Sorting Nexin 6, a Novel SNX, Interacts with the TGF-β Family of Receptor Serine-Threonine Kinases

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Running Title: Interaction of SNX6 with the TGF-B Family of Receptor

SUMMARY

Sorting nexins (SNX) comprise a family of proteins with homology to several yeast proteins, including Vps5p and Mvp1p, that are required for the sorting of proteins to the yeast vacuole. Human SNX1, 2, and 4 have been proposed to play a role in receptor trafficking and have been shown to bind to several receptor tyrosine kinases, including receptors for EGF, PDGF, and insulin as well as the long form of the leptin receptor, a GP130 associated receptor. We now describe a novel member of this family, SNX6, which interacts with members of the transforming growth factor-beta (TGF-B) family of receptor serine-threonine kinases. These receptors belong to two classes-type II receptors that bind ligand, and type I receptors that are subsequently recruited to transduce the signal. Of the type II receptors, SNX6 was found to interact strongly with ActRIIB and more moderately with wild-type and kinase defective mutants of TBRII. Of the type I receptors, SNX6 was found to interact only with inactivated TβRI. SNXs 1-4 also interacted with the TGF- β receptor family, showing different receptor preferences. Conversely, SNX6 behaved similarly to the other SNX proteins in its interactions with receptor tyrosine kinases. Strong heteromeric interactions were also seen among SNX1, 2, 4, and 6, suggesting the formation *in vivo* of oligometric complexes. These findings are the first evidence for the association of the SNX family of molecules with receptor serine-threonine kinases.

INTRODUCTION

The transforming growth factor (TGF- β) family is comprised of a large number of peptides, including the TGF-Bs themselves, activin/inhibin, the bone morphogenetic proteins (BMPs), the growth and differentiation factors (GDFs), glial-derived neurotrophic factor (GDNF), and Mullerian Inhibitory Substance (MIS) (1). Although there are no yeast TGF- β s, homologues have been identified in primitive metazoans, including C. elegans and Drosophila (2-4). With the exception of only GDNF, these ligands signal through heterotetrameric pairs of serine-threonine kinase receptors. Ligand first interacts with a type II receptor, which, following ligand binding, recruits a type I receptor (5). The type II receptors are constitutively active kinases, catalyzing phosphorylation both of themselves in an autocatalytic reaction and of the recruited type I receptor (6). Once bound to ligand and phosphorylated by the type II receptor, the type I receptor then transduces the signal to the intracellular signaling intermediates, including the recently described family of Smad proteins (7-13). In general, one or two closely related type I and one or two closely related type II receptors are utilized by each class of ligand. For example, TGF- β 1 and TGF- β 3 bind to the type II TGF- β receptor (T β RII), with subsequent recruitment of the type I TGF-B receptor (TBRI/ALK5 (activin-like kinase 5) (6). Similarly, activin binds to either ActRII or ActRIB, with ActRIB (ALK4) or possibly ActRI (ALK2) then joining the complex (14-19). BMPs typically bind to their type II receptor (BMPRII) and then recruit either BMPRIA (ALK3) or BMPRIB

(ALK6) (14;20), but they can also bind to the activin type II receptors, in which case they recruit ActRI (ALK2) (21).

Despite extensive work on characterization of the ligand binding affinities of the various receptors and determination of downstream signaling following ligand binding, relatively little is known about the trafficking of TGF- β receptors within the cell. Experiments in mink lung epithelial cells have shown that endogenous T β RI and T β RII move from the ER to the cell surface along independent, non-intersecting pathways, so that no heteromeric complex formation occurs prior to the expression of these receptors on the cell surface (22;23). For each receptor, surface binding of ligand both decreases receptor half-life (22;23) and also results in downregulation of receptor surface expression (24-27). The pathways available to individual receptors following internalization have not yet been characterized.

The sorting nexins are a family of cytoplasmic and membrane associated proteins that are hypothesized to function in the intracellular trafficking of plasma membrane receptors. The first sorting nexin, SNX1, was cloned in a yeast two-hybrid assay as an interactor with the cytoplasmic domain of the EGF receptor (28). SNX1 was subsequently shown to be homologous to Vps5p, a yeast protein essential for the correct targeting of carboxypeptidase Y and other soluble hydrolases from the *trans*-Golgi network through an endosomal/prevacuolar compartment to the yeast vacuole (29;30). Given this function for the yeast SNX1 homologue, SNX1 itself was proposed to target the EGF receptor for lysosomal degradation through an endocytic pathway. Four additional sorting nexins have subsequently been cloned (31). SNX2, SNX3, and SNX4 were identified in database searches through homology with SNX1. Studies with these

4

four proteins have shown that SNX1, SNX2, and SNX4 bind to multiple receptor tyrosine kinases including receptors for EGF, PDGF, and insulin, and to the long form of the leptin receptor (31). SNX1 additionally binds to the transferrin receptor. Furthermore, SNX1, SNX2, and SNX4 oligomerize with each other (31). SNX3 is distinguished from the other SNXs in that it does not associate with any of the receptors studied or with any of the other SNXs (31). The most recently reported sorting nexin, SNX5, was cloned using the yeast two-hybrid system as an interactor with the Fanconi anemia complementation group A protein (4).

