Molecular Biology of the Cell
Vol. 8, 1261–1271, July 1997

Syntaxin 6 Functions in trans-Golgi Network Vesicle Trafficking

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Submitted February 13, 1997; Accepted April 23, 1997
Monitoring Editor: Ari Helenius

The specific transfer of vesicles between organelles is critical in generating and maintaining the organization of membrane compartments within cells. Syntaxin 6 is a recently discovered member of the syntaxin family, whose constituents are required components of several vesicle trafficking pathways. To better understand the function of syntaxin 6, we generated a panel of monoclonal antibodies that specifically recognize different epitopes of the protein. Immunoelectron microscopy shows syntaxin 6 primarily on the trans-Golgi network (TGN), where it partially colocalizes with the TGN adapter protein AP-1 on clathrin-coated membranes. Additional label is present on small vesicles in the vicinity of endosome-like structures. Immunoprecipitation of syntaxin 6 revealed that it is present in a complex or complexes with α-soluble NSF attachment protein, vesicle-associated membrane protein 2, or cellubrevin and a mammalian homologue of VPS45, which is a member of the sec1 family implicated in Golgi to prevacuolar compartment trafficking in yeast. We show that mammalian VPS45 is found in multiple tissues, is partially membrane associated, and is enriched in the Golgi region. Converging lines of evidence suggest that syntaxin 6 mediates a TGN trafficking event, perhaps targeting to endosomes in mammalian cells.

INTRODUCTION

The ability to efficiently distribute classes of proteins to distinct membrane-bound organelles is a hallmark of eukaryotic cells and is a highly specific process mediated largely by vesicle trafficking (Palade, 1975). The routing of transport vesicles throughout the cell is controlled by three classes of proteins: those present on target membranes, those present on transport vesicle membranes, and a series of cytosolic proteins including ATPases and GTPases (Rothman and Wieland, 1996). The most intensely studied vesicle and target membrane proteins are members of the vesicle-associated membrane protein (VAMP)[1]/synaptobrevin, syntaxin, and Synaptosomal-associated protein of 25 kDa (SNAP-25) families. These proteins termed soluble *N*-ethylmaleimide-sensitive factor (NSF) attachment protein receptors (SNAREs) at least partially define the specificity of vesicle docking and membrane fusion throughout the secretory pathway (Bennett *et al.*, 1992; Sollner *et al.*, 1993).

One of the most detailed biochemical models of vesicle docking and fusion has been proposed for regulated exocytosis in nerve terminals (Bennett and Scheller, 1994). The hydrophilic protein n-sec1 binds to syntaxin 1 at the plasma membrane. n-sec1 probably serves to regulate protein interactions of syntaxin with other components including SNAP-25 and

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[1] Abbreviations used: GST, glutathione *S*-transferase; MPR, mannose-6-phosphate receptor; NSF, *N*-ethylmaleimide-sensitive factor; SNAP, soluble NSF attachment protein; SNAP-25, synaptosomal-associated protein of 25 kDa; SNARE, soluble NSF attachment protein receptor; VAMP, vesicle-associated membrane protein.

VAMP (Pevsner *et al.*, 1994). VAMP, syntaxin, and SNAP-25 form a core protein complex thought to be a central player in conferring the specificity of vesicle trafficking and/or membrane fusion. Although a particular sec1-like protein and SNARE partners act at a single transport step, other proteins such as NSF and α-SNAP are involved at several different steps throughout the secretory pathway and thus are general mediators of vesicle trafficking.

To determine other general and specific aspects of intracellular vesicle transport, different trafficking steps throughout the secretory pathway have been examined. The shuttling of vesicles between the endoplasmic reticulum (ER) and Golgi has been an area of intense investigation. ER to Golgi trafficking is mediated in part by syntaxin 5, which is localized to the *cis*-Golgi (Bennett *et al.*, 1993; Dascher *et al.*, 1994). Analogous to the syntaxin 1–n-sec1 pairing, syntaxin 5 binds to, and is thought to be regulated by, the sec1 family member rsly1 (Dascher and Balch, 1996). In addition syntaxin 5 is found in a series of complexes with four additional type II membrane proteins, GOS-28, rbet1, rsec22b, and membrin, that are localized to the ER and the *cis*-Golgi (Hay *et al.*, 1997). Analogous complexes have also been found in yeast (Lian and Ferro-Novick, 1993; Sogaard *et al.*, 1994). Although many features are conserved between synaptic vesicle targeting and ER to Golgi trafficking, it is unclear why there are so many different membrane proteins for the ER to Golgi trafficking step. Thus, although it appears that several trafficking steps use syntaxin, sec1, and VAMP family members, it also appears that each trafficking step may have its own eccentricities.

We are interested in examining other trafficking steps to determine how applicable the models of synaptic vesicle and ER to Golgi trafficking are to other vesicular transport steps. Syntaxin 6 is a recently discovered protein implicated in intracellular vesicle trafficking through its sequence homology with the syntaxin and SNAP-25 families, as well as its direct interaction with α-SNAP (Bock *et al.*, 1996). Syntaxin 6 shows homology to the syntaxin family member Pep12/VPS6, a yeast protein implicated in vacuole protein trafficking (Jones, 1977). Mutants of *Saccharomyces cerevisiae* that lack Pep12 accumulate 40- to 50-nm vesicles, have enlarged vacuoles, and are defective in the delivery of soluble hydrolases to the vacuole (Becherer *et al.*, 1996). By subcellular fractionation, the major pool of Pep12 was believed to be associated with endosomes, whereas a minor pool cofractionated with a late Golgi/trans-Golgi network (TGN) marker.

Another yeast protein implicated in Golgi to prevacuolar compartment trafficking is VPS45, identified in a mutant screen as a requirement for vesicle transport from the Golgi apparatus to the vacuole (Rothman and Stevens, 1986; Rothman *et al.*, 1989). Like Pep12, VPS45 is a class D type mutant required for the proper sorting of vacuolar hydrolases and for segregation of vacuoles into budding daughter cells (Cowles *et al.*, 1994; Piper *et al.*, 1994). Characterization of the VPS45 sequence revealed that this protein is a member of the vesicle trafficking protein gene family typified by sec1 (Pevsner, 1996). Although a genetic interaction between Pep12 and VPS45 exists, no direct biochemical data physically links these proteins.

