Structure and Membrane Binding Properties of the Endosomal Tetratricopeptide Repeat (TPR) Domain-containing Sorting Nexins SNX20 and SNX21*

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Background: SNX-PXB proteins SNX20 and SNX21 are poorly characterized members of the sorting nexin (SNX) family. Sorting nexins (SNX) orchestrate membrane trafficking and signaling events required for the proper distribution of proteins within the endosomal network. Their phox homology (PX) domain acts as a phosphoinositide (PI) recognition module that targets them to specific endocytic membrane domains. The modularity of SNX proteins conveys a wide variety of functions from signaling to membrane deformation and cargo binding, and many SNXs are crucial modulators of endosome dynamics and are involved in a myriad of physiological and pathological processes such as neurodegenerative diseases, cancer, and inflammation. Here, we have studied the poorly characterized SNX20 and its parologue SNX21, which contain an N-terminal PX domain and a C-terminal PX-associated B (PXB) domain of unknown function. The two proteins share similar PI-binding properties and are recruited to early endosomal compartments by their PX domain. The crystal structure of the SNX21 PXB domain reveals a tetratricopeptide repeat (TPR)-fold, a module that typically binds short peptide motifs, with three TPR α-helical repeats. However, the C-terminal capping helix adopts a highly unusual and potentially self-inhibitory topology. SAXS solution structures of SNX20 and SNX21 show that these proteins adopt a compact globular architecture, and membrane interaction analyses indicate the presence of overlapping PI-binding sites that may regulate their intracellular localization. This study provides the first structural analysis of this poorly characterized subfamily of SNX proteins, highlighting a likely role as endosome-associated scaffolds.

Results: We find the SNX-PXB proteins are localized to endosomes, and that the PXB domain has a tetratricopeptide repeat (TPR)-fold. The PXB domain has an atypical TPR-fold with conserved surfaces likely to mediate protein-protein interactions. The SNX-PXB proteins are endosome-associated scaffolds with conserved surfaces likely to mediate protein-protein interactions.

Conclusion: The PXB domain has an atypical TPR-fold with conserved surfaces likely to mediate protein-protein interactions. The PXB domain has an atypical TPR-fold with conserved surfaces likely to mediate protein-protein interactions. The PXB domain has an atypical TPR-fold with conserved surfaces likely to mediate protein-protein interactions. The PXB domain has an atypical TPR-fold with conserved surfaces likely to mediate protein-protein interactions.

Significance: SNX-PXB proteins are endosome-associated scaffolds with conserved surfaces likely to mediate protein-protein interactions. The PXB domain has an atypical TPR-fold with conserved surfaces likely to mediate protein-protein interactions.

The trafficking of proteins in eukaryotic cells is a tightly regulated process. A complex array of signaling proteins, molecular transporters, and lipids target transmembrane proteins to their appropriate location in the cell (1). Acting as a central hub in this process, the endosome is a multifunctional organelle regulating the sorting and delivery of biological regulators such as receptors, adhesion molecules, and ion channels (2), as well as acting as specialized platforms for cell signaling (3). One of the major protein families regulating endosomal function and organization are the sorting nexins (SNXs). All SNX proteins possess a phox homology (PX) domain that binds to phosphoinositide (PI) lipids contained in the membranes of different organelles, thus mediating their attachment to the cytoplasmic leaflets of endosomal compartments (4). Most members of the SNX family also incorporate a variety of distinct structural and functional domains, which are then targeted by the PX module to appropriate membranes within the endosomal network (5, 6). The SNX proteins can thus be classified into different subfamilies based on the structural arrangements of these different scaffolding, enzymatic, and regulatory domains (4, 5, 7). As the SNX proteins are established regulators of endosomal sorting and signaling, they have also been shown to play an important role in pathological states such as chronic and neurodegenerative diseases, pathogen invasion, and inflammation (reviewed in Ref. 5). Although the roles of many of the SNXs have been partly deciphered and attributed to particular cellular functions, many other members of the SNX family still remain poorly characterized. Given the increasing appreciation of their importance in maintaining cell homeostasis and driving pathogenesis, it is crucial to gain new molecular insights into the functions of the SNX family protein members that remain unexplored.

Although not all SNX proteins play a direct role in membrane trafficking, it is clear that the association of SNXs with membranes of the endocytic system is often coupled to protein-protein interactions that regulate recruitment of trafficking cargoes and signaling proteins. As just one example, the SNX-4.1/ezrin/radixin/moesin subfamily composed of the members...

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The abbreviations used are: SNX, sorting nexins; PI, phosphoinositide; PX, phox homology; PSGL-1, P-selectin glycoprotein ligand 1; TRP, tetratricopeptide repeat; SAXS, small angle x-ray scattering; MALLS, multi-angle laser light scattering; PDB, Protein Data Bank; PXB, PX-associated domain B.
SNX17, SNX27, and SNX31, possess both PSD95-discs-large- zonula occludens and 4.1/ezrin/radixin/moesin domains that associate with early endosomes through their PX domain, and couple transmembrane proteins with specific peptide motifs to the retromer complex to mediate endosomal trafficking (8–12). Synergistic action of the PX, PSD95-discs-large-zonula occludens, and 4.1/ezrin/radixin/moesin domains can reinforce binding avidity to the membrane microenvironment through coincidence detection (13).

One of the most poorly characterized SNX subfamilies are the so-called SNX-PXB proteins SNX20 and SNX21 (also called SNX-L) (5), where PXB stands for “PX-associated domain B.” SNX20 appears to be mainly expressed in macrophages and immune cells, whereas SNX21 appears to be expressed more broadly but with particularly high levels in fetal liver tissue (14, 15). A single study of the SNX20 molecule identified it as an endosomal protein that could associate with the cytosolic domain of the transmembrane adhesion receptor P-selectin glycoprotein ligand 1 (PSGL-1) (16). Overexpression of SNX20 caused a redistribution of PSGL-1 from the cell surface into endosomal organelles, suggesting a role in PSGL-1 trafficking. However, SNX20 knock-out mice showed no overt phenotypes related to endothelial cell adhesion, and therefore its precise function is still not clear. Down-regulation of SNX21 by siRNA also has a modest effect on the endocytic degradation of the epidermal growth factor receptor (17), further suggesting a role in the mechanism underlying this effect has also not been characterized in detail.