In this paper we report the cloning of a novel sorting nexin, SNX6. This molecule was identified in a yeast two-hybrid screen to identify binding partners of Smad1, but studies in mammalian cells have shown that the interaction with this Smad protein is very weak, and that, instead, SNX6 shows strong interactions with several members of the TGF- β family of receptor serine-threonine kinases, as well as with receptor tyrosine kinases shown previously to interact with other SNX proteins. We also show that SNX6 can hetero-oligomerize and colocalize intracellularly with SNX1, SNX2 and SNX4, and that these other SNXs also associate with members of the TGF- β receptor family. These studies are the first to document the interaction of sorting nexins with receptor serine-threonine kinases, thus broadening the range of receptor interactions with the SNX family.

EXPERIMENTAL PROCEDURES

Cell Culture—COS-1, COS-7, and HepG2 cells were maintained in Dulbecco's modified Eagle medium (DMEM) with 10% FBS and antibiotics (100 U/ml penicillin and 100 µg/ml streptomycin) (Life Technologies, Inc).

Cloning of SNX6—SNX6 was originally identified in a yeast two-hybrid screen using full length Smad1 as bait and a human fetal brain cDNA library. Full length human SNX6 was subsequently cloned from a heart cDNA library using standard techniques. The full-length human clone was sequenced in both directions with standard techniques.

Sequence Alignment, Domain Mapping, and Phylogenetic Analysis—Sequence alignment was performed using the CLUSTALW multiple sequence alignment program.

Tissue Distribution of SNX6—Human multiple tissue Northern blots (Clontech) were hybridized with a full-length SNX6 probe. The probe was ³²P-labeled using RadPrime DNA Synthesis (Life Technologies, Inc.) random primed DNA labeling kit. The blots were hybridized overnight at 65°C, then washed 3 times for 30 minutes each at 65°C. Washed blots were autoradiographed for several days at -80°C.

Construction of Epitope-Tagged SNX6 Constructs—5'-tagged full length and deletion constructs of SNX6 were all prepared using a PCR-based strategy. PCR primers for the gene sequences included BamHI and ClaI restriction sites. PCR products were gel-purified, restriction digested, and then ligated into the BamHI/ClaI sites in the vectors pEBB-Flag and pEBB-HA. Each construct was fully sequenced.

Transient Transfection, Immunoprecipitation, and Western Blotting—For the coimmunoprecipitation experiments COS-1 cells were plated in 100 mm dishes at 2×10^6

cells 24 hours prior to transfection. Cells were transfected for 4 hours in serum-free medium using 45 µL of LipofectAMINE (Life Technologies, Inc.), washed once with phosphate buffered saline and replenished with fresh medium. After 24 hours the medium was replaced with DMEM plus 0.2% FBS, and 48 hours after transfection the cells were lysed by the addition of 0.5 mL lysis buffer (25 mM HEPES, pH 7.5, 150 mM NaCl, 1% Triton X-100, 5 mM EDTA, and 10% glycerol) plus phosphatase and protease inhibitors. The cells were allowed to incubate in the lysis buffer for 20 minutes on ice and were then scraped into microfuge tubes. After high speed microfugation for 15 minutes, an aliquot of the lysate was removed for Western blotting, and the remainder immunoprecipitated for 2 hours with 0.4-1 μ g of an epitope-specific antibody and 35 μ L of protein G sepharose (80% suspension) (Amersham). Lysates and immunoprecipitates were then separated by sodium dodecyl sulfate-polyacrylamide gel electropheresis and transferred onto Immobilon-P membranes (Millipore) for blotting. Proteins were detected using horseradish peroxidase (HRP) conjugated primary or secondary antibodies and visualized by chemiluminescence (Pierce). Antibodies used were anti-c-myc mouse monoclonal 9E10 (hybridoma supernatant), anti-c-myc rabbit polyclonal A-14 (Santa Cruz), anti-HA mouse monoclonal 12CA5 (hybridoma supernatant), anti-HA rabbit polyclonal Y-11 (Santa Cruz), Horse radish peroxidase (HRP) conjugated anti-HA mouse monoclonal (Roche Biochemicals), anti-Flag mouse monoclonal M2 (Sigma), anti-T β RII rabbit polyclonal C16 (Santa Cruz), anti-TßRII goat polyclonal (R&D Systems), and anti-SNX2 rabbit polyclonal. The procedure for the immunoprecipitation of endogenous complexes from HepG2 cells varied only in that the immunoprecipitation and the primary antibody staining were performed overnight.

Indirect Immunofluorescence—COS-1 cells were plated at 3×10^5 cells onto 22 mm glass coverslips 24 hours prior to transfection. Cells were transfected for 4 hours in serum-free medium using 8 µL of LipofectAMINE (Life Technologies, Inc.), washed once with phosphate buffered saline and replenished with fresh medium. After 24 hours the serum was replaced with DMEM plus 0.2% FBS. 48 hours after transfection the cells were fixed in cold 3.5% paraformaldehyde for 5 minutes, permeabilized in cold absolute methanol for 2 minutes, and then incubated 5 minutes in 50 mM glycine to quench paraformaldehyde autofluorescence. The transfected constructs were then detected by incubation for 2 hours at room temperature with M2 mouse monoclonal anti-Flag antibody and Y-11 rabbit polyclonal anti-HA antibody. After washing in PBS (3 times for 5 minutes each), the coverslips were incubated for 2 hours at room temperature with fluorescein isothiocyanate (FITC)-conjugated goat anti-mouse IgG and rhodamine (TRITC)-conjugated goat anti-rabbit IgG secondary antibodies. The coverslips were then mounted in medium containing 4,6-diamino-2-phenylindole (DAPI) (Vector Labs). The cells were examined using a Leica laser scanning confocal microscope.