The mammalian counterpart of transport to the yeast vacuole is the trafficking of TGN-derived vesicles to endosomes and then to lysosomes. This pathway is best understood from studies on the transport of soluble lysosomal enzymes (Kornfeld and Mellman, 1989; Ludwig *et al.*, 1995). In the TGN, newly synthesized lysosomal enzymes bind to two types of mannose-6-phosphate receptors (MPRs). The cytoplasmic tails of these receptors contain signals that can bind the adapter protein AP-1, a prerequisite for the formation of clathrin-coated vesicles from the TGN (Pearse and Robinson, 1990). The MPRs and their ligands exit the TGN in AP-1/clathrin-coated vesicles, which then shed these coats before fusing with an endosomal intermediate (Ludwig *et al.*, 1991; Johnson and Kornfeld, 1992a). So far, the AP-1/clathrin-mediated pathway is the only known pathway in which TGN-derived vesicles directly fuse with endosomes. By contrast, segregation of vacuolar proteins in yeast does not appear to require clathrin (Payne *et al.*, 1988).

Although it is likely that syntaxin 6 mediates aspects of vesicle trafficking, it was unclear where within the secretory pathway the protein functions. In this report we generate a new series of monoclonal antibodies raised against syntaxin 6 and define the precise subcellular localization of the protein and its interactions with other vesicle trafficking proteins. Converging lines of evidence suggest that syntaxin 6 mediates a TGN vesicle trafficking event, probably transport from the TGN to the endosome.

MATERIALS AND METHODS

Monoclonal Antibodies

Monoclonal antibodies against syntaxin 6 were obtained from the corresponding hybridoma cell lines generated by fusion of NS-1 mouse myeloma cells with spleen cells from a BALB/c mouse immunized with a glutathione *S*-transferase (GST)-syntaxin 6 fusion protein (Lane *et al.*, 1986). This mouse's serum recognized a band of 29 kDa in rat brain postnuclear supernatant. The monoclonal antibodies were isotyped by using an enzyme-linked immunosorbent assay (ELISA) system (Boehringer Mannheim, Indianapolis, IN). Ascites fluid was produced by Joseph Beirao (Josman Laboratories, Napa Valley, CA). The monoclonal antibodies' epitopes were mapped to various regions of syntaxin 6 through an ELISA system. Fusion proteins encompassing distinct domains of syntaxin 6 were absorbed to a 96-well microtiter tray. Culture supernatants were then incubated in the microtiter wells with the different constructs and processed for ELISA by standard methods as summarized in Table 1 (Harlow, 1988).

Table 1. Construct–antibody binding

	Construct–antibody binding				
Antibody	1–165 aa	1–197 aa	1–235 aa	26–235 aa	165–235 aa
3D10	+	+	+	−	−
8F4	+	+	+	−	−
3D6	+	+	+	+	−
8E1	+	+	+	+	−
3H4	−	+	+	+	+
7E2	−	+	+	+	+

A crude purification of the immunoglobulin (Ig) fraction of the 3D10 ascites fluid was performed using a standard procedure of ammonium sulfate precipitation and DEAE chromatography (Harlow, 1988).

Polyclonal Antibodies

Anti-VPS45 antiserum was produced by immunizing a Swiss/Webster mouse with a GST-h-VPS45 fusion protein. The serum was affinity purified with GST-h-VPS45 fusion protein immobilized on cyanogen bromide-activated Sepharose beads (Sigma, St. Louis, MO). This antiserum recognized a single band of 70 kDa in rat brain postnuclear supernatant by Western blot analysis (1:2000 dilution). Anti-glutaraldehyde-fixed syntaxin 6 antiserum was prepared by incubating GST-syntaxin 6 fusion protein (1–2 $\mu g/\mu l$ in 0.1 M sodium phosphate buffer, pH 7.4) in 1% glutaraldehyde for 1 h at 4°C (Danbolt *et al.*, 1992). Sodium borohydride was then added to a final concentration of 0.1%. This fixed protein was then used as an antigen to immunize a BALB/c mouse. The resulting serum recognized a single band of 29 kDa in rat brain postnuclear supernatant. To localize AP-1, we used an affinity-purified rabbit anti-gamma-adaptin protein antibody, kindly provided by Dr. M.S. Robinson (University of Cambridge, Cambridge, United Kingdom). Anti-VAMP rabbit polyclonal was used at a 1:100 dilution (Pevsner *et al.*, 1994).

Immunofluorescence Microscopy

Indirect immunofluorescence localization was performed on NRK, FAO, COS, and PC12 cell lines as described previously (Bennett *et al.*, 1993). Antisera were used at the following dilutions: syntaxin 6 monoclonal culture supernatants undiluted; purified 3D10 ascites fluid at a 1:1000 dilution; anti-VPS45 at a 1:1000 dilution; and anti-myc polyclonal antiserum at a 1:300 dilution (Santa Cruz Biotechnology, Santa Cruz, CA). COS cells were transfected with full-length syntaxin 6 with an amino-terminal myc tag in the pCMV expression vector (Andersson *et al.*, 1989). Transfections were performed as described previously (Hay *et al.*, 1996).

Immunogold Labeling of Ultrathin Cryosections

PC12 cells of a low passage number (clone II-251, kindly provided by Dr. W.B. Huttner, University of Heidelberg, Heidelberg, Germany) were cultured in DMEM supplemented with horse serum and fetal calf serum in 10% CO_2. Cells were fixed for 2 h at room temperature in 1% formaldehyde and 0.2% glutaraldehyde in 0.1 M sodium phosphate buffer (pH 7.4) and then embedded in 10% gelatin in phosphate-buffered saline. The gelatin blocks were impregnated with 2.3 M sucrose and prepared for cryosectioning as described (Slot *et al.*, 1988). Multiple immunogold labeling was performed as described (Slot *et al.*, 1991), and then the grids were analyzed in a JEOL 1010 electron microscope.

To quantitatively analyze the subcellular distribution of syntaxin 6, sections that were immunogold labeled with antibody 3D10 were scanned in a random manner. Each gold particle encountered that was located within 30 nm of a membrane was subscribed to the compartment with which it was associated. In total, 1039 gold particles were counted in grids derived from two independent labeling assays. Finally, the percentage of total label that was found over a specific compartment was calculated. Syntaxin 6 labeling over cellular compartments not indicated (e.g., plasma membrane, large dense-core vesicles, or lysosomes) was around background level and therefore not included.

