of Iowa) and Transduction Laboratories (Lexington, KY), respectively. Cy3- and FITC-conjugated secondary antibodies were from Jackson ImmunoResearch (West Grove, PA). Anti-SNX1 antibodies were generated by immunizing a rabbit with bacterially synthesized mouse SNX1 fused with maltose-binding protein (MBP), and purified by affinity-chromatography (*24*). The Mouse Multiple Tissue Northern Blot was from Clontech. Cell culture reagents were obtained from Gibco BRL. Other chemicals were from Sigma, unless otherwise specified.

Mammalian Cell Culture and Yeast Experiments—The media used for cell culture were: DMEM supplemented with 10% fetal calf serum (FCS), NIH3T3, and BHK cells;

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Abbreviations: DMEM, Dulbecco's modified Eagles' medium; FITC, fluorescein isothiocyanate; MBP, maltose-binding protein; PCR, polymerase chain reaction; PX domain, phox homology domain; RT-PCR, reverse transcription polymerase chain reaction; SNX1, sorting nexin 1.

F12 medium containing 10% and 5% FCS, NRK and CHO, respectively; and DMEM supplemented with 10% FCS, 0.4 μM α-melanocyte–stimulating hormone (Calbiochem), and 0.5 mM isobutylmethylxanthine, B16 cells. All media contained non-essential amino acids, 100 units/ml penicillin, and 100 μg/ml streptomycin.

Yeast culture, transformation, and Western blotting were performed as described previously (*25*). The strains used were YPH499 (*26*) and MHS3-4D (YPH499, *vam3-Δ1::HIS3*) (*22*). Cells were observed under an Olympus BX50 Microscope equipped with differential interference contrast optics.

cDNA Cloning—Poly(A)$^+$ RNA extracted from a 13.5 dpc mouse (C57BL/6J) embryo was used to construct a cDNA library on a yeast expression plasmid, pKT10 (*27*). The library was introduced into yeast *Δvam3* cells by the modified lithium acetate method (*28*). Ura$^+$ transformants were selected in a synthetic medium comprising 2% glucose, 0.5% casamino-acids, 40 μg/ml tryptophan, and 10 μg/ml adenine. Candidate Vam$^+$ colonies were identified by red pigmentation due to the accumulation of *ade* fluorochrome in vacuoles, and vacuolar morphology was subsequently verified by fluorescence microscopy (*23*). Plasmids were recovered from the transformants and the cDNA fragment was sequenced.

Reverse Transcription (RT) Polymerase Chain Reaction (PCR)—Poly(A)$^+$ RNA from mouse (C3H/HeSnJ) liver and a BcaBEST™ RNA PCR Kit (Takara) were used to analyze the splicing variants of SNX1 after synthesizing first strand DNA with an oligo (dT) primer. PCR primers used were: A-Fw (–61–37 bp, numbered from the first letter of initiation codon of mouse SNX1 cDNA) and D-Rv (1926–1902 bp) for the full length; A-Fw and A-Rv (586–562 bp), region A of the cDNA; B-Fw (507–531 bp) and B-Rv (1119–1095 bp), region B; C-Fw (1040–1064 bp) and C-Rv (1599–1583 bp), region C; and D-Fw (1583–1599 bp) and D-Rv (1926–1902 bp), region D.

Immunofluorescence and Electron Microscopy—Fixed cells were permeabilized (*23*), incubated with the antibodies against SNX1, lamp-2, or EEA1, and then reacted with fluorescently labeled secondary antibodies. Samples were mounted on VectaShield (Vector Laboratories, Burlingame, CA) and visualized using a laser scanning confocal imaging system (LSM510, Carl Zeiss). A Hitachi H-7000 Electron Microscope was used for negative staining.

