Journal of Cell Science 112, 845-854 (1999)
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JCS0233

Syntaxin 11 is associated with SNAP-23 on late endosomes and the trans-Golgi network

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Accepted 15 January; published on WWW 25 February 1999

SUMMARY

SNARE proteins are known to play a role in regulating intracellular protein transport between donor and target membranes. This docking and fusion process involves the interaction of specific vesicle-SNAREs (e.g. VAMP) with specific cognate target-SNAREs (e.g. syntaxin and SNAP-23). Using human SNAP-23 as the bait in a yeast two-hybrid screen of a human B-lymphocyte cDNA library, we have identified the 287-amino-acid SNARE protein syntaxin 11. Like other syntaxin family members, syntaxin 11 binds to the SNARE proteins VAMP and SNAP-23 in vitro and also exists in a complex with SNAP-23 in transfected HeLa cells and in native human B lymphocytes. Unlike other syntaxin family members, no obvious transmembrane domain is present in syntaxin 11. Nevertheless, syntaxin 11 is predominantly membrane-associated and colocalizes with the mannose 6-phosphate receptor on late endosomes and the trans-Golgi network. These data suggest that syntaxin 11 is a SNARE that acts to regulate protein transport between late endosomes and the trans-Golgi network in mammalian cells.

Key words: SNARE, Syntaxin, SNAP-23, Protein transport, Endosome

INTRODUCTION

There have been considerable advances in recent years in understanding the regulation of vesicle-mediated protein transport in eukaryotic cells. The analysis of the genetics of protein secretion in yeast, the biochemistry of synaptic vesicle exocytosis, and the cell biology of vesicular transport in mammalian cells all point to the involvement of SNARE proteins in regulating protein transport (reviewed in Ferro-Novick and Jahn, 1994; Südhof, 1995; Bennett and Scheller, 1994; Rothman, 1994). There are SNARE proteins on vesicle membranes (termed vesicle SNAREs, or v-SNAREs) and SNARE proteins on target membranes (termed target SNAREs, or t-SNAREs). It was originally postulated that the v-SNARE/t-SNARE complex serves as a functional receptor for cytosolic proteins termed SNAPs and NSF, and it was the ATPase activity of NSF that ultimately led to membrane fusion (Söllner et al., 1993b). More recent data, however, suggests that the SNAREs themselves are capable of facilitating membrane fusion (Weber et al., 1998). This finding is consistent with data demonstrating that NSF (and SNAPs) function by rearranging and dissociating pre-formed SNARE complexes, thereby allowing subsequent rounds of v-SNARE/t-SNARE pairings that can ultimately lead to the fusion of opposing vesicle and target membranes (Söllner et al., 1993a,b; Hanson et al., 1995; Hayashi et al., 1995; Ungermann et al., 1998).

Söllner et al. identified two different target membrane-associated proteins in macromolecular SNARE complexes isolated from rat brains, one being syntaxin and the other SNAP-25 (unrelated to cytosolic SNAPs; Söllner et al., 1993a). Both syntaxin and SNAP-25 bind to the vesicle-associated v-SNARE VAMP, and studies using a variety of clostridial neurotoxin proteases have highlighted the importance of each of these proteins in synaptic vesicle docking and fusion with presynaptic membranes (reviewed in Südhof, 1995; Niemann et al., 1994). Since SNAP-25, an essential component of the neuronal SNARE complex, is expressed almost exclusively in neuronal and neuroendocrine cells, it has been assumed that a non-neuronal SNAP-25 homologue exists which can facilitate regulated exocytosis in all tissues of the body. We have recently identified SNAP-23 as this ubiquitously expressed non-neuronal homologue (Ravichandran et al., 1996). SNAP-23 is approximately 60% identical to SNAP-25 and is able to functionally replace SNAP-25 in the process of regulated exocytosis in a pancreatic cell line (Sadoul et al., 1997). SNAP-23 is also required for efficient biosynthetic protein transport and transcytosis (Low et al., 1998a) as well as transferrin recycling (Leung et al., 1998) in polarized epithelial cells, providing additional evidence that SNAP-23 functions to facilitate protein transport in vivo.