Immunoprecipitations

Anti-syntaxin 6 monoclonal 3D10 immunobeads were produced by incubating purified 3D10 ascites fluid with protein G-Sepharose beads (Pharmacia Biotech, Uppsala, Sweden) at a ratio of 4 mg of antibodies to 1 ml of beads. The antibody was cross-linked to the beads with dimethyl pimelimidate using standard procedures (Harlow, 1988). Frozen rat brains (Harlan Bioproducts, Indianapolis, IN) were homogenized in 6 ml of homogenization buffer (50 mM Tris, pH 8.0, 150 mM NaCl, 1 mM dithiothreitol) per brain. Postnuclear supernatant was obtained by centrifuging the homogenate at 1000 × *g* for 5 min twice. Crude membranes were harvested by centrifuging the postnuclear supernatant at 20,000 × *g* for 15 min twice. Crude membranes were resuspended in the homogenization buffer at a concentration of 10 mg/ml. Membranes were solubilized by adding an equal volume of 2% Triton X-100 in homogenization solution to these crude membranes followed by a 1-h incubation at 4°C. Unsolubilized membranes were pelleted by centrifugation at 20,000 × *g* for 15 min. This extract was then precleared with 200 μl of protein G-Sepharose per ml of extract. The precleared extract was then mixed with the 3D10 or control immunobeads overnight in a ratio of 5 μl of beads to 1.5 ml of extract. The immunobeads were washed four times in 150 mM NaCl, 50 mM Tris (pH 8.0), and 0.5% Triton X-100, and material was eluted with SDS sample buffer.

Protein distribution and membrane extraction analyses were performed as described previously (Bock *et al.*, 1996). Western blotting of these samples was carried out by using affinity-purified anti-VPS45 serum (1:2000 dilution). Sucrose-gradient floatation analysis of VPS45 was performed as described previously (Hsu *et al.*, 1996).

RESULTS

Monoclonal Antibodies against Syntaxin 6

To further study the function of syntaxin 6, we produced monoclonal antibodies against a GST-syntaxin 6 fusion protein. After three immunizations, the mouse serum detected a band of the expected molecular weight, 29 kDa, on Western blots of postnuclear supernatants from rat brain tissue. We screened approximately 1000 hybridomas first by ELISA with the recombinant fusion protein and second by Western blotting against rat brain postnuclear supernatant (Figure 1A). Six independent hybridomas were recovered and cloned.

All six monoclonal antibodies contained IgG1 heavy chain and κ light chain. Each of the monoclonal antibodies was then tested for binding to a series of constructs encompassing various domains of the cytosolic portion of syntaxin 6. At least three different epitopes were recognized by the set of antibodies. Monoclonal antibodies 3D10 and 8F4 bind to the amino-terminal 25 amino acids, 3D6 and 8E1 bind to amino acids

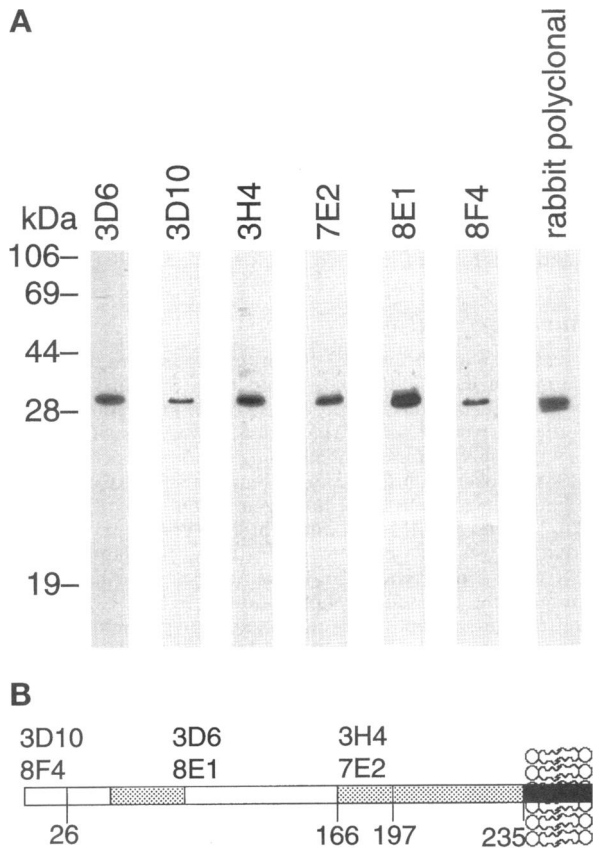

Figure 1. Syntaxin 6 monoclonal antibodies recognize different epitopes. Six independent hybridomas were isolated and characterized. (A) The culture supernatant was analyzed by Western blotting against postnuclear supernatant from rat brain tissue. A band at 29 kDa was detected at the predicted molecular weight of syntaxin 6. A band of the same size was detected with the previously characterized rabbit polyclonal serum. (B) Fusion protein constructs were used to map the epitopes for the six antibodies on syntaxin 6. Stippled boxes indicate predicted α-helical regions that may participate in coiled-coil protein interactions.

26–166, and 3H4 and 7E2 bind to amino acids 166–197 (see MATERIALS AND METHODS and Figure 1B).

Syntaxin 6 in the TGN

We had shown previously that a polyclonal antibody raised against syntaxin 6 stained the Golgi region in FAO cells in culture (Bock *et al.*, 1996). We performed immunofluorescence microscopy on NRK cells using six monoclonal antibodies; all displayed specific staining in the Golgi region. We investigated the immunolocalization of syntaxin 6 in FAO, NRK, and PC12 cell lines with monoclonal antibody 3D10. In all of these cells, the immunoreactivity was localized in a juxtanuclear area, again consistent with our previous study (Figure 2). At this level of resolution, only very faint staining was observed outside the Golgi region