In this study, we report the crystal structure of the domain-swapped C-terminal PXB domain of SNX21, demonstrating that it possesses a tetratricopeptide repeat (TPR)-fold and suggesting that this SNX subfamily is likely to be involved in protein-protein interactions. The topology of the SNX21 TPR domain is highly unusual, suggesting a potential autoinhibitory conformation and bearing some structural similarity to the TPR domain of the functionally unrelated yeast protein Fis1p. This provides structural clues that allow us to speculate on the function and regulation of this domain in SNX21. The solution structures of the SNX-PXB proteins determined by small angle x-ray scattering (SAXS) reveal a compact globular architecture, which may facilitate coincidence detection of PIs and potential transmembrane protein cargoes. Mutagenesis and liposome pelleting assays demonstrate the ability of these proteins to couple transmembrane protein cargoes. Mutagenesis and liposome pelleting assays demonstrate the ability of these proteins to couple transmembrane protein cargoes. Mutagenesis and liposome pelleting assays demonstrate the ability of these proteins to couple transmembrane protein cargoes.

Experimental Procedures

**Antibodies**—The following primary antibodies were used: mouse monoclonal anti-Myc tag (9B11) antibody (Cell Signaling Technology, 2272); mouse anti-EEA1 antibody (BD Transduction Laboratories, 610457), mouse anti-human CD107a/LAMP-1 antibody (BD Pharmingen, 555798), mouse monoclonal anti-human CD8a purified clone OKT8 antibody (eBioscience, 14-0086-80), rabbit polyclonal c-Myc antibody (Novus Biologicals, NB600-336), and anti-CD8 (18). Donkey anti-mouse coupled to Alexa Fluor® 488 or 647 and donkey anti-rabbit coupled to Alexa Fluor® 488 or 647 (Life Technologies) were used as secondary antibodies for immunofluorescence. The IRDye®800CW donkey anti-rabbit IgG and IRDye®680LT goat anti-mouse IgG were purchased from LI-COR Biosciences.

**Mammalian Expression Plasmids—**pcDNA-3.1-n-myc-SNX20 was generated by inserting amplified murine SNX20 cDNA using primers BamHI, forward CCG GATCC GCA AGT CCA GAG CAT CCT and XbaI, reverse CCC TCTAGA TCA GGA CAG ATA CTC CCG into the BamHI/XbaI sites of pcDNA-3.1-n-myc backbone. pcDNA-3.1-n-myc-SNX21 was generated by inserting amplified murine SNX21 cDNA using PCR primers infusion_1, AGAAAGACCTG GGAATCC GCC TCG CCG CTC CTA CAC CGG and infusion_2, GCCC TTC-TAGA CTCGAG TTA GTC CAG CAC CTC TTT GAT into the BamHI/XbaI sites of pcDNA-3.1-n-myc backbone. pcDNA-3.1-n-myc-SNX20-R313Q and pcDNA-3.1-n-myc-SNX21-R331Q were generated by site-directed mutagenesis using a QuikChange II kit (Stratagene) in accordance with the manufacturer’s instructions. CD8-P-selectin and CD8-PGSL-1 were cloned by ligating the P-selectin and PGSL-1-amplified cdNA flankned with restriction sites corresponding to BamHI (5′) and Xhol (3′) enzymes into a BamHI/Xhol-digested pcDNA-3.1-n-GFP-CD8 expression vector.

**Recombinant Protein Expression and Purification—**cdNAs corresponding to residues 1–313 of SNX20 (mouse), 93–363 of SNX21 (mouse), and 231–363 of SNX21 (mouse) were cloned using circular polymerase extension cloning. The SNX-PXB genes were inserted into a phUE vector yielding a N terminally His₆-ubiquitin-tagged protein containing a ubiquitin-protease cleavage site. Site-directed mutagenesis was carried out using the QuikChange II kit (Stratagene) with 100 ng of parental DNA for all experiments. Proteins were expressed in the Escherichia coli BL21(DE3) strain overnight at 20 °C and purified using a two-step procedure involving affinity chromatography followed by gel filtration. Proteins were first purified on a 5-ml nickel-nitrilotriacetic acid gravity column and eluted with 300 mM imidazole in a buffer containing 25 mM Tris (pH 8.0), 500 mM NaCl, 20 mM imidazole, and 10% (v/v) glycerol. The ubiquitin tag was cleaved off for 12 h by dialysis into an imidazole-free buffer in the presence of ubiquitin protease, and the His₆-ubiquitin was separated from the proteins by elution through a nickel-nitrilotriacetic acid gravity column. As a second step, proteins were gel filtered in a buffer containing 25 mM Tris (pH 8.0), 500 mM NaCl, and 1 mM DTT using a Sepharose S200 16/60 column attached to an AKTA system (GE Healthcare).

**Protein Crystallization, Data Collection, and Structure Determination—**SNX21-(231–363) (PXB) fractions were pooled and concentrated to ~20 mg/ml. Eight 96-well crystalization hanging-drop screens were set-up using a Mosquito Liquid Handling robot (TTP LabTech) at 20 °C. Diffraction quality crystals were obtained using the sitting drop vapor diffusion method and a buffer containing 10% (w/v) PEG 8000, 20% (v/v) ethylene glycol, 0.1 M MES/imidazole (pH 6.5), 0.02 M sodium formate, 0.02 M ammonium acetate, 0.02 M trisodium...
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citrate, 0.02 M sodium potassium L-tartrate, and 0.02 M sodium oxamate. The derivative crystal was produced by soaking for 1 h in the crystallization solution supplemented with 10 mM ethyl-
HgCl₂. Crystals were then transferred into a cryoprotectant
solution composed of mother liquor supplemented with 20%
glycerol and cooled to 100 K prior to data collection.