Cell Fractionation—Cells were washed with phosphate-buffered saline, then lysed in 10 mM HEPES-KOH buffer, pH 7.4, containing 1 mM phenylmethylsulfonyl fluoride and the Complete™ proteinase inhibitor cocktail (Boehringer Mannheim). Homogenization was performed by ten passages through a syringe equipped with a 26G needle. Postnuclear supernatant was mixed with an equal volume of 2% Triton X-100 or 0.4 M Na_2CO_3 (pH 11.3), incubated for 30 min at 4°C, and then centrifuged at 100,000 ×*g* for 30 min. The resulting supernatant and pellet were analyzed immunochemically after precipitation with 10% trichloroacetic acid. The postnuclear supernatant was also centrifuged at 100,000 ×*g* for 30 min without the addition of Triton X-100 or Na_2CO_3, and the resulting supernatant (400 μg protein in 200 μl) was subjected to Diol300 gel filtration chromatography (Shimadzu).

Yeast Two-Hybrid Analysis—The two-hybrid analysis was conducted with Matchmaker two-hybrid system 3 (Clontech). The cDNA fragment for the entire coding region of SNX1 was inserted into pGBKT7 and pGADT7 to generate fusion genes with the DNA-binding domain and activation domain of Gal4p, respectively. Parts of SNX1 cDNA coding Met-1–Arg-271 (amino terminal half), Ala-163–Ser-521 (carboxyl terminal half), and Ala-163–Arg-271 (PX domain) were fused, respectively, in frame with the GAL4-BD in pGBKT7. The resulting plasmids and control vectors were introduced into a yeast strain AH109 (*MATa ade2 his3 leu2 lys2 trp1 ura3 gal4 gal80 LYS2::GAL1-HIS3 GAL2-ADE2 URA3::MEL1-lacZ*). The interactions of the expressed proteins were assayed by the growth of transformants on medium lacking both adenine and histidine.

Purification of SNX1—The coding region of SNX1 was introduced into the *Eco*RI–*Sal*I sites of MBP (maltose binding protein)-expression vector pMAL-c2 (New England BioLabs). The resulting plasmid, pMAL-SNX1, produces a fusion protein with the amino acid sequence of MBP-"linker peptide (IEGRISEF)"-SNX1 (Factor Xa cleaves between R and I within the linker). The MBP-SNX1 fusion protein was affinity-purified using amylose-resin (New England BioLabs), followed by ammonium sulfate precipitation (33–45% saturation). The fusion protein was incubated with Factor Xa (New England BioLabs, 10 μg Factor Xa/mg MBP-SNX1) for 16 h at room temperature, and the resulting SNX1 was obtained using a Diol300 gel column.

RESULTS

Rescue of Yeast vam3 Mutant by SNX1—We searched for a mouse protein(s) which can rescue the yeast *vam3* mutant having a defect in vacuolar syntaxin (*19, 29*), and found that mouse syntaxin 7 can carry out the Vam3 function (*23*). In this study, we observed that a cDNA encoding a protein homologous to human sorting nexin 1 (SNX1) could partially rescue the mutant. The identity between the human and mouse proteins (521 amino acid residues) was 93.5%. Human SNX1 was identified as a molecule which

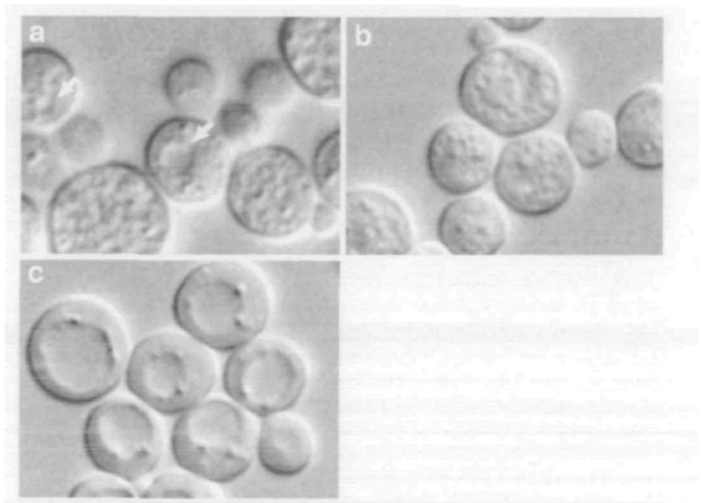

Fig. 1. **SNX1 weakly complements the yeast *vam3* mutant.** SNX1-expression plasmid pKT-SNX1 (a) could partially restore the vacuolar morphology (arrows) of *Δvam3* cells. The results of a control experiment with vector plasmid pKT10 (b) in *Δvam3* and the wild-type (c) are also shown. Photographs were taken under a differential interference contrast microscope.