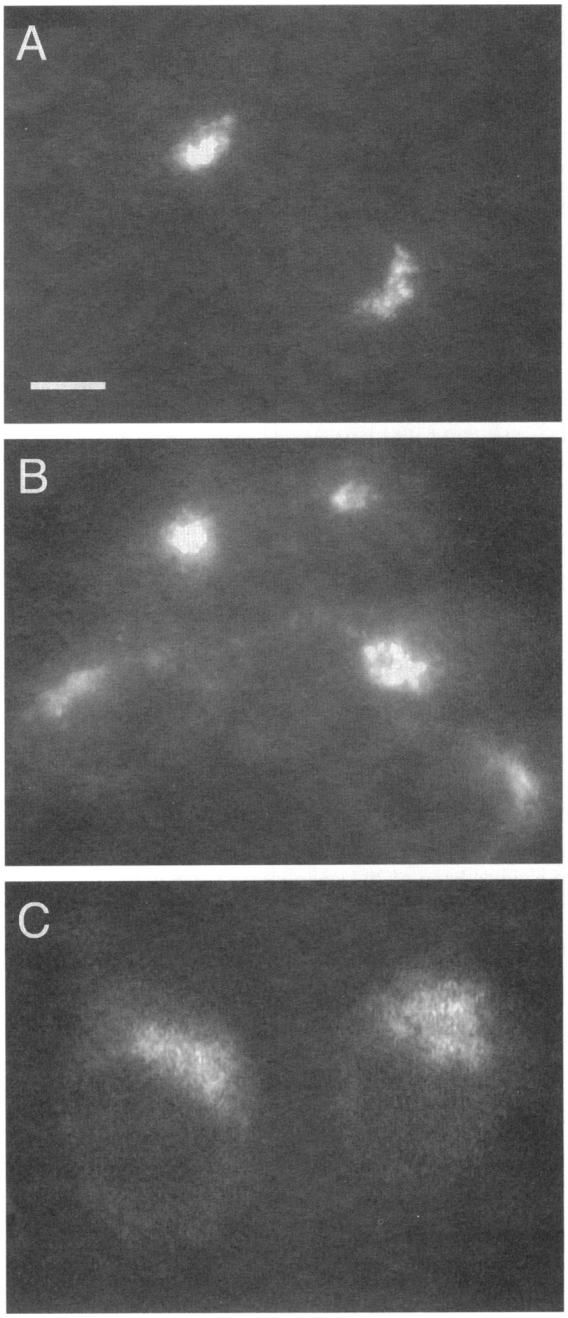

Figure 2. Syntaxin 6 localizes to the Golgi region. Immunofluorescence microscopy illustrating the staining of syntaxin 6 with purified ascites fluid from monoclonal antibody 3D10 (1:1000 dilution). A distinctly Golgi staining pattern was observed in fixed and permeabilized NRK (A), FAO (B), and PC12 (C) tissue culture cell lines. Bars: A, 2.4 μm; B, 2.4 μm; and C, 1.1 μm.

and no reactivity was observed with nonspecific Ig or secondary antibody alone. Thus a number of antibodies recognizing distinct epitopes of syntaxin 6 all stain the Golgi region in a variety of cell types.

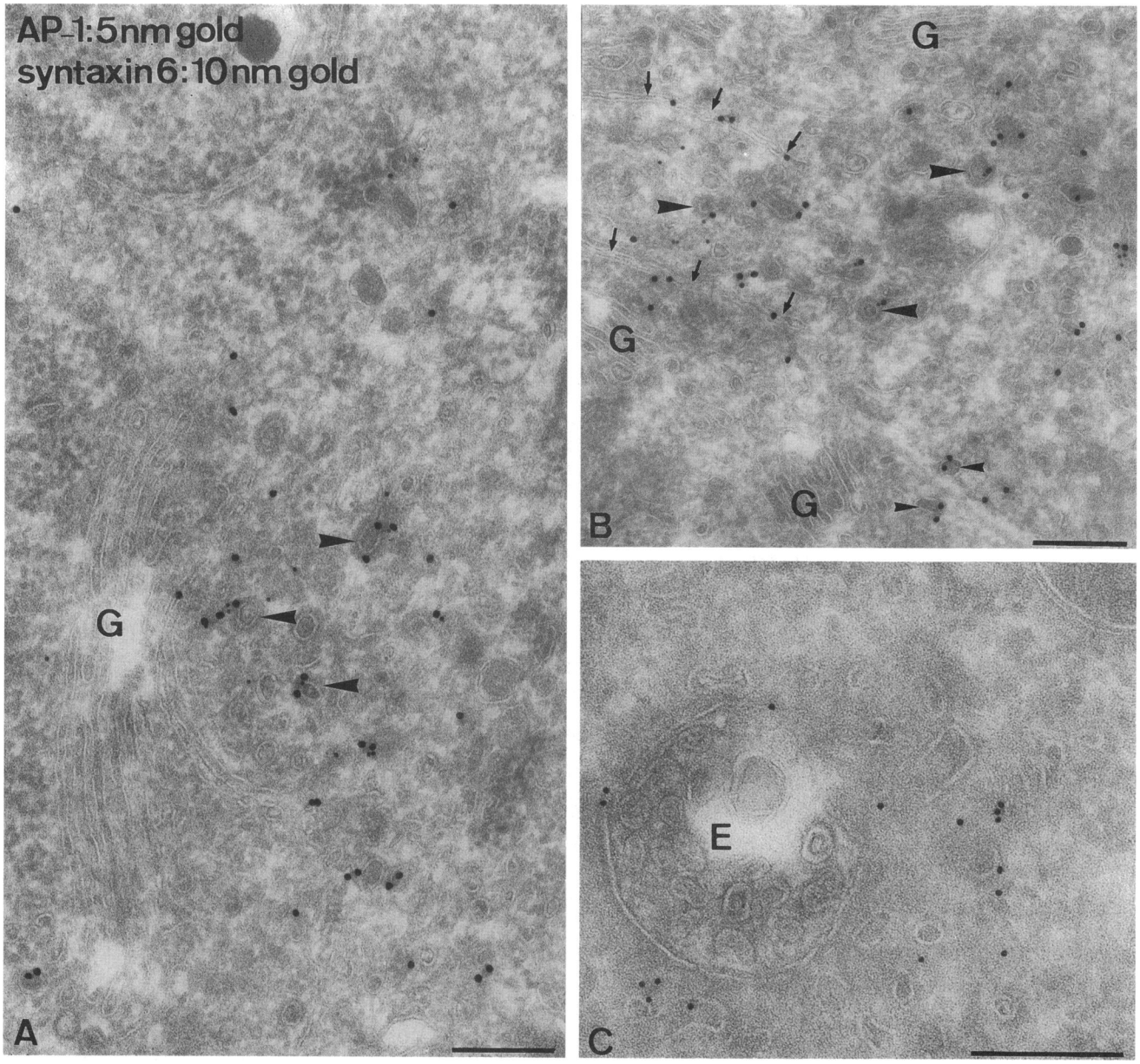

Figure 3. Double immunogold labeling of AP-1 (5-nm gold) and syntaxin 6 (10-nm gold) in PC12 cells. Antiserum against glutaraldehyde-fixed fusion protein was used to localize syntaxin 6. (A and B) Colocalization of syntaxin 6 and AP-1 over clathrin-coated TGN membranes (large arrowheads). (B) Syntaxin 6 labeling was also found over noncoated tubular (arrows) and vesicular (small arrowheads) membrane profiles, which were devoid of AP-1 label. (C) Syntaxin 6-positive/AP-1 negative vesicles in close vicinity to an endosome (E). Note that the limiting membrane of the endosome is only weakly stained and that the internal vesicles are devoid of label. G, Golgi complex. Bars, 200 nm.