Native data were collected at the UQ ROCX diffraction facility
on a Rigaku FR-E Superbright generator with Osmic Vario-
Max HF optics and Rigaku Saturn 944 CCD detector. Deriva-
tive data were collected at 100 K at beamline MX-2 (UQ ROCX
and Australian Synchrotron, Table 1) and integrated and scaled
with MOSFLM (19) and SCALA (20). The SNX21 PXB domain
structure was solved by single isomorphous replacement with
anomalous scattering (SIRAS) using CRUNCH2 and BP3 for
heavy atom location, SOLOMON (21) for density modification
and phase improvement, and BUCCANNEER for automated
model building as implemented within the CRANK automated
platform in CCP4i (22). After phase determination, BUCCANNEER
was able to automatically build 271 residues of a total of 288. A
model was built using COOT (23) followed by repeated refine-
ment and model building with PHENIX.REFINE and COOT.
All structural figures were generated using PyMOL.

Multi-angle Laser Light Scattering—Samples (20–50 μl) of
purified truncated SNX20 and SNX21 proteins were injected
onto either a Superdex 200 HR 10/30 size-exclusion column
(GE Healthcare), with a flow rate of 0.5 ml/min, or Wyatt
WTC-030N5 SEC protein silica column (5
(5
(5

GE Healthcare), with a flow rate of 0.5 ml/min, or Wyatt
WTC-030N5 SEC protein silica column (5 μm 300 Å 4.6 × 300
mm), with a flow rate of 0.2 ml/min. The chromatography sys-
tem was coupled to a three-angle light scattering detector
(mini-DAWN TRISTAR) and a refractive index detector (Opti-
lab DSP, Wyatt Technology). Data were collected every 0.5 s.
Data analysis was carried out using ASTRa software.

Small-angle X-ray Scattering—The molar masses and mono-
dispersity of homogeneous solutions of truncated SNX20 and
21 proteins were confirmed using MALLS. SAXS data were
collected at the SAXS/WAXS beamline at the Australian Syn-
chrotron from a concentration series of each protein between
2.5 and 7.5 mg/ml. Scattering data were measured in a q range
between 0.035 and 0.6 Å⁻¹ at 12 keV with a 1.6-m camera
length. Data were measured on a Pilatus 1 M camera (Dectris)
and absolute scaled using distilled water. For each concentra-
tion, 20 1-s exposures were background-corrected, averaged,
and scaled using ScatterBrain software. All further processing
was carried out using the ATSAS program suite (24). PRIMUS
was used for initial data evaluation, with Kratky plots were used
to assess the folded state of the proteins. The radius of gyration
\( R_g \) values listed in Table 2 were determined using the Auto\( R_g \)
function, with data quality of ≥90%. The consistency of \( R_g \) val-
ues with increasing concentration, together with inspection of
Guinier plots for each data set, confirmed the absence of aggre-
gation or interparticle interference. Similar \( R_g \) values were
obtained by GNOM, the program used to calculate the pair
distance distribution function (P(r)) and maximum diameter
(Dmax) of the SNX20 and SNX21 proteins. Normalized scat-
ttering intensity \( I(q) \) values were estimated using extrapola-
tions of the Guinier plot in the linear region \( q_{max} \times R_g ≤ 1.3 \).
Molecular masses were determined by evaluating the scattering
data on an absolute scale and also by comparison with a second-
ary protein standard (glucose isomerase).

Ab initio free atom modeling was performed using the pro-
gram GASBOR with a total of 40 independent simulations car-
rried out for each protein. The accuracy of each model was
assessed by the normalized residual coefficient, \( \chi \), which pro-
vides a value for the goodness of fit. Low values (close to 1.0)
indicate good agreement of the models with the experimental
data. The 40 individual GASBOR models were averaged using
DAMAVER and filtered based on occupancy and volume to
generate restored \( ab \) initio shapes of each protein. Rigid body
modeling of the SNX 20 and 21 proteins was performed using
either SASREF (SNX20Δ50) or BUNCH (SNX21Δ93RQ) with
partial scattering amplitudes computed by the program
CRYSOL. Models for SNX20 and SNX21 PX domains were
generated using the structure of kinesin16B (PDB code 2V14) and
cytokine-independent survival kinase (PDB code 1XTN) as tem-
plates, respectively (using Swiss-model), and used in combination
with the monomeric domain of the SNX21TPR crystal structure
(PDB code 4YMR). CRYSOL was used to evaluate the fitting of
the theoretical curve of SNX21TPR to the experimental scattering
data. The atomic models generated were docked into the SAXS-
base models using the program SUPCOMB20.

Liposome Pelleting Assays—PLs were protonated using HCl
and mixed with POPC (1-palmitoyl-2-oleoylphosphatidy-
choline) lipids (POPC-phosphatidylethanolamine:PL mixed
as 70–30:10%) or Folch lipids (Folch:PL mixed as 90:10%). Li-
posomes were prepared using a 100-nm membrane for extrusion
to obtain small unilamellar vesicles. The assay was conducted
using 20 μg of protein diluted in a 1 mg/ml of liposome solution
in assay buffer (25 mM HEPES (pH 7), 150 mM NaCl, 1 mM
DTT) to a total volume of 100 μl. After a 20-min incubation at
room temperature, the liposomes were pelleted by ultracentri-
fugation at 100,000 × g using a benchtop ultracentrifuge
(Optima TL centrifuge, rotor TLA100.3). The supernatant was
collected and the pellet was resuspended in 50 μl of assay buffer
to be analyzed by SDS-PAGE and Coomassie staining.

Isothermal Titration Calorimetry—Isothermal titration calor-
imetry experiments were performed on a microcal iTC200
instrument in 50 mM Tris (pH 8.0), 100 mM NaCl. Phospholip-
id lipids (diC₈-PtdIns3P, diC₈-PtdIns(3,4)P₂, diC₈-PtdIns(3,5)P₂,
diC₈-PtdIns(4,5)P₂, diC₈-PtdIns(3,4,5)P₃, diC₈-PtdIns4P, diC₈-
PtdIns) were dissolved in the respective buffers at 500 μM
and titrated into 15 μM SNX20 protein solutions in 13 3.1-
μl aliquots at 25 °C. Data were processed using ORIGIN to extract
the thermodynamic parameters \( \Delta H \), \( K_a (1/K_d) \), and the stoichi-
ometry \( n \). \( \Delta G \) and \( \Delta S \) were derived from the relationship:
\( \Delta G = -RT \ln K_a \) and \( \Delta H = T \Delta S \).