Luciferase Functional Assays—HepG2 cells were plated at 3×10^5 cells per well in 6-well plates 24 hours prior to transfection. For the 3TP-Lux assays the cells were transfected with the 3TP-Lux reporter, pSV- β -gal to normalize transfection efficiency, the sorting nexin, and pCDNA3 to normalize the amount of transfected DNA. For the FAST1/ARE assays the cells were transfected with FAST1, the 3A-Luc reporter, pSV- β gal, the sorting nexin, and pCDNA3. Cells were transfected for 8 hours in serum-free medium using 8 μ L of LipofectAMINE (Life Technologies, Inc.), washed once with phosphate buffered saline and replenished with fresh medium. After 24 hours the serum was replaced with DMEM plus 0.2% FBS, and the cells were treated with either 5 ng/ml of TGF- β 1 or 50 ng/ml Activin A for 24 hours. The cells were then lysed, and the luciferase and β -gal activities determined. All assays were performed in triplicate. The β -gal values were used to correct the luciferase values for transfection efficiency.

RESULTS

SNX6 is a member of the sorting nexin family—Sorting nexin 6 was first cloned from a yeast two-hybrid screen to detect proteins interacting with Smad1, a signaling intermediate in the BMP pathway(32). However, attempts to confirm this interaction in a mammalian system demonstrated that SNX6 associated only weakly with Smad1. Subsequent database searches noted the precise sequence for sorting nexin 6 and identified this clone as a member of the sorting nexin family. The amino acid sequence alignment for sorting nexins 1-6 is presented in Fig 1A, while a schematic sequence alignment of these same sorting nexins is depicted in Fig. 1B. The PX domains, which are aligned, are indicated by the white boxes, and the predicted coiled coil regions are represented by the smaller black boxes. Each protein contains only one PX domain but a variable number of coiled coils. SNX3, unlike the others, has no coiled coil regions, which may contribute to its general lack of association with other proteins. SNX5 shows the greatest similarity to SNX6 (66% identity at the amino acid level), and these two proteins structurally resemble each other with short N-terminal regions and a relatively long spacer region between the PX domain and the first coiled coil.

Tissue Distribution of SNX6—Northern blot analysis of SNX6 using a human multiple tissue blot and full length SNX6 as probe disclosed widespread expression of the gene (Fig. 1C). Expression was highest in heart, skeletal muscle, and placenta, with little expression noted for lung or liver. Two transcripts were detected—one of 3.0 kb and one of 2.2 kb. The intensity for the smaller transcript was always greater than that for the larger.

SNX6 associates with TGF- β Receptor Family Members—Since SNX6 interacted only weakly with Smad family members (data not shown) and since other SNX family members had been shown to interact with cell surface receptors, we investigated whether SNX6 might interact directly with receptors of the TGF- β family. Initially, type I receptors (ALK1-ALK6) and the type II TGF-B receptor (TBRII) were assessed for their ability to bind SNX6. The kinase-deficient (KD) mutants of these receptors were utilized in order to maximize the likelihood of observing what might otherwise be a transient association. Immunoprecipitation of Flag-tagged SNX6 followed by blotting for the HAtagged receptors demonstrated strong binding of SNX6 to the KD TβRII (Fig. 2A, lane 9) and to KD ALK5 (TBRI) (lane7), with much weaker associations evident with KD ALK1 (lane 2) and KD ALK6 (lane 8). A panel of type II receptors was also tested for interaction with SNX6 (Fig. 2B). Wild type (WT) type II receptors were chosen for this panel, with KD T β RII also included for comparison with the previous experiments. SNX6 associated more strongly with ActRIIB than with KD T β RII, and showed only a weak association with WT TBRII. It did not interact with ActRII or BMPRII. Attempts were then undertaken to map the interacting regions of the proteins. Deletion mutants of SNX6 were designed to isolate the effects of particular motifs of the protein, especially

the PX or the coiled coil domains (Fig. 2C). SNX6 deletion mutants were tested with KD T β RII in coimmunoprecipitation experiments, and in these studies the PX domain alone (1-252) bound the receptor as strongly as the full-length protein, while the coiled coil domain showed only a weak association with the receptor (Fig. 2D).

SNX6 associates with Receptor Tyrosine Kinases—Other sorting nexins have previously been shown to interact with receptor tyrosine kinases. To investigate associations of SNX6 with receptor tyrosine kinases, COS7 cells were cotransfected with various receptors and HA-tagged SNX6. Each receptor was then immunoprecipitated, and Western blotting of the immunoprecipitates was performed for the HA-tagged SNX6. Sorting nexin 6 associated with each receptor examined, the EGF receptor, the insulin receptor, and the PDGF receptor, and with the long form of the leptin receptor (Fig. 3).