The most basic form of the SNARE hypothesis proposes that distinct v-SNARE/t-SNARE combinations regulate the entire array of intracellular transport steps utilized in cells. In agreement with this hypothesis, a variety of syntaxin isoforms have been localized to specific intracellular organelles in many

diverse cell types. Syntaxins 1-4 are present primarily on the plasma membrane (Bennett et al., 1993; Low et al., 1996), syntaxin 5 and syntaxin 16 are present on the Golgi apparatus (Bennett et al., 1993; Tang et al., 1998b), syntaxin 6 (Bock et al., 1996; 1997a) and syntaxin 10 (Tang et al., 1998c) are present on the trans-Golgi network (TGN), and syntaxin 7 is present on early endosomes (Wong et al., 1998). Although many unique SNAREs with specific locations have been identified, we are only beginning to understand the mechanism by which these proteins regulate the specificity of particular vesicle-mediated transport reactions. In addition, there is evidence that a class of small GTPases termed rabs regulate SNARE complex assembly and membrane fusion (reviewed in Novick and Zerial, 1997), adding an additional layer of complexity to the problem of maintaining organelle integrity in the cell.

The ubiquitous t-SNARE SNAP-23 was originally isolated as a syntaxin 4 binding protein using the yeast two-hybrid system. In this current study, we have identified syntaxin 11 based on its ability to bind to SNAP-23 in vitro and in vivo. Interestingly, immunofluorescence microscopy reveals that syntaxin 11 is present in perinuclear structures which significantly colocalize with the mannose 6-phosphate receptor on late endosomes and the TGN, suggesting that syntaxin 11 regulates traffic either into or out of these compartments.

MATERIALS AND METHODS

cDNA cloning of syntaxin 11

The entire coding region of human *SNAP-23* was subcloned into the GAL4-DNA binding domain vector pGBT9 and used to screen a human B lymphocyte cDNA library in the GAL4-activation domain vector pACT I using the yeast two-hybrid system (Ravichandran et al., 1996). Of 12 positive clones, five independent clones consisted of a novel in-frame syntaxin homologue. We generated a 324-bp probe derived from the coding region of this clone to screen a human placenta λgt11 cDNA library (Clontech) to isolate a full-length cDNA of this syntaxin. A total of 6×10^5 phage were plated and transferred to Hybond nylon membranes (Amersham). The membranes were hybridized with the [γ-^{32}P]dCTP-labeled syntaxin 11 probe in 7% SDS, 0.5 M $NaP0_4$, 1% bovine serum albumin, 1 mM EDTA overnight at 68°C. The filters were sequentially washed at 68°C with 6× SSC (SSC is 15 mM sodium citrate, 150 mM NaCl, pH 7.0) containing 0.5% SDS, 2× SSC containing 0.1% SDS, and 0.5× SSC containing 0.1% SDS and were exposed to x-ray film. Positive plaques were extracted and re-plated to obtain single plaques. Among five positive clones, four contained identical open reading frames and one of these (clone A1) was sequenced completely on both strands by automated sequence analysis using an Applied Biosystems 373 DNA Sequencer. This clone, encoding human *syntaxin 11*, was subcloned into the mammalian expression vector pcDNA3 (Invitrogen) by adding *Eco*RI and *Xho*I sites at the 5′- and 3′-ends of the cDNA coding region using the polymerase chain reaction. Xpress-epitope tagged syntaxin 11 was generated by excising the insert from pcDNA3-*syntaxin 11* and ligating it into the *Eco*RI and *Xho*I sites of pcDNA3.1/His C (Invitrogen). A mutant lacking the carboxy-terminal 13 amino acids of syntaxin 11 was generated using the polymerase chain reaction by the introduction of a stop codon. The sequence of wild-type and mutant pcDNA3-*syntaxin 11* was confirmed by automated sequence analysis. pcDNA3-*SNAP-23* has been described previously (Ravichandran et al., 1996; Low et al., 1998b).

In vitro binding assays

Recombinant GST fusion proteins were prepared as described previously (Ravichandran et al., 1996). Full-length GST-SNAP-23 was generated by the polymerase chain reaction using human *SNAP-23* (Ravichandran et al., 1996) as the template. The sequence of the construct was confirmed by automated sequence analysis. [^{35}S]Methionine-labeled in-vitro transcribed and translated syntaxin 11 was prepared using the TNT Quick T7 translation kit (Promega). The translated material was incubated for 2 hours at 4°C with 3 μg of each GST fusion protein immobilized on glutathione-Sepharose (Pharmacia), washed extensively in a buffer of 20 mM Hepes, 100 mM NaCl, 1 mM $MgCl_2$, 0.1% Triton X-100, 1 mg/ml bovine serum albumin, pH 7.0, containing 0.5 mM phenylmethylsulfonyl fluoride, 0.1 mM Nα-p-tosyl-L-lysine chloromethyl ketone and 5 mM iodoacetamide. The Sepharose beads were then washed once in the above buffer without Triton X-100 and bovine serum albumin and analyzed by SDS-PAGE and fluorography as described (Ravichandran et al., 1996).