Cell Culture and Transfection—Human epithelial HeLa (CCL-
2) cells were maintained in complete Dulbecco’s modified
Eagle’s medium (DMEM) (Life Technologies) supplemented
with 10% (v/v) fetal bovine serum (FBS) and 2 mM L-glutamine.
Cells were incubated in humidified air/atmosphere (5% CO₂) at
37 °C. Cells were transfected with Lipofectamine 2000 as per
the manufacturer’s instructions (Life Technologies). 0.8 μg of
DNA and 2 μl of Lipofectamine 2000 was used per well of a
24-well plate.
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Immunofluorescence Imaging—Cells were grown on glass coverslips, fixed with 4% formaldehyde in 250 mM HEPES, and permeabilized with Triton X-100 (0.25% in PBS) before incubation with antibodies. Cells on coverslips were incubated with primary antibodies in 1% BSA in PBS for 1 h, followed by wash in PBS and a 1-h incubation with secondary antibodies with or without DAPI (all in 1% BSA in PBS). After washing in PBS, coverslips were rinsed in water and mounted on glass slides using Dako Fluorescent Mounting Medium. Fluorescent images were acquired using Zeiss LSM 710 with Plan-Apochromatic ×63/1.4 Oil DIC objective confocal microscope, operated by ZEN2009 acquisition software.

Cell Lysis and Immunoprecipitation—HeLa cells on 10-cm dishes were transfected using Lipofectamine 2000 (Life Technologies). 16 h post-transfection the cells were washed twice with cold PBS and lysed in TK lysis buffer containing 50 mM HEPES, 150 mM NaCl, 10 mM Na2P2O7, 30 mM NaF, 2 mM Na3VO4, 10 mM EDTA, 0.5% Nonidet P-40, 0.5 mM 4-benzene-sulfonyl fluoride hydrochloride and protease inhibitor mixture. Lysates were centrifuged at 13,300 rpm for 10 min at 4 °C, and the resulting supernatant was subjected to BCA assay to determine protein concentrations. For co-immunoprecipitation, equivalent amounts of protein samples were pre-cleared with 30 μl of protein G-conjugated agarose beads (50% slurry in PBS) for 1 h at 4 °C. The cleared supernatants were then subjected to CD8 or Myc immunoprecipitation by using mouse monoclonal anti-CD8 or anti-myc antibody coupling with Protein G-conjugated agarose beads overnight at 4 °C. The beads were then washed three times with lysis buffer, before bound proteins were eluted by boiling the beads for 5 min in SDS-PAGE protein loading buffer.

SDS-PAGE and Western Blotting—Equal amounts of cell lysates or immunoprecipitation samples resuspended in SDS-PAGE protein loading buffer were resolved on SDS-PAGE gels and transferred onto a PVDF membrane (Immobilon-FL, Millipore) using a semi-dry transfer apparatus (Bio-Rad) according to the manufacturer’s instructions. After blocking in diluted Odyssey blocker (1:1 ratio in PBS) at room temperature for 1 h, membranes primary antibodies in diluted Odyssey blocker with 0.1% Tween 20 overnight at 4 °C. Membranes were washed in PBST three times for 5 min each wash, followed by the incubation of membranes with IRDye® secondary antibodies for 1 h at room temperature. Fluorescence intensities were detected by LI-COR Odyssey Infrared Imaging System (LI-COR Biosciences). For the detection of less sensitive signals, the enhanced chemiluminescence (ECL) detection kit (Super Signal ECL substrate, Thermo Scientific) was used.

Results

SNX20 and SNX21 Share Conserved Domain Architectures—Based on sequence alignment and secondary structure prediction analysis, the SNX20 and SNX21 proteins have a similar domain architecture and share ~40% similarity in their protein sequence. Both SNX20 and SNX21 have a predicted N-terminal disordered region, followed by a PX domain closely linked by a short sequence to the C-terminal PXB domain we defined previously (5) (Fig. 1, A and B). The helical repeats of the latter are weakly predicted to form a TPR-fold, a domain typically involved in protein-protein interactions (25). Interestingly, the very N terminus of SNX21 is predicted to include a short conserved stretch of ~10 residues with α-helical secondary structure that is absent in SNX20, and its conservation across various species suggests a potentially unique role in SNX21 (Fig. 1B). As almost all other structural elements in both SNX20 and SNX21 align perfectly, it is clear these two proteins will share a similar tertiary structure and likely have related and perhaps redundant cellular functions. In phylogenetic analyses of the SNX20 and SNX21 proteins we were not able to trace the ancestry of these proteins further back than the Insecta class where only a single homologous protein is found (Fig. 1, C and D). This suggests the PXB domain was likely acquired late in eukaryotic evolution, and subsequently this ancestral PXB domain-containing protein underwent gene duplication and neofunctionalization that created two paralogues in chordates. SNX21 shows a slightly higher similarity to the ancestral Drosophila melanogaster protein compared with SNX20, although the difference is not significant enough to be confident of stating whether SNX20 or SNX21 is the closest functional relative of the insect protein.