Other SNX proteins show unique patterns of interaction with TGF-β Receptor Family Members—Given the strong associations of SNX6 with various receptors within the TGF-β family, we examined the associations between these receptors and the other sorting nexins. The interactions of SNX1-SNX4 were determined for the panel of KD type I receptors, with the results tabulated in Table 1. SNX3 showed no interaction with any of the KD receptors, and SNX1 showed a weak interaction with only KD ALK4 and KD ALK5. SNX2 and SNX4 demonstrated more robust associations, with SNX2 interacting with KD ALK4, KD ALK6, and KD TβRII and with SNX4 interacting with KD TβRII and more weakly with KD ALK6. Since SNX6 bound strongly to KD ALK5, we then tested the interaction of this receptor with the other SNXs. KD ALK5 bound to varying degrees to all SNXs except SNX3, with SNX6 showing the strongest interaction, and SNX1, SNX2, and SNX4 showing more modest levels of association (Fig. 4A). A similar experiment was then performed to assess the binding of the various SNXs with ActRIIB, which had been shown to interact most strongly with SNX6 (Fig. 4B). Interestingly, SNX1, 2, and 6 also interacted strongly with this type II receptor. As expected, SNX3 did not interact, but surprisingly neither did SNX4. Finally, in order to demonstrate the physiologic relevance of these interactions we sought an association of an endogenous SNX with an endogenous receptor serine-threonine kinase. HepG2 cells were used since they have both abundant SNX2 and detectable levels of TβRII. Immunoprecipitation for the receptor using an antibody against the extracellular domain coimmunoprecipitated a portion of the endogenous SNX2 (Fig. 4C). Comparable experiments using an antibody directed against the cytoplasmic portion of the receptor failed to show a similar interaction (data not shown), suggesting that the antibody disrupts the association of the sorting nexin with the cytoplasmic region of the receptor.

SNX6 forms homo-oligomeric and hetero-oligomeric complexes with other SNXs As SNX proteins have been shown to complex with each other, we assessed the ability of SNX6 to form homo-and hetero-oligomeric complexes by cotransfecting epitope-tagged SNX expression vectors into COS1 cells, followed by immunoprecipitation and Western blot analysis of cell lysates. Homomeric interactions were tested by cotransfecting HA and FLAG-tagged SNX6 into COS1 cells. Following immunoprecipitation with anti-FLAG antibody, Western blotting with anti-HA antibody demonstrated a strong homomeric association (Fig. 5A) that was not affected by treatment with TGF-β1 (data not shown). Similar studies were carried out to determine whether heteromeric interactions could be detected between SNX6 and the other sorting nexins. Interaction

studies using HA-SNX6 and Myc-tagged SNX1-SNX4 showed strong associations of SNX6 with SNX1, SNX2 and SNX4, but no association with SNX3 (Fig. 5B).

To map the regions of the protein that mediate the association between SNX6 and the other sorting nexins, deletion constructs of SNX6 were tested for their ability to interact with either SNX1 or SNX4. In contrast to the selective interaction of the PX domain with T β RII, these experiments showed that all regions of the molecule retained considerable ability to interact (Fig. 5C and data not shown). Only SNX6 1-72, the extreme amino terminus lacking both the PX and coiled coil domains, did not associate. The relative uniformity of expression of the various deletion constructs suggests that the degree of association with each region was similar and that both the PX domain and the coiled coil regions may contribute to the hetero-oligomerization.

Indirect Immunofluorescence of the sorting nexins—Indirect immunofluorescence was utilized to assess the intracellular localization of SNX6 and to seek further evidence for the association of SNX6 with other sorting nexins. Both SNX6 (fig. 6 A, E, I, M) and sorting nexins 1-4 (Fig. 6 B, F, J, N) localized specifically to the cell cytoplasm in a diffuse pattern. This cytoplasmic localization for SNX6 was not altered by cotransfection of a constitutively active T β RI to stimulate TGF- β signaling pathways or a constitutively active BMPRIA to stimulate BMP signaling (data not shown). Cotransfection of SNX6 and sorting nexins 1, 2, and 4, showed nearly complete overlap of the two staining patterns, as indicated by the yellow color in the images (Fig. 6 D, H, P). Surprisingly, cotransfection of SNX3 with SNX6 also showed considerable, though not complete, colocalization (Fig. 6 L). For this pair of proteins, the overlap is strongest at the periphery of the cell, in contrast to the uniform overlap seen with the other SNXs.

Luciferase Functional Assays—One question not vet answered by the previous experiments was whether alteration of the levels of SNX6 within a cell might perturb TGF-β signal transduction pathways. To explore this issue, functional assays using TGF- β responsive reporter genes linked to luciferase expression were performed in HepG2 cells. These assays measure the ability of a ligand to induce gene expression, and they thereby assess the functional capacity of the entire signaling pathway. Any disturbance along the pathway, including a possible shift in cell surface expression of the TGF- β or activin receptors, might be expected to change the flux through the pathway and quantifiably modify the read-out. The FAST1/ARE assay uses the 3A-Luc reporter, a construct generated by the tandem repeat of an activin response element identified in the promoter for the *Mix2* gene. The assay, which is sensitive to both TGF- β and activin, measures a direct, Smad2-specific response. Assays using this reporter with stimulation by TGF-β demonstrated a 5-fold reduction in luciferase activity with increasing amounts of SNX6 (Fig. 7A), and similar assays with stimulation by activin showed an 8-fold decrease (Fig. 7B). For both ligands the reduction in luciferase values occurred in a stepwise, dose-dependent fashion. 3TP-Lux, a segment of the PAI-1 promoter downstream of three TPA-responsive elements, is also sensitive to both TGF- β and activin. In assays using this reporter, SNX6 reduced the stimulation by TGF- β about 50% (Fig. 7C). Interestingly, the basal, unstimulated luciferase readings for both reporters diminished at an even greater rate with increasing SNX6, with the FAST1/ARE system showing a 12-fold reduction, and the 3TP-Lux system a 6-fold decrease. As a control, similar assays were performed using increasing amounts of SNX3, the one sorting nexin that bound none of the tested receptor serine-threonine kinases. In contrast