To precisely define the subcellular distribution of syntaxin 6, we performed immunogold labeling of ultrathin cryosections of PC12 cells. Identical subcellular localization patterns were obtained with the monoclonal antibodies, the previously described rabbit polyclonal antibody, and an antibody raised in mice against glutaraldehyde-fixed recombinant syntaxin 6 fusion protein (our unpublished data and Figure 3). Although the labeling densities varied, the reproducibility of the syntaxin 6 localization pattern using distinct antibodies illustrates the specificity of this localization pattern. The highest labeling density was obtained with the antibody raised against the glutaraldehyde-fixed protein, which was used for the quantitation discussed below.

Approximately 69% of the syntaxin 6 labeling was in the TGN, i.e., tubulovesicular membranes at the trans side of the Golgi complex (Figure 3, A and B). Syntaxin

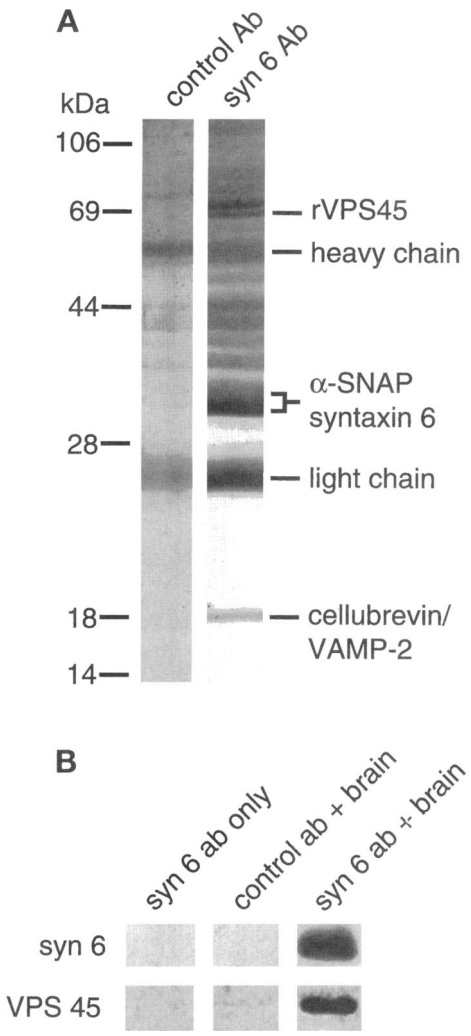

Figure 4. Immunoprecipitation of syntaxin 6 containing complexes. Crude membranes from rat brain were isolated and solubilized with 1% Triton X-100. The solubilized membranes were incubated with protein G-Sepharose beads with either syntaxin 6 monoclonal antibody 3D10 or a nonspecific mouse Ig. The immunobeads were washed, eluted with SDS protein sample dye, resolved by SDS-PAGE, and either stained with Coomassie blue (A) or transferred to nitrocellulose (B). (A) The marked bands were excised from the gel and subjected to internal amino acid sequencing. (B) The nitrocellulose blots were probed with anti-syntaxin 6 or anti-h-VPS45 antibodies. No band was present in a control precipitation with 3D10 beads in buffer alone or with nonspecific Ig beads incubated with the brain extract. When the 3D10 beads were used to precipitate from the brain extract, a band of 29 kDa could be seen with the syntaxin 6 antibody and a band at 70 kDa could be seen with the h-VPS45 antibody.

6 label was associated with long tubular membranes, which often exhibited an electron-dense coating, characteristic for the presence of clathrin. Double immunogold labeling showed colocalization of syntaxin 6 and AP-1 over these coated membranes (Figure 3, A and B). Three percent of all syntaxin 6-representing gold particles were found over coated vesicles that, at least in the plane of the section, were not associated with the TGN, and a further 20% of the label was associated with noncoated vesicles. Finally, 8% of the syntaxin 6 label was associated with endosomes, i.e., tubulovacuolar compartments with a variable number of internal vesicles (Figure 3C). Most of this label was found over small vesicles in close vicinity to the endosomes. Only occasionally label over the limiting membrane of the endosome was observed, whereas the internal vesicles were virtually devoid of label.

Table 2. Amino acid sequence from syntaxin 6 immunoprecipitations

Protein	Sequence	Corresponding amino acids
VPS45	LESIADMK	305–312
	LVSAVVEYGGK	422–432
	DAVAITK	444–450
α-SNAP	AIAHYEQSADYYK	141–153
	VAGYAAQLEQYQK	168–180
	LLEAHEEQNVDSYTESVK	247–264
Syntaxin 6	DQMSASSVQALAERK	104–118
Cellubrevin/ VAMP-2	VLERDQK	40–46
	LSELDDRADALQAGASQFETSAAK	47–70

Proteins Interacting with Syntaxin 6

A key to understanding previously characterized vesicle trafficking steps has been the elucidation of protein complexes. We wanted to determine whether syntaxin 6 was associated with a protein complex, and if so, to determine what were the components of this complex. To this end, we immunoprecipitated syntaxin 6 from Triton X-100-solubilized rat brain membranes (Figure 4A). Twenty micrograms of monoclonal antibody 3D10 are capable of absorbing all of the syntaxin 6 from approximately 5 mg of solubilized brain membrane protein. The immunoprecipitation is specific because syntaxin 6 did not bind to immunobeads containing nonspecific Ig. We excised the major bands of 18, 29, 31, and 70 kDa visible by Coomassie blue staining. These gel slices were digested with the protease LysC and a series of resulting peptides were sequenced (Table 2).

The sequences from band p18 correspond to 34 contiguous amino acids present in the closely related proteins VAMP-2 and cellubrevin. VAMP-2 has been demonstrated to act as a v-SNARE in exocytosis. On the other hand, a role for cellubrevin has been suggested in vesicle recycling to endosomes (McMahon *et al.*, 1993). Bands p29 and p31 both produced sequences corresponding to syntaxin 6 and α-SNAP. These bands migrated quite near each other on the gel and

likely cross-contaminated each other when the slices were excised. Nonetheless, these data are consistent with our previous in vitro binding study, showing a direct interaction between these two proteins. The sequences from band p70 correspond to the predicted amino acid sequence of hVPS45 (Pevsner *et al.*, 1996). hVPS45 is the human homologue of yeast VPS45, which was identified in a mutant screen as a protein required for vesicle transport from the Golgi apparatus to the vacuole in yeast. A genetic interaction has been shown between yeast VPS45 and Pep12. The demonstration that syntaxin 6 specifically immunoprecipitates VPS45 strengthens the argument that syntaxin 6 and Pep12 are functionally related.