The SNX21 C-terminal PXB Domain Is a Tetratricopeptide Repeat (TPR)-fold—As SNXs for the most part couple the PX domain with other modules, they possess a wide range of functions including phosphoinositide kinase activity, phospholipase activity, and protein-protein interactions among others (4, 5, 7). Based on our structural predictions, we accordingly designed a number of full-length and truncated constructs that were used in subsequent structural and biochemical experiments (Fig. 2A). Multi-angle laser light scattering (MALLS) studies of these recombinant proteins indicated that SNX20Δ50 and SNX21Δ93 are both monomeric in solution, whereas the SNX21 PXB domain (SNX21Δ230) appears to form a stable homodimer. To gain insight into the possible functions of the SNX20 and SNX21 proteins, we crystallized and determined the structure of the C-terminal SNX21 PXB domain by single isomorphous replacement and anomalous scattering (SIRAS) at a resolution of 2.4 Å (Fig. 2B; Table 1). The structure shows the PXB domain does indeed consist of TPR α-helical repeats. Clear electron density reveals that the crystallized protein forms a domain-swapped dimer, linked between helices α4 and α5, stabilizing the two molecules in the asymmetric unit (Fig. 2B). This unconventional domain-swapped conformation correlates with the MALLS data that showed the SNX21 PXB/TPR domain to be dimeric in solution (Fig. 2A). In addition, the domain-swapped SNX21 PXB/TPR crystal structure showed excellent agreement with SAXS data, indicating that the domain-swapped organization of this protein is not due to its crystalline arrangement but represents a low energy, stable assembly adopted by the recombinant production in solution (Fig. 2C; Table 2).

Although the SNX21 PXB domain forms a domain-swapped dimer, because both full-length SNX20 and SNX21Δ93 are monomeric, PXB dimerization is unlikely to be biologically relevant. We believe this low-energy pairing of the PXB domain forms during recombinant expression in E. coli, due to the lack of the N-terminal PX domain. Notably, neither the PXB domain of SNX20, nor the isolated PX domains of either
SNX20 or SNX21 are soluble when expressed in E. coli, suggesting an intimate stabilizing association between the PX and PXB domain in the full-length proteins (further confirmed by SAXS studies described below). Therefore we based our further analyses on the extrapolated SNX21 TPR domain monomer (Fig. 3A). The SNX21 TPR domain exhibits the typical helix-turn-helix motif common to TPR proteins. The first six α-helices (α1-α6) correspond to three typical TPR repeats, whereas the seventh and final C-terminal α-helix (α7) folds in an atypical conformation in both protomers, traversing back onto the core structure (Fig. 3, A and B). The exposed surface where a α7 capping helix would normally pack against helices α5 and α6 is predominantly hydrophilic (including exposed Arg, Glu, and Asn side chains), and does not show the expected hydrophobic nature required for typical α7 packing. We deduce that the unusual α7 conformation is therefore a natural result of small sequence alterations in SNX21 protein precluding this normal arrangement.

Each TPR repeat consists of an inner helix and an outer helix that forms a concave structure, which has almost exclusively been shown to promote protein-protein interactions (25). The α7 helix itself is highly conserved, and the amino acids lining the concave groove beneath the helix also form a hydrophobic surface that is conserved in evolution among SNX21 proteins.
This region of the protein may function in establishing hydrophobic contacts with protein partners. However, the presence of the H92517-helix completely obscures this hydrophobic surface. Although highly speculative, the exposed loop between H92516 and H92517 contains serine, threonine, and proline residues, suggesting some degree of flexibility and a potential for phosphorylation-induced regulation.

The SNX21 PXB/TPR Domain Shares Structural Similarities to the Yeast Protein Fis1p—In almost all TPR domains, the extended surface shaped by the TPR repeats forms an interface for controlling interactions with various protein partners (25). For example, the concave surface of the TPR domain often associates with short linear peptide motifs in their protein partners, as seen for the Hop TPR2A domain in complex with an Hsp90 peptide (26) (PDB code 1ELR) (Fig. 4A). All TPR domains possess an odd number of H9251-helices, where the last helix forms a final capping structure at the C terminus of the protein. In SNX21, however, the final odd helix H92517 instead adopts a unique conformation that in fact folds back across the surface of the TPR repeats, obscuring the putative concave surface (Fig. 3C). This suggests that this region of the protein may function in establishing hydrophobic contacts with protein partners. However, the presence of the α7-helix completely obscures this hydrophobic surface. Although highly speculative, the exposed loop between α6 and α7 contains serine, threonine, and proline residues, suggesting some degree of flexibility and a potential for phosphorylation-induced regulation.

TABLE 1
Data collection, phasing and refinement statistics for SNX21 PXB domain

|                               | Native                   | Mercury derivative | Wavelength (Å) | 1.5418 | 1.5418 |
|--------------------------------|--------------------------|--------------------|----------------|--------|--------|
| Space group                    | P212121                  | P212121            |                |        |        |
| Cell dimensions                | 100.9, 100.9, 63.6       | 100.6, 100.6, 64.3 | a, b, c (Å)    | 90, 90, 120 | 90, 90, 120 |
| Resolution (Å)                | 21.5-2.40 (2.53-2.40)    | 21.55-2.63 (2.78-2.63) | Rmerge         | 0.088 (0.330) | 0.141 (0.613) |
| Rmerge (Å)                     | 10.9 (2.8)               | 9.8 (2.8)          |
| Total number reflections       | 60,610                   | 97,578             |
| Total unique reflections       | 13,890                   | 11,386             |
| Completeness (%)              | 93.0 (66.4)              | 99.8 (100.0)       |
| Multiplicity                   | 4.4 (2.9)                | 8.6 (7.8)          |
| Wilson B factor                | 47.6                     | 59.1               |

Phasing (SIRAS)

| Number Hg sites found | 4                      |
| Figure of merit       | 0.42                   |
| Figure of merit after density modification | 0.73 |
| Number of residues automatically built | 271 of 288 |

Refinement

| Resolution (Å) | 21.5-2.4 (2.59-2.40) |
| No. reflections/No. Rfree | 13873 (1932)/692 (123) |
| Rmerge/Rfree | 0.230 (0.295)/0.267 (0.332) |
| No. atoms | 2,048 |
| Protein | 2,048 |
| Solvent | 21 |
| Average B-factor (Å²) | 48.3 |
| Root mean square deviations | 0.008 |
| Bond lengths (Å) | 1.37 |
| Bond angles (°) | 1.37 |
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region of the TPR domain likely to promote interactions. For example, aligning the SNX21 and Hop/Hsp90 structures highlights the fact that the α7 helix of SNX21 occludes this typical ligand-binding interface (Fig. 4A). In Hop TPR2A, the Hsp90 motif is able to bind in the groove formed by the three core TPR repeats, because the C-terminal helix (corresponding to α7 in SNX21) is folded in the typical fashion parallel to and capping the last TPR repeat.