to SNX6, SNX3 had no effect on reporter activity (data not shown), suggesting that the reduction in the basal values is not non-specific, but likely due to interference with autocrine stimulation of the reporters by TGF- β .

DISCUSSION

In this paper we show for the first time associations between members of the sorting nexin family and receptor serine-threonine kinases. Each SNX showed a different pattern of interaction with these receptors of the TGF-β family. Concerning the type I receptors, SNX6 alone bound strongly to ALK5. Likewise, only SNX2 interacted appreciably with ALK4. Differences in association patterns for SNX binding were again evident in their interactions with the type II receptors. TβRII associated with SNX2, SNX4, and SNX6, but not with SNX1 or SNX3, while ActRIIB interacted strongly with SNX1, SNX2, and SNX6 but not with SNX3 or SNX4. In contrast to the distinct associations of SNXs with receptor serine-threonine kinases, the pattern of SNX6 binding to the receptor tyrosine kinases was similar to that of the other sorting nexins. All SNXs (except SNX3) tested to date have interacted at least to a degree with the tested receptor tyrosine kinases (28;31), and SNX6 reproduced this pattern, binding the EGF receptor.

Studies of the association of endogenous SNX2 with T β RII confirmed that the above findings have physiologic relevance. We feel that the somewhat low stoichiometry of the interaction results from the confluence of several features of sorting nexin biology.

First, the sorting nexins are capable of binding to a range of different receptors, but probably only one or two simultaneously. In a cell only a minority of the sorting nexin molecules is likely interacting with a given receptor. Similarly, most cells express multiple different sorting nexins, so that at any given time one type of receptor may be interacting with multiple sorting nexins. Finally, sorting nexins may have other roles in the cell so that only a small proportion is bound to receptors in the normal physiologic state. Although the function of the sorting nexins is not yet clear, the intracellular localization of these molecules to vesicles and their strong interactions with receptors suggest that, as a part of their roles within the cell, the different sorting nexins are involved in the targeting of receptors to intracellular trafficking pathways.

A characteristic structural feature of the sorting nexins is the phox homology (PX) domain, a roughly 100 amino acid motif of uncertain function found in components of the NADPH oxidase system, the sorting nexins and their orthologs, and the phosphatidylinositol 3-kinases (33). This domain is typically found in the middle of the molecule or nearer the amino terminus. All of the sorting nexins except for SNX3, which consists almost entirely of a PX domain with only minimal amino and carboxy terminal extensions, also have at least one coiled coil domain near their carboxy terminus. Heteromeric interactions of SNX6 with other sorting nexins appear to be mediated roughly to an equal degree through the PX domain or the coiled coil regions of SNX6. The receptors can also associate with either domain of SNX6, although the interaction seems to be stronger with the PX domain. The involvement of these domains in interactions between SNX6 and another SNX or between SNX6 and a receptor suggests a model in which a receptor interacts with the PX domain of SNX6 while another sorting

16

nexin associates through the coiled coil domain, forming a linear trimeric complex. More probably, additional SNX molecules interact through both their PX and coiled coil domains, allowing larger complex formation, likely including multiple receptor molecules.

The function of the sorting nexins is at this time still unknown. Mutation of the yeast homologs results in protein missorting, suggesting that they are involved in intracellular trafficking (29;30). In mammalian cells overexpression of SNX1 was noted to accelerate EGF receptor degradation, implying a role for this protein in endosomal transport. Our reporter assays add further evidence that these molecules play a role in receptor trafficking pathways. The linear decrease in signal with increasing SNX6 clearly indicates that the sorting nexin is interfering with TGF- β signaling. While these assays provide no direct measure for any single component of this signaling pathway, when examined in the context of the previous coimmunoprecipitation data these results suggest that the increasing amounts of SNX6 within a cell alter plasma membrane receptors. The sorting nexin may be binding to and sequestering receptor, fostering increased receptor degradation, or directly inhibiting receptor function. Despite these findings, definitive evidence for a specific function remains elusive.