binds to plasma membrane receptors (*6, 7*). The introduction of mouse SNX1 weakly restored the vacuolar morphology of the *vam3* mutant: approximately 10% of the total *vam3* cells expressing SNX1 showed vacuolar structures significantly larger than those of the mutant carrying a control vector plasmid (Fig. 1). SNX1 exhibits limited homology with Vam3p, indicating that the partial rescue may not reflect genetic complementation. This notion prompted us to study mouse SNX1 in more detail.

Tissue Distribution and Splicing Variant of SNX1—SNX1 transcripts of 2.2 kb were detected in mouse tissues on Northern blotting (Fig. 2a). The transcript amount was high in testis and kidney; intermediate in heart, brain, spleen, lung, and liver; and the lowest in skeletal muscle (Fig. 2a).

Two human splicing variants, SNX1 and SNX1A, have been reported, SNX1A having a deletion of 65 amino acids (Fig. 2b, shaded region) (*7*). However, we found no splicing variants as to the coding region of mouse liver mRNA (Fig. 2a). Amplification of the entire transcript by RT-PCR yielded fragments of 2.0 and 1.8 kbp, the former being consistent with the size of the cDNA (Fig. 2b, lane E). Sequencing analysis revealed that they code for the same SNX1 molecule (data not shown). Furthermore, we performed RT-PCR analysis of the four regions (regions A–D, Fig. 2b) of the cDNA, and found a splicing variant only as to the 3′-untranslated region.

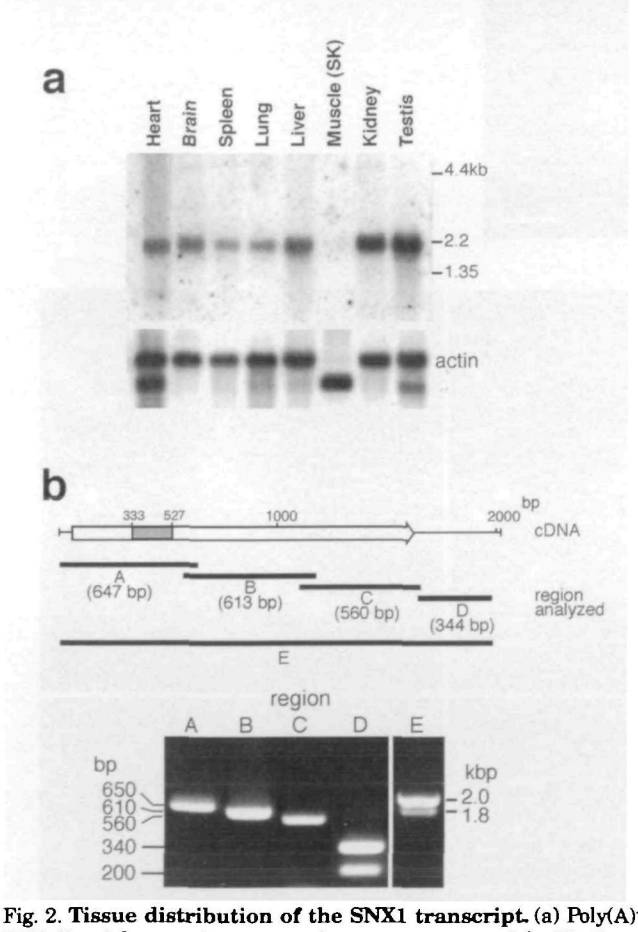

Fig. 2. **Tissue distribution of the SNX1 transcript.** (a) Poly(A)$^+$ RNA (2 μg) from various mouse tissues was processed for Northern blotting using a probe of the SNX1 coding region. (b) RT-PCR analysis of the mouse SNX1 transcript. RT-PCR was performed using poly(A)$^+$ RNA from C3H/HeSnJ mouse liver. Sets of primers were designed to amplify fragments corresponding to regions A, B, C, and D. Upper line represents mouse SNX1 cDNA, where the SNX1 coding region is indicated by an open box. Shaded box corresponds the region deleted in human SNX1A. The results of the gel electrophoresis of RT-PCR products are shown with the estimated sizes. Two DNA fragments of 2.0 and 1.8 kbp were amplified using primers for the full-length SNX1 transcript (lane E). A splicing variation was found only in the 3′-untranslated region (lane D).