The SNX21 conformation suggests a potential self-regulatory interaction by the C-terminal α7-helix. Interestingly, we noticed a similar occlusion of the concave TPR surface in only one other TPR-containing protein: the yeast mitochondrial fission protein Fis1p. Fis1p is a mitochondrial membrane protein with a single-pass C-terminal transmembrane sequence, and a cytoplasmic N terminus recruiting the dynamin-related Dnm1p protein that mediates fission at the mitochondrial division site via adaptors Mdv1p and Caf4p (27). This cytosolic domain is a TPR-fold with an N-terminal extension shaped in a short α-helix, which traces back across the concave surface of the TPR domain, in a similar location to the SNX21 α7 helix (28, 29) (Fig. 4B). Fis1p expression in FIS1−/− yeast strains rescues mitochondrial fission-deficient phenotypes, whereas complementation with Fis1pΔN (lacking the N-terminal α-helix) does not (28). This indicates that the N-terminal helix plays a crucial role in Fis1p function in yeast, and structural studies have provided a mechanistic explanation. Yeast Fis1p effectors Mdv1 and Caf4 both associate in a hairpin fashion by clamping two interaction areas on each side of Fis1p (29) (Fig. 4C, left). This interaction is stabilized by two structural elements: a helix from Caf4p stacks against the N-terminal extension of Fis1p, and a conserved groove on the top part of the Fis1p TPR domain accommodates the stretch of sequence linking the two Caf4p helices. This binding groove is conserved in Fis1p, and most interestingly a similarly located and highly conserved groove is also seen in SNX21 (Fig. 4C). In SNX21 this groove is acidic in nature suggesting the potential to bind positively charged sequences (Fig. 4C).

SNX20 and SNX21 Adopt a Globular and Compact Architecture—To define the overall architecture of the SNX-PXB proteins in solution, we used SAXS to examine both SNX20 and SNX21 proteins with N-terminal deletions to remove the predicted disordered regions (Fig. 5; Table 2). The scattering curves and pair distribution functions (Fig. 5A) indicate that SNX20Δ50 and SNX21Δ93 possess a relatively compact globular conformation in solution. Models for SNX20 and SNX21 PX domains were generated using the structure of kinesin16B (30) (PDB code 2V14) and cytokine-independent survival kinase (31) (PDB code 1XTN) as templates, respec-

### Table 2

Summary of MALLS and SAXS analyses of the SNX-PXB proteins

| Protein          | MALLS (Sequence, kDa) | SAXS (Sequence, kDa) | SNX20Δ50 (Sequence, kDa) | SNX21Δ93 (Sequence, kDa) |
|------------------|-----------------------|----------------------|-------------------------|--------------------------|
| SNX20 PXB/TPR    | 29.3                  | 28.9                 | 40.8*                   | 40.6                     |
| SNX20Δ50 SNX21Δ93| 30.6                  | 33.2                 | 42.1                    |                          |

Ab initio analysis

χ (GASBOR)

0.36

0.38

0.70

Rigid-body modeling/ validation

χ²

0.435

0.794

1.21

CRYSOL

BUNCH

SASREF

* Including a 10-kDa ubiquitin tag.
tively (using Swiss model), and in combination with the mono-meric domain of the SNX21 TPR crystal structure were used by
the program BUNCH to perform rigid body modeling. Com-
bining these two structural modules by rigid body refinement
allowed us to obtain a full-length solution structure that was
compared with the SNX20\textsubscript{H9004} and SNX21\textsubscript{H9004} SAXS enve-
lopes. The modeled domains were refined against the scattering
data with excellent scores, indicating that their architecture
and orientation in solution reflects their true conformation
(Fig. 5B, Table 2). This derived hybrid structure overlays well
with the \textit{ab initio} models calculated by the program GASBOR,
and reveal that the PX and PXB/TPR domains of SNX20 and
SNX21 are closely packed to form a compact tertiary structure.
Altogether, this data suggests that the PX and PXB/TPR
domains of the SNX-PXB proteins are tightly packed against
each other, and are likely to use this rigid architecture to simul-
taneously bind PIs and protein partners by increasing binding
avidity.

\textbf{Structural Studies of SNX20 and SNX21}

\textbf{The SNX-PXB Proteins Localize to Early Endosomes at Steady
State—}The localization of the SNX proteins to specific regions
of the endocytic system is critical to their function. Previous
studies of SNX20 indicated an endosomal localization of the
protein in COS and CHO cells (16). Here we compared the
localization of both SNX20 and SNX21 in HeLa cells using
N-terminal myc-tagged myc-SNX20 and myc-SNX21 con-
structs (Fig. 6A). These experiments confirm the localization of
SNX20 on early endosomal EEA1-positive punctate structures,
but not late endosomal LAMP1-positive compartments indi-
cating a predominantly early endosomal distribution (Fig. 7A).
SNX21 shows an almost identical distribution indicating the
two paralogues are both early endosome-associated SNX family
members.