Recently, the sorting nexins have been identified as part of a molecular complex termed the retromer complex which may facilitate retrograde transport from endosomes back to the *trans*-Golgi network. A core retromer complex, consisting of Vps35p, Vps29p, and Vps26p, was first identified in yeast and shown to function in the retrieval of Vps10p (the carboxypeptidase Y receptor) from endosomes to the Golgi (34). Subsequent work demonstrated that Vps5p, the SNX1 homolog, and Vps17p, another PX domain containing protein, also form part of this complex. In particular, these experiments suggested that a core complex of Vps35p, Vps29p, and Vps26p provide the receptor specificity, after which Vps5p and Vps17p assemble onto the membrane to promote vesicle formation (35). The human orthologs of Vps35p, Vps29p, and Vps26p have recently been cloned and investigated (36). These binding experiments demonstrate multiple interactions among these proteins, again suggesting that Vps35p forms the core of a molecular complex. But the precise mechanism by which the cargo of a given transport vesicle is chosen still remains undetermined. Given the differing specificities the sorting nexins display for the receptor serine-threonine kinases, the probability seems high that these molecules, rather than the core retromer complex, either serve to select the proteins to be conveyed within particular transport vesicles or associate with and provide a marker for those proteins that actually do regulate this specificity.

In conclusion, we have cloned a novel sorting nexin, SNX6, that interacts strongly with specific members of the receptor serine-threonine kinase and receptor tyrosine kinase families and that colocalizes and associates with other sorting nexins. These findings demonstrate for the first time the interaction of sorting nexins with a family of receptor serine-threonine kinases, both enlarging the scope of receptors which interact with this class of proteins and showing a higher degree of specificity than that noted for the receptor tyrosine kinases. The results of these experiments further suggest that the sorting nexins function in a multimeric complex to enable the specific selection and transport of molecules within the intracellular vesicular transport pathways. Whether the levels of this family of proteins are regulated to modulate physiological responses or to play a role in disease pathogenesis remains to be determined.

18

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FOOTNOTES

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¹The abbreviations used are: SNX, sorting nexin; TGF- β , transforming growth factor- β ; EGF, epidermal growth factor; PDGF, platelet-derived growth factor; ActRIIB, activin type IIB receptor; TGF- β RII, transforming growth factor- β type II receptor; TGF- β RI, transforming growth factor- β type I receptor; BMP, bone morphogenetic protein; GDF, growth and differentiation factor; GDNF, glial-derived neurotrophic factor; MIS, Mullerian Inhibitory Substance; ALK, activin-like kinase; ActRIB, activin type IB receptor; BMPRII, bone morphogenetic protein type II receptor; PX domain, phox homology domain; FITC, fluorescein isothiocyanate; TRITC, rhodamine; DAPI, 4,6diamino-2-phenylindole; KD, kinase-deficient Fig. 1. The sorting nexins share common PX and coiled coil domains. (A) Multiple sequence alignment of sorting nexins 1-6 was performed using the CLUSTALW program, and the aligned sequences are shown. (B) Sorting nexins 1through 6 are depicted schematically, with the white boxes representing the PX domain and the solid boxes representing coiled coil regions. The initial amino acids for the PX domains are aligned vertically, and the remainder of each protein is drawn to scale. The amino acid positions within each protein are indicated by the numbers. (C) A multiple tissue Northern blot was probed with full length SNX6. The numbers adjacent to the blot represent the size in kilobases of each transcript.

Fig. 2. Sorting nexin 6 associates with type I and type II TGF-β receptors. (A) COS-1 cells were transfected with FLAG-tagged SNX6 and HA-tagged kinasedeficient (KD) type I receptors or KD type II TGF-β receptor. Cell lysates were immunoprecipitated with an anti-FLAG antibody and blotted the receptor with an anti-HA antibody to demonstrate association of KD ALK1, ALK5, ALK6, and TGF-β type II receptors with SNX6 (top row). The middle and bottom rows are control Western blots to show expression of HA-receptors and FLAG-SNX6, respectively. (B) COS-1 cells were transfected with FL-SNX6 and HA-tagged receptors (lanes 1-4) or HA-SNX6 and FLtagged receptors (lanes 5-8). Lanes 1-4 were immunoprecipitated with an anti-FLAG antibody and lanes 5-8 with an anti-HA antibody to immunoprecipitate SNX6. Lanes 1-4 were then blotted with an anti-HA antibody and lanes 5-8 with an anti-FLAG antibody to demonstrate association of the sorting nexin with receptors (top). SNX6 interacts most strongly with ActRIIB, moderately with KD TGF- β RII, and weakly with WT TGF- β RII. No association was seen with ActRII or BMPRII. (C) The deletion constructs of SNX6 are schematically depicted with reference to the full-length molecule. 73-406, 153-406, and 253-406 delete all or part of the PX domain (white box). 1-152 and 1-252 delete the coiled coil domains (solid boxes) and part of the linker region. 1-72 deletes the coiled coil regions and nearly the entirety of the PX domain. (D) COS-1 cells were transfected with HA-T β RI KD and the FL-tagged deletion constructs of SNX6. Cell lysates were immunoprecipitated with an anti-FLAG antibody and blotted for T β RI KD using an anti-HA antibody. T β RI KD associated more strongly with PX domain (1-152 and 1-252) than with the coiled coil regions (253-406). The middle and bottom rows represent control Western blots for the immunoprecipitations.

Fig. 3. **Sorting nexin 6 interacts with receptor tyrosine kinases.** COS-7 cells were transfected with HA-tagged SNX6 and untagged receptor constructs. Cell lysates were immunoprecipitated with the appropriate anti-receptor antibody and then blotted for SNX6 with anti-HA antibody to demonstrate association of SNX6 with each receptor (top row). The middle and bottom rows are control Western blots to show expression of HA-SNX6 and receptors, respectively. Odd numbered lanes represent controls in which HA-SNX6 was not expressed.