The broad expression spectrum of SNX1 shown by Northern blotting (Fig. 2a) was confirmed at its product level. The antibody against the SNX1 recognized a single 68-kDa protein band in lysates of mouse tissues (Fig. 3a), and cell lines from mouse (NIH3T3), rat (NRK), and hamster (CHO, BHK) (Fig. 3b). Thus, SNX1 is a ubiquitously expressed protein.

Association of SNX1 with Early Endosomes—Upon centrifugation of an NIH3T3 or NRK cell lysate at 100,000 ×*g*, about 20 and 80% SNX1 was recovered in the pellet and supernatant fraction, respectively (control, Fig. 3c). Incubation of the lysate with 1% Triton X-100 or 0.2 M Na_2CO_3 (pH 11.3) released essentially all SNX1 into the soluble fraction (Fig. 3c), suggesting that SNX1 is a soluble protein able to associate with membrane compartments.

Anti-SNX1 antibodies stained numerous small organelles in cultured cells. They were abundant in the perinuclear region (Fig. 4), and essentially all of them were also stained with antibodies against EEA1, a marker protein for early endosomes (*30, 31*) (Fig. 5, a–c). In contrast, the distribu-

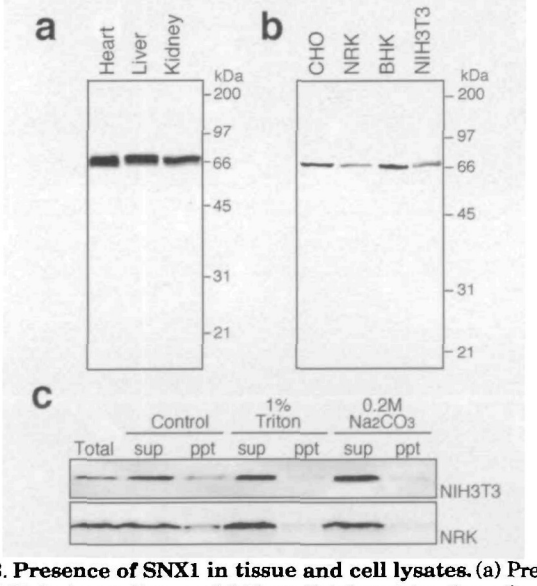

Fig. 3. **Presence of SNX1 in tissue and cell lysates.** (a) Presence of SNX1 in heart, liver, and kidney. Total proteins from the three mouse tissues were subjected to gel electrophoresis in the presence of sodium dodecylsulfate, then Western blotting using affinity-purified anti-SNX1 antibodies. (b) Presence of SNX1 in cell extracts. Lysates of CHO, NRK, BHK, and NIH3T3 cells were analyzed by Western blotting using anti-SNX1 antibodies. (c) Association of SNX1 with the membrane fraction. Postnuclear supernatants of NIH3T3 and NRK lysates were incubated in the presence of 1% Triton X-100 or 0.2 M Na_2CO_3 (pH 11.3), followed by centrifugation at 100,000 ×*g* for 30 min. The resulting supernatants (sup) and pellets (ppt) were subjected to Western blotting with anti-SNX1 antibodies. About 20% (estimated by densitometry) of SNX1 was recovered in the pellet when incubated without Triton X-100 or Na_2CO_3 (control, ppt).