The membranes of the early endosomal compartment are
naturally enriched in PtdIns3P at steady state, and the struc-
tural basis for canonical PtdIns3P recognition by PX domains is
now relatively well understood, involving a key arginine residue

\begin{figure}
\centering
\includegraphics[width=\textwidth]{figure4.png}
\caption{Potential ligand binding sites in the SNX21 PXB/TPR domain. A, the SNX21 PXB/TPR domain (blue) is aligned with the Hop TPR2A domain-Hsp90 peptide complex (Hop TPR2A, orange; Hsp90 peptide, yellow sticks). The cores of the two TPR domains superpose closely, whereas the atypical SNX21 C-terminal \( \alpha7 \) helix lies across the canonical binding interface that mediates Hsp90 binding in Hop TPR2A. The directions of the Hop TPR2A and SNX21 \( \alpha7 \) helices are indicated by dashed arrows to highlight the different orientations. B, structural alignment of SNX21 (blue) and yeast Fis1p TPR domains (yellow; PDB code 2PQR) highlights the structural conservation of the TPR cores, whereas the C-terminal \( \alpha7 \) of SNX21 and the N-terminal \( \alpha0 \) of Fis1p-fold against the concave surface from opposite directions. C, left, the Fis1p-Caf4p complex structure shows the binding region of Caf4p forms two \( \alpha \)-helices that associate with opposite faces of the TPR domain, whereas the linker regions bind within a conserved groove at the top of Fis1p TPR domain highlighted in surface view. Right, the SNX21 TPR also possesses a similar conserved groove at the same position (dashed ellipse). This conserved surface groove on the SNX21 TPR domain is acidic, as represented by electrostatic charges plotted on its surface (dashed ellipse).}
\end{figure}
that contacts the 3-phosphate group (Arg<sup>113</sup> in SNX20 and Arg<sup>161</sup> in SNX21) (5). Using isothermal titration calorimetry we find that SNX20 binds PtdIns3P headgroups with a $K_d \approx 25 \mu M$, whereas a mutation in the canonical PtdIns3P-binding pocket (R113Q) abolishes this interaction (Fig. 6B). As expected, when SNX20 and SNX21 protein constructs are mutated at the 3-phosphate binding arginine residues of their PX domains they show a diffuse cytoplasmic localization in HeLa cells and are no longer recruited to endosomal membranes (Fig. 6A).

**The SNX-PXB Proteins Interact with PtdIns3P and PtdIns(4,5)P<sub>2</sub>-containing Membranes in Vitro via Overlapping Pockets—** Although cellular experiments confirm the binding of SNX20 and SNX21 to PtdIns3P-enriched early endosomes in cells we also tested their qualitative interactions with membranes in vitro using liposome-pelleting assays with full-length SNX20 and SNX21Δ93 (Fig. 7A). Both SNX20 and SNX21Δ93 display quite similar binding profiles to PI-containing liposomes. SNX20 and SNX21 were both found to bind PtdIns3P-containing liposomes as predicted from their sequences and endosomal localization (5). PtdIns3P-enriched liposomes pelleted slightly higher levels of SNX21, suggesting a tighter interaction than with SNX20. Surprisingly, however, a substantial amount of protein was also found in the pellet fraction of liposomes containing PtdIns(4,5)P<sub>2</sub>. This appeared to be selective as proteins were not bound to the similar isoform PtdIns(3,4)P<sub>2</sub>. This data also correlates with the fact that the SNX-PXB proteins...
bind very strongly to Folch lipids, a brain-extracted lipid mixture containing multiple species of PIs but only relatively low levels of PtdIns3P (Fig. 7A). In our experience, proteins that bind PtdIns3P alone are not fully pelleted by Folch liposomes in these assays. In liposome pelleting assays using SNX20 and SNX21 proteins with mutations in the canonical PtdIns3P-binding site (R113Q and R161Q, respectively), we see complete loss of binding to PtdIns3P-containing liposomes, however, binding to Folch lipids and PtdIns(4,5)P2 remains relatively unaffected (Fig. 7B). This suggests that the canonical binding pocket of the SNX20 and SNX21 PX domains is able to associate with PtdIns3P normally, but that a second PtdIns(4,5)P2 binding site exists somewhere within the proteins. Notably, we were not able to detect binding to the soluble PtdIns(4,5)P2 headgroup by isothermal titration calorimetry (not shown) suggesting this interaction occurs preferentially within a membrane environment.

Surface representations of the SNX20 and SNX21 PX domain homology models (generated for our SAXS experiments) suggest the presence of additional basic, positively charged putative phospholipid binding sites, some of which overlap to some extent with the canonical PtdIns3P-binding site (Fig. 7C). We next mutated these clusters of positively charged residues in SNX21/H9004 to glutamine to test their potential role in PI interactions. As described above SNX21/H9004 (R161Q) was perturbed in binding PtdIns3P but was still pelleted by Folch liposomes. In fact we found that all mutant proteins were somewhat perturbed in binding to PtdIns3P in this system, however, the
greatest effects were seen for the canonical R161Q site, and also for cluster 2 (residues Arg187-Lys188-Arg189) (Fig. 7E). Similarly, this cluster 2 mutant, and R161Q to a lesser extent, also reduced binding to both Folch and PtdIns(4,5)P2. Cluster 2 residues lie in the extended loop connecting helices H9251 and H9252 of the PX domain. Typically this loop is highly flexible, and adjusts its conformation to embrace the bound canonical PtdIns3P headgroup (5). Our data therefore suggests that the PI-binding pocket is competent for binding to PtdIns3P in the usual manner, but can also accommodate PtdIns(4,5)P2, although the precise orientation of PtdIns(4,5)P2 in the binding pocket is unknown and will require further high-resolution structural analyses to define. Altogether, our data indicates that PtdIns(4,5)P2 binding is mediated by an overlapping binding pocket to the canonical PtdIns3P cavity on the PX domain of the SNX-PXB proteins.

**No Evidence for Stable Association of SNX20 or SNX21 with PSGL-1**—The major question regarding the SNX-PXB proteins is what is the function(s) of these apparently conserved endosomal scaffolds? Previously it was reported that SNX20 could interact with the cytoplasmic domain of the PSGL-1 receptor, and it was observed that overexpression of PSGL-1 caused a redistribution of PSGL-1 to a predominantly cell-surface location to SNX20-decorated endosomal compartments (16). We attempted to confirm these findings using a CD8-PSGL-1 fusion reporter, where the cytoplasmic tail of PSGL-1 is fused to the lumenal and transmembrane domains of CD8. In immunofluorescence experiments, we observe that CD8-PSGL-1 is localized at the plasma membrane of HeLa cells and also decorates intracellular membrane compartments (Fig. 8A). When the CD8-PSGL-1 reporter is co-expressed with myc-SNX20 or myc-SNX21 we do observe colocalization of the proteins at early endosomal compartments, however, we do not see any change in CD8-PSGL-1 cellular distribution relative to control experiments as was reported for SNX20 previously (16) (Fig. 8B). In conjunction with this we also do not find stable association with CD8-PSGL-1 using either immunoprecipitations (Fig. 8C) or GST-tagged PSGL-1 pulldowns with purified SNX-PXB proteins (not shown). Therefore, whereas our data shows that both SNX20 and SNX21 share overlapping early endosomal localization with PSGL-1, we do not see evidence for a stable interaction with PSGL-1 in this context.