Fig. 4. Sorting nexins 1-4, 6 show different specificities of interaction with a kinasedeficient type I TGF-β receptor and with the type II receptor ActRIIB. (A) COS-1 cells were transfected with HA-T β RI and either myc-SNX1-4 or FL-SNX6. The sorting nexin was immunoprecipitated using either an anti-myc or anti-FLAG antibody, and receptor association was detected with an anti-HA antibody (top). SNX1, 2, 4, and 6 associated to varying degrees with this type I receptor. (B) COS-1 cells were transfected with FL-ActRIIB and either myc-SNX1-4 or HA-SNX6. The sorting nexin was immunoprecipitated using either an anti-myc or anti-HA antibody, and receptor association was detected with an anti-FLAG antibody (top). SNX1, 2 and 6 associated strongly with this type II receptor. The middle and bottom rows represent control Western blots for the immunoprecipitations. (C) HepG2 cells were plated at 6 x 10⁶ cells and allowed to grow overnight. The lysates were precipitated with either control IgG (goat), a rabbit polyclonal anti-T β RII generated against the cytoplasmic portion of the receptor (cT β RII) or a goat polyclonal anti-T β RII generated against the extracellular portion of the receptor (eT β RII). The immunoprecipitates were detected either with cT β RII or a rabbit polyclonal anti-SNX2.

Fig. 5. **SNX6** associates with itself and with other sorting nexins. (A) COS-1 cells were transfected with FL- and HA-tagged SNX6. Cell lysates were immunoprecipitated with an anti-HA antibody and blotted for SNX6 with an anti-FLAG antibody to demonstrate the homomeric association of SNX6 with itself (lane 3). (B) COS-1 cells were transfected with HA-SNX6 and myc-tagged SNX1-4. Cell lysates were immunoprecipitated with an anti-HA antibody and blotted for SNX6 with SNX1-4 with an anti-myc antibody to show the heteromeric associations of SNX6 with SNX1, SNX2, and SNX4 (lanes 2,3, and 5). (C) COS-1 cells were transfected with myc-SNX1 and the FL-tagged

deletion constructs of SNX6. Cell lysates were immunoprecipitated with an anti-FLAG antibody and blotted for SNX1 using an anti-myc antibody. SNX1 associated strongly with each of the deletions except 1-72, the smallest (lane 6). The middle and bottom rows represent control Western blots for the immunoprecipitations.

Fig. 6. **SNX6 colocalizes with other sorting nexins.** COS-1 cells were transfected with HA-SNX6 and myc-SNX1 (A-D), myc-SNX2 (E-H), myc-SNX3 (I-L), or myc-SNX4 (M-P) constructs, fixed in 3.5% paraformaldehyde, permeabilized with methanol, stained, and analyzed with laser scanning confocal microscopy. The myc-tagged sorting nexins were detected using a monoclonal anti-myc antibody and a FITC-conjugated goat anti-mouse IgG secondary antibody (A, E, I, M). HA-SNX6 was detected using a rabbit polyclonal anti-HA antibody and a TRITC-conjugated goat anti-rabbit IgG secondary antibody (B, F, J, N). DAPI staining (C, G, K, O) highlights the location of nuclei. The FITC, TRITC, and DAPI images were overlapped (D, H, L, P) to demonstrate at least partial colocalization for SNX6 with sorting nexins 1-4.

Fig. 7. **SNX6 inhibits TGF-\beta and activin signaling.** HepG2 cells were transfected with a 3A-Luc reporter plasmid, FAST1, and increasing amounts of SNX6. The cells were serum-starved for 24 hours, during which time half were treated with 5 ng/ml TGF- β 1 (A) or 50 ng/ml Activin A (B). (C) HepG2 cells were transfected with the 3TP-Lux reporter plasmid and increasing amounts of SNX6. The cells were serum-starved for 24 hours, during which time half were treated with 5 ng/ml TGF- β 1. For each assay betagalactosidase values were used to normalize for transfection efficiency, and the results are presented as relative luciferase values. Error bars indicate standard deviations.