Although VAMP-2, cellubrevin, and α-SNAP have been previously characterized, mammalian VPS45 has not. Our first step to understand the function of VPS45 was to generate an antiserum by immunizing a mouse with the GST-h-VPS45 fusion protein and affinity purifying the antibodies by using the fusion protein bound to cyanogen bromide-activated Sepharose beads. With this antiserum, we detected a band of 70 kDa in the syntaxin 6 but not control precipitations (Figure 4B). This result confirms the amino acid sequencing results. Although these results do not prove a direct interaction, they do demonstrate that syntaxin 6 is associated in a complex with mammalian VPS45. Although there was no syntaxin 6 detectable in the supernatant from the 3D10 immunoprecipitation, approximately 50% of the total VPS45 present in crude membranes was not immunoabsorbed (our unpublished observations). This implies that either the addition of Triton X-100 disrupts the interaction of VPS45 with the syntaxin 6 complex or there is an additional route by which VPS45 is able to associate with membranes.

Tissue Distribution and Subcellular Localization of VPS45

To confirm the specificity of the VPS45 antiserum and determine the tissue distribution pattern of mammalian VPS45, we probed Western blots of postnuclear supernatant from various rat tissues with the affinity-purified antibody. The antibody recognized a predominant band of approximately 70 kDa, which is near 65 kDa, the predicted molecular weight of h-VPS45 (Figure 5). Mammalian VPS45 has its highest protein expression in brain, kidney, pancreas, lung, and spleen. These results are in agreement with the previously published RNA tissue distribution analysis (Pevsner *et al.*, 1996), and coincide with the protein distribution of syntaxin 6 (Bock *et al.*, 1996).

The n-sec1 immunoreactivity is found throughout the cytoplasm with a slight enrichment at the plasma membrane, where its binding partner syntaxin 1 is localized (Garcia *et al.*, 1995). On the basis of these

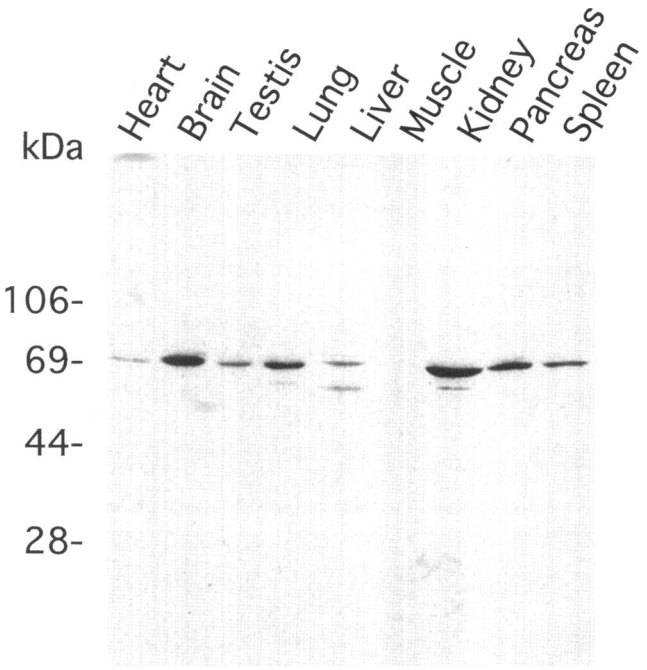

Figure 5. Mammalian VPS45 is widely expressed. Postnuclear supernatants from rat heart, brain, testis, liver, lung, kidney, skeletal muscle, pancreas, and spleen were analyzed by Western blot with affinity-purified anti-h-VPS45 mouse serum. A single band was detected at 70 kDa, which is near the predicted molecular weight for h-VPS45.

observations, we hypothesized that VPS45 might also have a cytosolic appearance with an enrichment in the Golgi region or near endosomes. To test this hypothesis, we performed immunofluorescence microscopy on fixed COS cells with the anti-VPS45 antiserum. A punctate staining pattern was seen throughout the cytoplasm with a distinct juxtanuclear enrichment characteristic of the Golgi region (Figure 6A). When we transfected COS cells with a myc-epitope–tagged syntaxin 6, the tagged epitope primarily colocalized with the VPS45 staining around the Golgi region (Figure 6, B and C). It could be that VPS45 binds to syntaxin 6 at the TGN and acts as a chaperone as the two proteins move onto clathrin-coated vesicles and then onto endosomes.

VPS45 Associates with Membranes

Although mammalian VPS45 is predicted to be a soluble protein, its staining pattern in immunofluorescence microscopy indicated a membrane association. We predicted that VPS45 would behave as a peripheral membrane protein through its interaction with syntaxin 6, just as n-sec1 does through its interaction with syntaxin 1. To test this hypothesis, we performed several experiments. First, we examined the distribu-