**Discussion**

Although the functions of many of the mammalian SNX proteins have been studied, revealing roles in a variety of signaling and cellular trafficking processes (4, 5, 7), a large number still remain very poorly characterized. Two of these proteins, SNX20 and SNX21, belong to the SNX-PXB subfamily, and to gain insights into the possible functions of these SNX proteins we have examined both their structure and membrane binding properties in vitro and in situ. Overall our data indicates that...
SNX-PXB proteins will possess a compact modular structure, which will likely be employed to engage multiple membrane-associated ligands including lipid headgroups and transmembrane proteins at the endosomal compartment.

The PX domain is generally employed by SNX family proteins to bind specifically to the PI lipid PtdIns3P, leading to the large majority of SNX proteins being localized at the PtdIns3P-enriched early endosomal compartment (5, 32). Although we find that both SNX20 and SNX21 are localized to endosomal compartments, as was also shown previously for SNX20 (16), we discovered an unexpected ability to recognize both PtdIns3P and PtdIns(4,5)P2. This is promoted by a
large positively charged binding pocket, with overlapping specificity for these two phospholipids. It should be noted that our results differ somewhat from previous experiments conducted by Schaff et al. (16) where lipid-strip assays suggested SNX20 could bind to numerous PI species other than the two we identified. The presence of two distinct binding pockets in the same PX domain has previously been described, for example, in phospholipase D1 (33). In this case a second distinct lipid binding pocket is formed by a cluster of cationic residues that bind with moderate affinity to anionic lipids and the secondary messenger phosphatidic acid. Simultaneous association with both distinct binding pockets increases the efficiency of phospholipase D1 PX domain-mediated membrane targeting. The mechanistic basis and more importantly the functional significance of the binding of SNX-PXB proteins to PtdIns(4,5)P₂ is still unclear, especially given the obvious lack of plasma membrane recruitment of SNX20 and SNX21 where PtdIns(4,5)P₂ levels are highest. However, it suggests a potential for regulating the localization of SNX-PXB proteins to different membrane domains, or following stimulation-induced changes in PI levels.

Our findings confirmed that the previously defined C-terminal “PXβ” domain of the SNX20 and SNX21 proteins possesses a TPR-fold, containing three TPR α-helical repeats. TPR scaffolds are composed of a sequential array of paired α-helices that form inherently curved structures, with concave surfaces typically employed to bind associated ligands. More than 100 TPR structures deposited in the PDB show that these domains exhibit different surface properties, promoting selectivity for targets. The amino acid insertions that modify the topology of the loops and length and angles of the helices give rise to impressive variations in structure, and thus make this module a highly versatile binding scaffold (reviewed in Ref. 34). The usual topology of TPR proteins is supported by an odd number of helices, the final one capping the hydrophobic core of the last TPR repeat for solubility. In our case, the three SNX21 TPR repeats are not capped in the usual fashion; instead the atypical helix α7-folds back to cover the hydrophobic concave groove of the TPR domain. In this conformation, helix α7 covers the region normally involved in heterotypic protein-protein interactions. Although Fis1p and SNX20/SNX21 are functionally unrelated, their similar topologies lead us to speculate that the mechanistic properties of Fis1p may provide some clues to the potential binding regions of the SNX20/SNX21 proteins. By comparison with the yeast TPR protein Fis1p (28), we suggest one possibility is that membrane-targeted SNX20/SNX21 proteins may engage transmembrane ligands through an unusual binding surface similarly to the binding of the Caf4p adaptor to Fis1p (Figs. 4C and 9). A contrasting alternative model is that the atypical α7 helix could play a potential regulatory role, whereby ligand binding or perhaps signal-induced conformational changes such as phosphorylation of the αα-α7 linker region cause a movement in α7 allowing ligand association with the conserved hydrophobic pocket buried beneath.

This speculation regarding ligand binding is predicated on the assumption that the TPR domains of SNX-PXB proteins are involved in modulating protein-protein interactions (as is typically the case), either with soluble effectors or transmembrane cargo proteins. However, defining the mechanism of ligand attachment to SNX20 or SNX21 will first require identification of SNX20- and SNX21-associated proteins. Schaff et al. (16) proposed a role for SNX20 in trafficking of the inflammatory adhesion receptor PSGL-1 after identifying an interaction with PSGL-1 in a yeast two-hybrid screen. Unfortunately we were not able to replicate these reported interactions between PSGL-1 and SNX-PXB proteins, although we do observe an overlapping cellular distribution of the proteins. We note that a confusing aspect to this previous study is that the region of SNX20 binding to PSGL-1 identified in two-hybrid screens was a C-terminal fragment of the PX domain plus the PXβ domain, however, peptide array studies suggested the PX domain alone was responsible for binding. Thus the molecular basis for PSGL-1 interaction with SNX20 remains to be firmly established, and whether SNX20 or SNX21 contribute to PSGL-1 trafficking remains unclear.

In conclusion, we show that the SNX20 and SNX21 constitute a subfamily of SNX proteins possessing both a PX domain and C-terminal PXβ/TPR domain. These proteins show the clear hallmarks of being membrane-associated scaffolding proteins, with a potential to mediate interactions with ligands.
including transmembrane proteins that require transport within the endosomal system, cytosolic effectors, or proteins regulating SNX-PXB function. Our work provides the first molecular insights into these proteins, and a foundation for understanding the mechanisms by which they influence endosomal function.

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