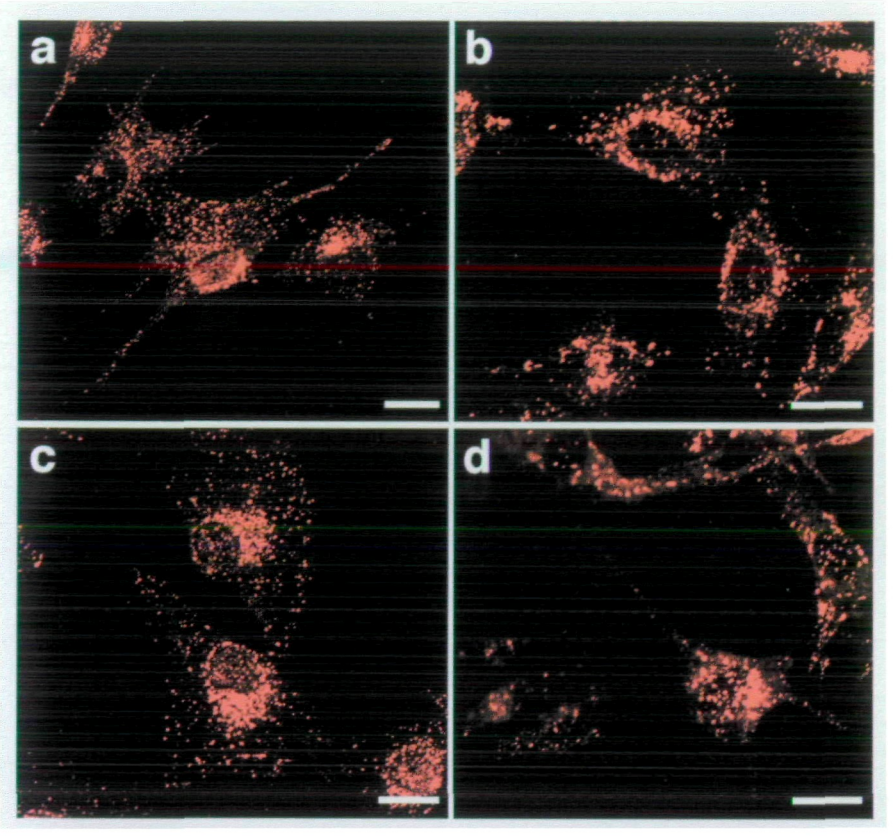

Fig. 4. **Intracellular localization of SNX1 in different cell lines.** Cells were fixed, permeabilized, and stained with anti-SNX1 antibodies, then incubated with Cy3-labeled anti–rabbit IgG and processed for confocal microscopy. The results for four cell lines are shown: (a) NIH3T3; (b) CHO; (c) NRK; (d) B16 mouse melanoma cell line. Bars indicate 20 μm.