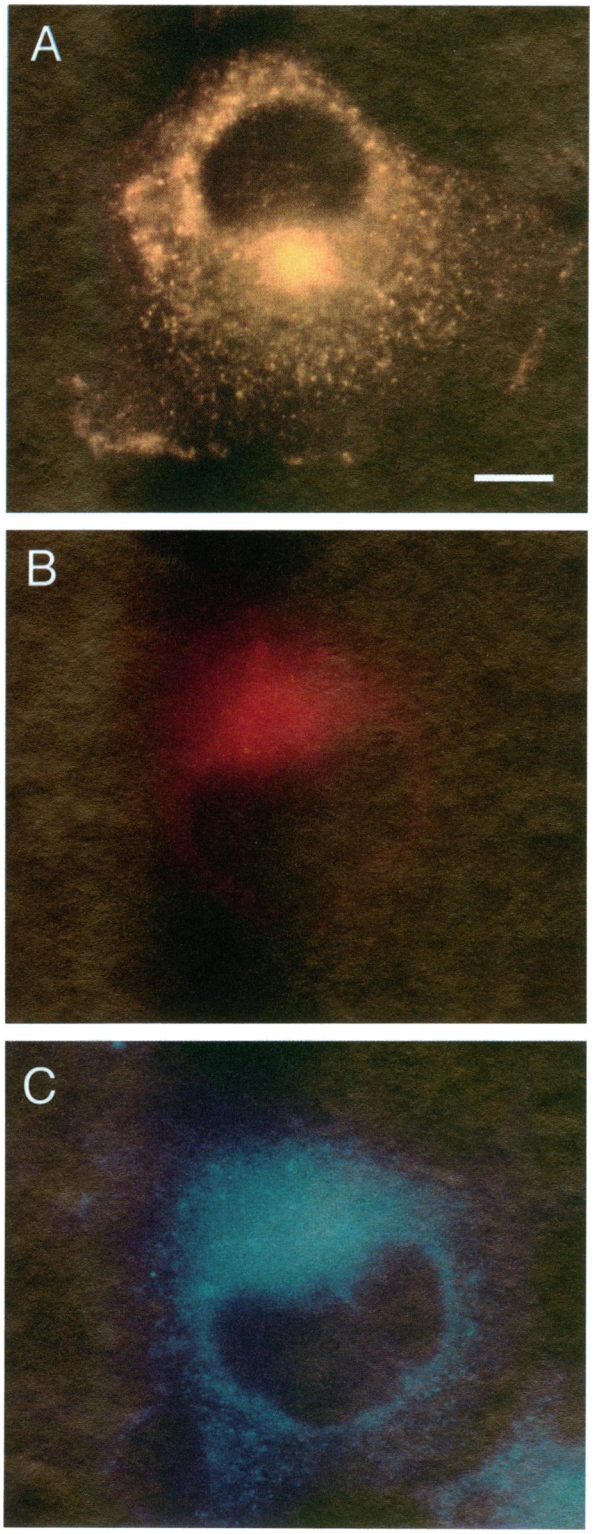

Figure 6. VPS45 is enriched in the Golgi region. (A) Immunofluorescence microscopy on fixed and permeabilized COS cells with the h-VPS45 antiserum (Cy3) illustrates a punctate staining pattern throughout the cytoplasm with a juxtanuclear enrichment characteristic of the Golgi region. In COS cells transfected with a myc-epitope–tagged syntaxin 6, the staining with anti-myc (Texas Red; B) overlapped with the VPS45 pattern (FITC; C). Bars: A, 2.6 μm: B and C, 3.1 μm.

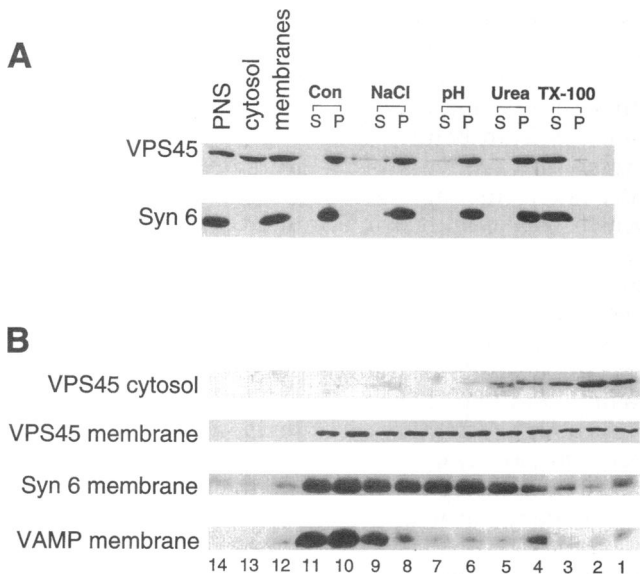

Figure 7. VPS45 associates with membranes. (A) Rat brain membrane extraction. Rat brain homogenate was subjected to a 1000 × *g* spin to yield postnuclear supernatant (PNS). This was then centrifuged at 100,000 × *g* to yield cytosol and membrane fractions. PNS was also spun at 20,000 × *g* and the resulting membrane pellet was resuspended in homogenization buffer (50 mM Tris, pH 8.0, 150 mM NaCl, 1 mM dithiothreitol, 0.3 μM phenylmethylsulfonyl fluoride). Then an equal volume of the extraction solution was added to each sample to yield the following final concentrations of extracting agents: con, only water added, 1.5 M NaCl, 1.5 M urea, 0.2 M Na_2CO_3, pH 11.2, and 2% Triton X-100. The samples were then incubated at 4°C for 30 min and centrifuged at 100,000 × *g*. The resulting supernatants (S) and pellets (P) were examined by anti-VPS45 or anti-syntaxin 6 Western blot analysis. (B) Sucrose floatation of brain membranes. Crude rat brain membranes were placed at the bottom of a 27–52.5% sucrose gradient and centrifuged at 100,000 × *g*, which allows membranes to float into the gradient. The presence of syntaxin 6 and VPS45 was monitored by Western blot analysis. The migration of synaptic vesicle membranes was also monitored with an anti-VAMP antibody. Fraction 1 corresponds to the bottom of the gradient.

tion of VPS45 in rat brain soluble and membrane fractions. The integral membrane protein syntaxin 6 was present exclusively in the membrane fraction, and VPS45 was present in both cytosolic and membrane fractions (Figure 7A). High salt, high pH, and urea did not extract significant amounts of VPS45 from the membranes; however, Triton X-100 extracted all VPS45 from the membranes. These data are consistent with VPS45 having a stable high-affinity interaction with a membrane receptor.

To confirm that VPS45 associates with membranes instead of being a constituent of a large protein aggre-

gate that pelleted during centrifugation, sucrose floatation of brain membranes was carried out. Crude membranes were isolated from a freshly dissected rat brain. A sucrose gradient was then layered on top of these membranes and subjected to centrifugation at 100,000 × *g* for 16 h. Fractions were removed from this gradient and analyzed by SDS-PAGE and Western blotting. In this kind of gradient, membranes should float up into the gradient and carry associated proteins with them. Cytosolic, denatured, or cytoskeletal-associated proteins will remain at the bottom of such a gradient. As controls the integral membrane proteins syntaxin 6 and VAMP were found to float into this gradient. The VPS45 present in the crude membrane preparation did in fact float, but the VPS45 present in the cytosol preparation did not (Figure 7B). The broader syntaxin 6 and VPS45 peaks compared with the VAMP peak may be explained by the presence of syntaxin 6 and VPS45 on a more heterogeneous population of membranes (i.e., TGN and coated and noncoated vesicles) than VAMP, which is found primarily on homogenous synaptic vesicles. Thus, these results demonstrate that mammalian VPS45 is stably associated with membranes, probably in part with a syntaxin 6-containing complex.

DISCUSSION

Syntaxin 6 localizes primarily to the TGN, where it is found on noncoated and AP-1/clathrin-coated membranes. Although the relative concentration of syntaxin 6 in clathrin-coated buds/vesicles cannot be determined by immunoelectron microscopy because the labeling efficiency may be affected by the coat itself, the colocalization of syntaxin 6 and AP-1 on coated membranes suggests that syntaxin 6 is actively sorted into transport vesicles. Only a few transmembrane proteins have been localized in TGN clathrin-coated vesicles including MPRs, major histocompatibility complex class II molecules, and lysosomal membrane proteins. All these proteins have identified signals in their cytoplasmic tail that are required for proper sorting from the TGN to endosomes (Trowbridge *et al.*, 1993; Hunziker and Geuze, 1996). AP-1 binds to these signals and then initiates the formation of a clathrin coat. There are two known sorting motifs: a tyrosine-based YXXØ (where Y is tyrosine, X is any amino acid, and Ø is an amino acid with a bulky hydrophobic group), or the dileucine motif LL (Johnson and Kornfeld, 1992; Letourneur and Klausner, 1992). Syntaxin 6 has several potential lysosomal sorting sequences including LL at amino acids 31–32 and 123–124, and YGRL at amino acids 140–143. What is the destination of these TGN-derived syntaxin 6-containing vesicles? The most straightforward conclusion is that these vesicles are destined to fuse with endosomes. Indeed, in addition to its presence on coated membranes, syntaxin 6 can also be found on clear vesicles in the vicinity of endosomes. These vesicles may have shed their clathrin coat in preparation for fusion with endosomes. In summary the morphological evidence we present supports a role for syntaxin 6 in the targeting and fusion of TGN-derived vesicles with endosomes.