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Α

SNX1 SNX2 SNX3 SNX4 SNX5 SNX6	1 1 1 1 1	HQSPKITTSLLPIN-NGSKENGIHEEQDQEPQDLFADATVELSLDSTQNNQKKVLAKTIISLSPQEATNSSKPQPTYEELEE HLQNQPSLPAEDISANSNGPKPTEVVLDDDREDLFAEATEEVSLDGLKGNLSYPRNLPLQSHLSLLHSAPRIESKSMSAPVIFDRSREEIEE
SNX1 SNX2 SNX3 SNX4 SNX5 SNX6	82 94 21 56 61 62	EEQEDQFDLTVGITDEKIGDGMNAYVAYKVTTONSIDL - FRSKQFAVKRRFSDFLGLYEKISEKHSQNGFIVPPSPEKSLIGMTKVKVGKEDS EANGDIFDIEIGVSDEKVGDGMNAYMAYRVTHKTSDSM - FSKSEFSVKR-FTDFLGHTTIPTTYLHVVIFVATSSRKSIVGMTKVKVGKEDS AYGPPSNFLEIDVSNEQTVGVGRGRFTYEHRVKTNLEI - EKLKESTVRRYSDFEWLRSELERE SKVVVPPLPGKAELRQLPFRGDDGI KKIEISVSEAEKRTGRNAMNM - QETYTAYLIENSVEHTDGOSVLTDSLWRRYSEFELERSVILVYYP HIVVPPLPEKRAEFVWKTSADN - PEFSVTRQHEDFVWLHDTLIE - TTDYAGLIIPPAPTKED - EDGPREKMQK LGE GE GSMTKEEFAKMMQELEAEY - NEFSVTRQHEEFIWLHDSFVE - NEDYAGVIIPPAPPRED - EDGSREKLQK LGE GE GSMTKEEFTKMKQELEAEY -
SNX1 SNX2 SNX3 SNX4 SNX5 SNX6	175 186 110 146 133 134	SSAEELEKRRAALERYLQRIVNHPTMLQDPDVREFLEK - EELPRAVGTQTLSGAGLLKMFNKATDAVSKMTIKMNESDIWFEEKLOEVECEEQRL SSTEFVEKRRAALERYLQRTVKHPTLLQDPDLROFLES - SELPRAVNTQALSGAGILRMVNKAADAVNKMTIKMNESDIWFEEKLOEVECEEQRL FDDNFIEERKQGLEQFINKVAGHPLAQNERCLHMFLQD - EIIDKSYTPSKIRHA MDPDFVERRRIGLENFLLRIASHPILCRDKIFYLFTCOEGNWKETWNETGFQLKADSRLKALNATFRVKNPDKRFTDLKHYSDELOSVISHLLRV - LAVFKKTVSSHEVFLQRLSSHPVLSKDRNFHVFLEYDQDISVRRKNTKEMFGGFFKSVVKSADEVLFTGVK - EVDDFFEDEKNFLINYYNRI - LAIFKKTVAMHEVFLCRVAAHPILRRDLNFHVFLEYNQDISVRGKNKKEKLEDFFKNMVKSADGVIVSGVK - DVDDFFEHERTFLLEYNNRV
SNX1 SNX2 SNX3 SNX4 SNX5 SNX6	269 280 241 224 225	RKLHAVVET - LVNHRKELALNTAQFAKSJAMLGSSEDNTALSRALSQLAEVEEKIEQLHQEQANNDFFLAELLSDVIRLLAIVRAAFDQRMKTW RKLHVSVEA - LVCHRKELSANTAAFAKSAAMLGNSEDHTALSRALSQLAEVEEKIDQLHQEQAFADFYMFSELLSDVIRLIAAVGGVFDHRMKCW RARVADRLYGVYKVHGNYGRVFSEWSAIEKEMGDGLQSAGHHMDVYASSIDDILEDEEHYADQLKEVIFYABALRAVCRKHELMQVDDEMAAQDI KDSCVKADK - MTRSHKNVADDYIHTAACHSMA - LEEPTVIKKYLKVAELFEKLRKVEGRVSSDEDIKITELLRYVMLNIEAAKDLYRRTKAI KDASAKSDR - MTRSHKNSAADDYNRIGSSLYALG - TODSTDICKFFLKVSELFDKTRKIEARVSADEDIKLSDLKYVLRESQAAKDLYRRSRS
SNX1 SNX2 SNX3 SNX4 SNX5 SNX6	363 374 336 317 318	QRWQDAQATDOKKREAEARLLWANKPDKLQQAKDETLEWESRVTQYERDEERISTVVRKEVIREEKEKSKDFKNHVIKYLETLL QKWEDAQITLLKKREAEAKMMVANKPDKIQQAKNEIREWEAKVQQGERDEQISKTIRKEVGREEKERVKDFKTVILKYLESLV ASKKQQCEELVTGTVRTFSLKGMTTKLFGQETPEQREARIKVLEQINEGEQQLKSKNLEGREFVKNAWADIERFKEQKNRDLKEALISMAVMQI IDYENSNKAMDKARLKSKDVKLAEAHQQECCOKFEQLSESAKEELINFKRKVAAFRKNLTEMSELEI VDYENANKALDKARAKNKDVLQAETSQQLCCOKFEKISESAKQELIDFKTRVAAFRKNLVELAELEL













D

IP SNX6 (Flag) Blot TBRI (HA)

Blot TßRI KD (HA)

Blot SNX6 (Flag)







78 1 2 3 4 5 6





Α

WC WC WC WC WC HASH IP SNX (Myc or Flag) Blot TBRI KD (HA) Blot TßRI KD (HA) **Blot SNX** (Myc, Flag) 3 1 2 6 4 5



Western IP $eT\beta RII$

В



В











	Receptor							
	ALK1 KD	ALK2 KD	ALK3 KD	ALK4 KD	ALK5 KD	ALK6 KD		
SNX1	-	-	-	weak	-	-		
SNX2	-	-	-	moderate	moderate	-	strong	
SNX3	-	-	-	-	-	-	g∕by ∎	
SNX4	-	-	-	-	weak	weak	strong	
SNX6	weak	-	-	-	strong	moderate	n strong Se 29, 201	

Table 1. Association of SNX with kinase deficient type I and type II receptors

Sorting nexin 6, a novel SNX, interacts with the TGF-beta family of receptor serine-threonine kinases

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