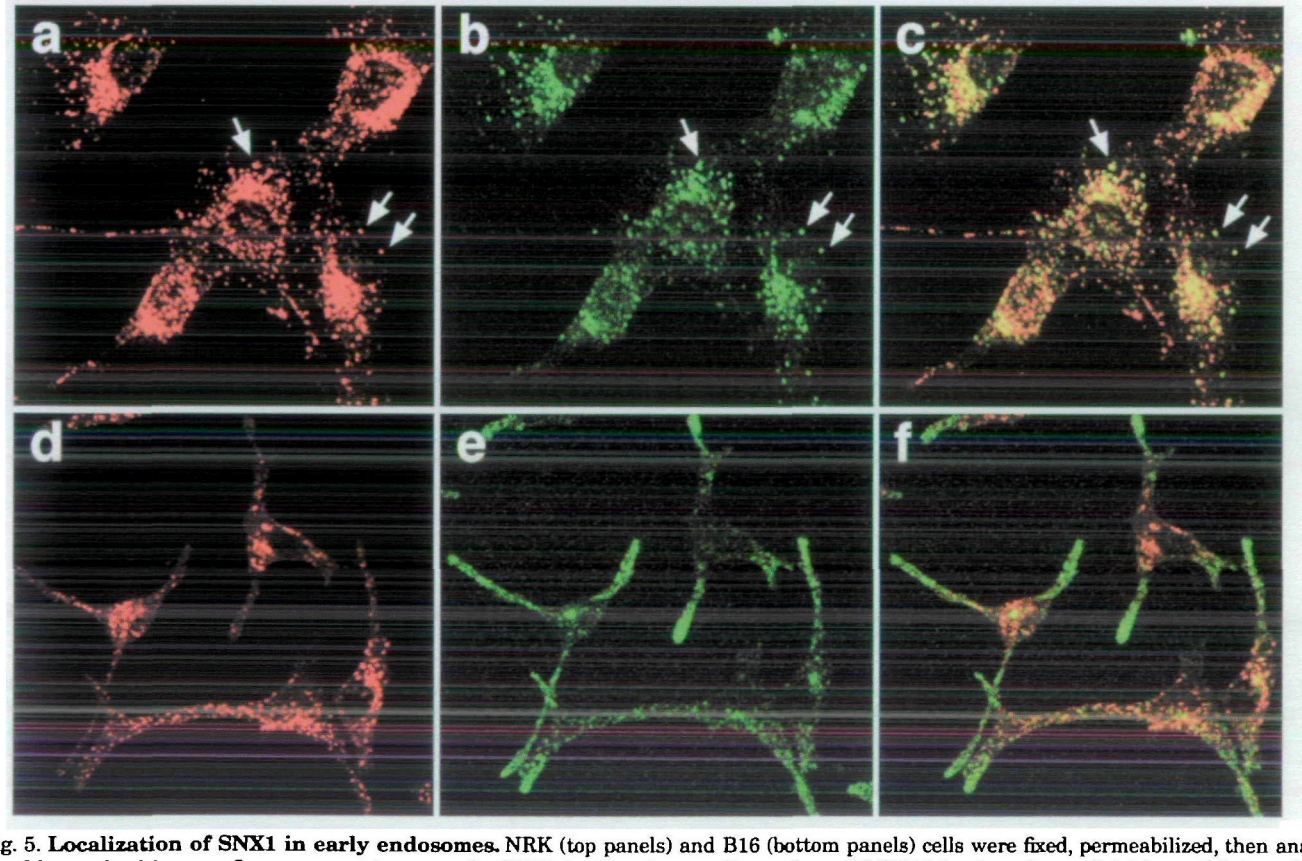

Fig. 5. **Localization of SNX1 in early endosomes.** NRK (top panels) and B16 (bottom panels) cells were fixed, permeabilized, then analyzed by confocal immunofluorescence microscopy for SNX1 (a, d) and organelle markers: (b) EEA1 (early endosome); (e) lamp-2 (late endosome, lysosome). The signals were merged: c, for a and b; f, for d and e. The regions of co-localization of SNX1 and EEA1 are indicated by arrows (a–c).