Complementing the morphological evidence, two phylogenetic parallels with yeast vacuolar trafficking are also consistent with syntaxin 6 mediating TGN to endosome trafficking. First, syntaxin 6 shows significant homology to yeast Pep12, a molecule implicated in Golgi to vacuolar trafficking. Second, we have demonstrated herein that mammalian VPS45 specifically immunoprecipitates with syntaxin 6. Although in yeast the transport step involving Pep12 and VPS45 may need to be more precisely refined, it is likely that they are involved in vacuolar trafficking from the Golgi to the endosome-like prevacuolar compartment.

In addition to the morphological and phylogenetic evidence, syntaxin 6 is also implicated in trafficking to endosomes through its interaction with cellubrevin or VAMP-2. The function of VAMP-2 in nonneuronal cells has not been as well studied as has cellubrevin's because until recently it was not realized how extensive VAMP-2's tissue distribution was (Rossetto *et al.*, 1996). However, in nonneuronal cells VAMP-2 has an endosomal-like immunofluorescent staining pattern. It is possible that VAMP-2 plays a role in trafficking to endosomes in nonneuronal cells. Cellubrevin has been more extensively studied in nonneuronal cells and appears to mediate recycling of transferrin receptor-containing vesicles through endosomal intermediates (Galli *et al.*, 1994) and has been shown to be present on endosomes (Daro *et al.*, 1996). It is interesting to speculate that cellubrevin serves at the endosome as the receptor for syntaxin 6-containing vesicles. Thus morphologic, phylogenetic, and biochemical evidence suggest that syntaxin 6 mediates TGN to endosome trafficking. Further work is required to test this hypothesis, including a definition of the cargo present in the syntaxin 6-containing vesicles.

The presence of syntaxin 6 on vesicles contrasts with other syntaxin family members, which are thought to reside on target membranes: syntaxin 1, 2, 3, and 4 on the plasma membrane and syntaxin 5 on the *cis*-Golgi. This defined the proteins as t-SNAREs. Recent evidence has shown that approximately 20% of both syntaxin 1 and SNAP-25 are in fact present on vesicles, although the bulk of the proteins localize to target membranes as originally proposed (Tagaya *et al.*, 1995; Walch-Solimena *et al.*, 1995; Gaisano *et al.*, 1996). Thus, it remains possible that syntaxin 6 is a TGN receptor for incoming vesicles and that a low level of the protein resides on transport vesicles. Although if this were the case, a role for an interaction between syntaxin 6 and cellubrevin or VAMP-2 is more difficult to imagine.

However, if syntaxin 6 functions on the vesicles observed in the electron micrographs we present and cellubrevin or VAMP-2 does in fact serve as its target membrane receptor on endosomes, we are forced to reevaluate the protein structure dividing lines between v- and t-SNAREs. Perhaps some syntaxin family members function in particular trafficking steps on target membranes, for example, in synaptic transmission, and other members function in other trafficking steps on vesicle membranes. It may be the case in general that vesicle docking and membrane fusion, as is clearly the case in homotypic fusion, need not be a polarized process. The important function of docking may be to bring the two specific lipid bilayers into close apposition. Once the actual fusion is triggered, the asymmetry demarcating the target and vesicle membranes may be irrelevant.

A fundamental question that must be addressed if syntaxin 6 is to be deemed a vesicular syntaxin is: does syntaxin 6 belong in the syntaxin family? Syntaxin 6 is the most divergent member of the mammalian syntaxin family identified thus far; however, it is clearly significantly more related to syntaxins than to any other proteins. Although it is about 50 amino acids shorter than the other syntaxins, its topology is characteristic of the syntaxin family. As with all other syntaxins, syntaxin 6 has a carboxyl-terminal membrane anchor directly preceded by a region predicted to form coiled-coils, and an amino-terminal region predicted to form coiled-coils. Moreover, syntaxin 6 binds α-SNAP; however, although α-SNAP binds to the carboxyl-terminal coiled-coil on syntaxin 1, it binds to the amino-terminal region, perhaps the coiled-coil, on syntaxin 6. The presence of α-SNAP in the syntaxin 6 immunoprecipitations is also distinct from analogous syntaxin immunoprecipitations. To see α-SNAP in syntaxin 1 and 5 immunoprecipitations exogenous α-SNAP in combination with nonhydrolyzable ATP had to be added. The syntaxin 6 immunoprecipitations had neither of these components nor any EDTA to inhibit ATPase activity. Another emerging hallmark of syntaxins is that they interact with sec1 family members (see below). In this article, we have demonstrated such an interaction between syntaxin 6 and mammalian VPS45, although we do not yet know if this represents a direct binding. Thus although syntaxin 6 does not behave as a "classical" syntaxin family member in all aspects, it is clearly related to this family by sequence homology, protein topology, and protein interactions. Perhaps the nonclassical nature of syntaxin 6 should not be a surprise because the homology of syntaxin 6 to both a syntaxin family member (Pep12) and SNAP-25 has already blurred the distinction between these gene families.

Lastly, the TGN is a spectacularly complex arrangement of tubulovesicular membranes, which serves as a major sorting point for many vesicle trafficking events. From the TGN, proteins can be sorted to endosomes, lysosomes, and distinct domains of the plasma membrane. In addition to these anterograde pathways, retrograde trafficking also occurs. The recognition of a vesicle trafficking protein within the TGN, syntaxin 6, thus holds the potential to resolve the street signs at this major crossroads within the cell.

ACKNOWLEDGMENTS

We specially acknowledge Dr. Shu-Chan Hsu for all her biochemical advice. We thank Karen Peterson for hybridoma culturing, Richard Winant of the Stanford PAN facility for amino acid sequencing and discussions, Tineke Broers-Vendgrig for assistance in the preparation of ultrathin cryosections, Drs. Jesse Hay and Andrew Bean for insightful advice, and Richard Lin for critical reading of the manuscript.

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