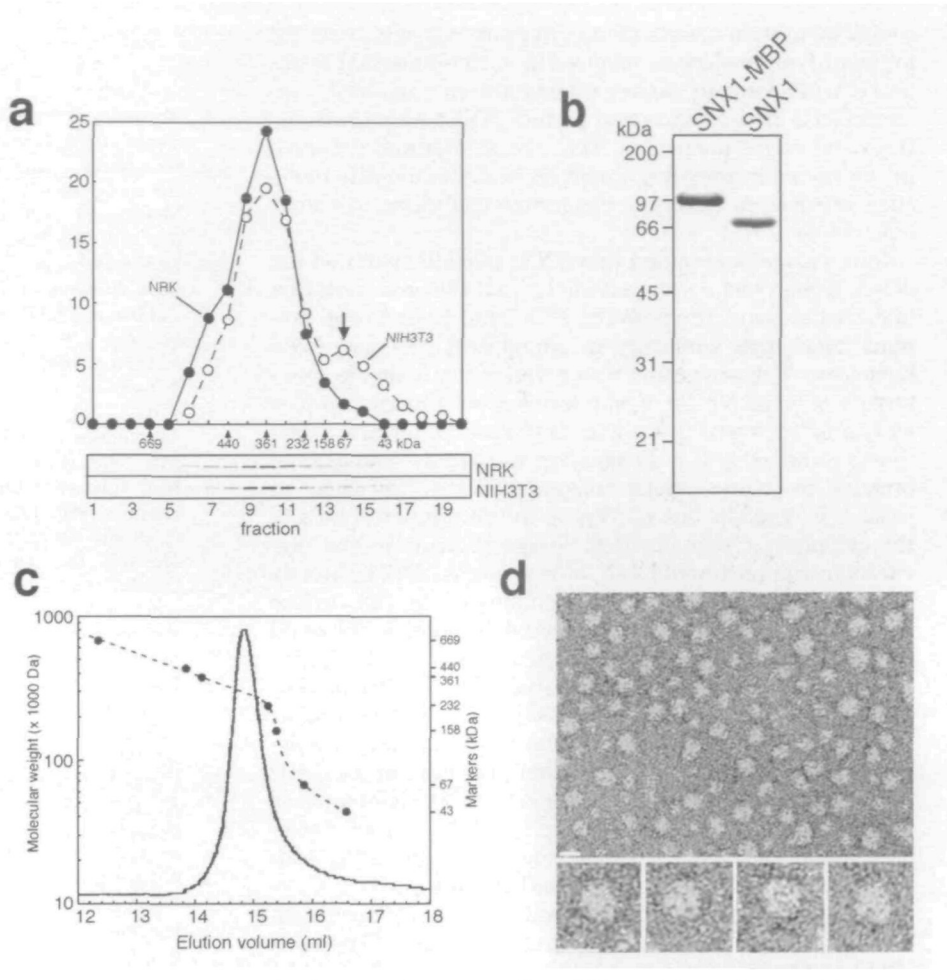

Fig. 6. **Formation of the SNX1 homo-oligomer.** (a) Gel filtration of NRK and NIH3T3 cell extracts. Post-nuclear supernatants obtained from NRK (closed circles and solid line) and NIH3T3 (open circles and dashed line) cells were centrifuged at 100,000 ×*g* for 30 min, and the resulting supernatants (400 μg protein in 100 μl) were applied to a Diol300 gel filtration column. Fractions collected were subjected to Western blotting using antibodies against SNX1 (lower panels). The amount of SNX1 in each fraction was determined with NIH Image software (version 1.62) (upper panel). Arrow indicates the fraction corresponding to a monomeric form of SNX1. The positions of marker proteins are shown (arrowheads): 669 kDa, thyroglobulin; 440 kDa, ferritin; 361 kDa, F_oF_1 ATPase; 232 kDa, catalase; 158 kDa, aldolase; 67 kDa, serum albumin; 43 kDa, ovalbumin. (b) Gel electrophoresis of the purified SNX1. The purified recombinant SNX1 or SNX1-MBP fusion protein was subjected to gel electrophoresis and stained with Coomassie Brilliant Blue. (c) Gel filtration of the purified SNX1. The purified recombinant SNX1 (4 μg) was applied to a Diol300 column calibrated with standard proteins indicated by closed circles. The solid line shows the elution of the recombinant SNX1. Positions of marker proteins are shown (closed circles). (d) Electron microscope images of the SNX1 homo-oligomer. The purified recombinant SNX1 was negatively stained with uranyl acetate. Higher magnification of representative SNX1 particles is shown in the lower panels. The bar indicates 20 nm.

tion of lamp-2, a marker protein for late endosomes and lysosomes (*32*), was clearly different from that of SNX1 in mouse melanoma B16 cells, in which lamp-2–positive compartments are located in the dendric arbors of the cells (Fig. 5, d–f). These results indicated that SNX1 is localized to the early endosomes but not to the late endosomes and lysosomes.

Formation of an Oligomeric Complex of SNX1—We examined the molecular sizes of SNX1 in NRK and NIH3T3 cells. The supernatants of NRK and NIH3T3 cell lysates were subjected to gel filtration, and the eluates were assayed for SNX1 (Fig. 6a). Most SNX1 was recovered in fractions corresponding to ~320 kDa in molecular size. Only a small portion (<5% of total SNX1 applied) was found in a fraction corresponding to a monomeric form (Fig. 6a, arrow).

We examined the possibility of homo-oligomer formation of SNX1 using the yeast two-hybrid system. The yeast cells (Ade^- His^-) could grow in the absence of adenine and histidine when SNX1 fusion proteins with Gal4-activation and DNA-binding domains were introduced. This results suggest that SNX1 could form a homo-oligomer. With deletion of the amino (Met-1–Val-162) or carboxyl-terminal (Ala-272–Ser-521) half of SNX1, there was no positive growth, indicating the importance of the entire region for homo-oligomer formation.

The purified recombinant MBP-SNX1 (fusion protein with MBP) and SNX1 were recovered in fractions corresponding to ~97 and ~60 kDa, respectively, on gel electrophoresis in the presence of sodium dodecylsulfate (Fig. 6b). However, upon gel filtration without the detergent, the purified SNX1 was eluted in fractions corresponding to ~300 kDa, slightly smaller than the size found for cell extracts, and no apparent peak for a monomer was detected (Fig. 6c). Since the calculated molecular size of SNX1 is 59 kDa, the SNX1 homo-oligomer is most likely a pentamer. As shown in Fig. 6d, the purified SNX1 was a spherical particle with an average diameter of approximately 15 nm, this being consistent with the molecular size determined on gel filtration.

DISCUSSION

Yeast *vam* mutants show defects in vacuolar morphology and protein transport to the vacuole (*19, 29*). The *VAM* gene products are localized on the vacuolar membrane, and thought to regulate the docking and fusion of the vacuolar membranes (*19, 25, 33–35*). The *Arabidopsis thaliana* vacuolar (AtVam3p) and mouse late endosomal (syntaxin 7) syntaxins can complement *vam3* mutants (*22, 23*). The

complementation assays of *vam* mutants are highly useful for identifying molecules involved in vacuo-lysosomal membrane trafficking in higher organisms. In this study, we identified a mouse endosomal protein, SNX1 which restores the yeast *vam3* phenotype. Thus, yeast mutants defective in the vacuolar assembly would be useful to identify molecules involved in endocytic membrane trafficking in higher eukaryotes.

It was surprising to find that SNX1 partially restored the defect of the yeast *vam3* mutant, but not the *vps5* mutation (data not shown), although the SNX1 and yeast Vps5p proteins show high similarity in amino acid sequence (57% homology). This suggested that SNX1 is not a simple counterpart of yeast Vps5p. Vps5p forms a multimeric complex with Vps17p, Vps26p, Vps29p, and Vps35p, termed a retromer complex, and is involved in retrograde transport of proteins from prevacuolar compartments to late Golgi in yeast (*17*). Vps35p, but not Vps5p in the complex, binds to the cytoplasmic domain of the cargo proteins in the prevacuolar compartments (*17, 36*), whereas SNX1 directly interacts with the cytoplasmic domains of the cell surface receptors (*6, 7*). Possibly due to this difference, SNX1 could not complement the *vps5* mutation.

The SNX1 and Vam3 proteins show only limited similarity: a domain of Vam3p (Ser-31–Gln-111) is 20% similar with SNX1 (Ala-424–Gln-505). The *vam3* mutants are defective in membrane transport from pre-vacuolar compartments and late Golgi to the vacuole (*19, 33*). Expression of mouse SNX1 may disturb the function of the retromer complex at the pre-vacuolar compartments in yeast cells, leading to the partial rescue of the *vam3* mutant.

Immunochemical studies showed that SNX1 is co-localized with a marker protein of early endosomes. The anti-SNX1 antibody did not stain plasma membrane, late endosomes and lysosomes. This is the first direct observation of endogenous SNX1 localization. In this regard, Kurten *et al.* (2001) recently showed that overexpressed GFP-SNX1 could be localized to the early endosomes (*37*). Previous studies observed that overexpression of SNX1 accelerated degradation of EGF receptor (*6, 38*). Taken together, these suggest that SNX1 regulates endocytic trafficking, possibly at the sorting step of recycling receptors.

Yeast two-hybrid analysis showed that SNX1 could form a homo-oligomer. The recombinant SNX1 forms a homo-oligomer (~300 kDa), possibly a pentamer, judging from the molecular weight calculated from the amino acid sequence and measured by gel filtration. Consistent with this, we found an SNX1 oligomer (320–380 kDa) but no apparent monomeric form in NRK and NIH3T3 cells, suggesting that SNX1 forms a stable oligomer in cytosol. The slight difference in size suggests the presence of additional component(s) in the complex *in vivo*. We also observed particles of the recombinant SNX1 (~15 nm in diameter) under electron microscopy. It would be of interest to determine whether or not other SNX family proteins (*7–10*) also form oligomeric structures, and whether the PX domain is involved in the complex formation.

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