Antibodies

Syntaxin 11-specific antiserum was generated by immunizing rabbits with a synthetic peptide corresponding to the first 15 amino acids of syntaxin 11 coupled to maleimide-activated keyhole limpet hemocyanin via a carboxy-terminal cysteine. The keyhole limpet hemocyanin-peptide complex was emulsified in complete Freund's adjuvant immediately prior to injection into rabbits. Booster immunizations were made every other week using the same antigen emulsified in incomplete Freund's adjuvant. The rabbits were bled 1 week after each immunization and were screened by immunoblotting using B lymphocyte membranes. The SNAP-23 antiserum raised against the amino-terminal 17 amino acids of human SNAP-23 has been described previously (Low et al., 1998b). The anti-Xpress monoclonal antibody was obtained from Invitrogen. The anti-human transferrin receptor monoclonal antibody (clone T56/14) was from Calbiochem and the anti-human LAMP 1 monoclonal antibody (clone H4A3) was from the Developmental Studies Hybridoma Bank, University of Iowa, Iowa City, IA. Tissue culture supernatant of the anti-bovine 300 kDa mannose 6-phosphate receptor monoclonal antibody 2G11 was the generous gift of Dr Suzanne Pfeffer, Stanford, CA (Dintzis et al., 1994) and was used at a 1:10 dilution. Fluorescein isothiocyanate-conjugated goat anti-rabbit IgG and indodicarbocyanine (Cy5)-conjugated goat anti-mouse IgG secondary antibodies were obtained from Jackson ImmunoResearch Laboratories, Inc. These antibodies were affinity-purified so as to eliminate cross-reactivity with other species immunoglobulins.

Cell culture and immunoprecipitation

The human B lymphoblastoid cell line JY was maintained in culture as described (Anderson and Roche, 1998). HeLa cells were maintained in DMEM supplemented with 10% fetal bovine serum. Subconfluent HeLa cells were transiently transfected using the calcium-phosphate method as described previously (Anderson and Roche, 1998), or using Lipofectamine Plus reagent (Life Technologies) according to the manufacturer's protocol. We have not observed significant differences in syntaxin 11 expression using either of these protocols. The cells were analyzed approximately 36 hours after transfection. Transiently transfected HeLa cells in 10 cm Petri dishes were labeled with 0.5 mCi [^{35}S]methionine for 1 hour at 37°C. Cells were lysed in 1% Triton X-100 in Tris-buffered saline on ice, nuclei and other debris were removed by centrifugation, and specific immunoprecipitations using antibodies bound to protein A-agarose were performed as described previously (Anderson and Roche, 1998). Immunoprecipitated proteins were separateded by 10.5% SDS-PAGE and analyzed by immunoblotting or fluorography as indicated.

Blotting

A nylon membrane containing poly(A^+)RNA from multiple human

tissues was obtained from Clontech. The membrane was hybridized with [γ-^{32}P]dCTP-labeled probes in QuickHyb (Stratagene) for 1 hour at 68°C. The syntaxin 11 probe encoded amino acids 1-287 of human syntaxin 11. The blot was then sequentially washed in 6× SSC/0.5% SDS at 37°C, 42°C and 68°C and twice in 2× SSC/0.5% SDS at 68°C. Following exposure of the blot to x-ray film, the membrane was stripped according to the manufacturer's protocol and was re-hybridized with a [γ-^{32}P]dCTP-labeled glyceraldehyde-3-phosphate dehydrogenase (GAPDH) probe using the conditions described above.

Proteins from multiple human tissues were obtained from Clontech. Protein samples were solubilized by incubation at 100°C in SDS and analyzed by SDS-PAGE and immunoblotting using the syntaxin 11 antiserum (1:2000) as described previously (Anderson and Roche, 1998).

Subcellular fractionation

Adherent cells were harvested by trypsinization, and both adherent and non-adherent cells (approximately 1×10^7 cells) were resuspended in 1 ml hypotonic buffer (10 mM Tris, 10 mM KCl, 1 mM EGTA, 0.5 mM $MgCl_2$, pH 7.4) and homogenized using a ball bearing cell homogenizer (clearance 10 μm) at 4°C. The homogenate was centrifuged at 3000 ***g*** at 4°C to obtain a post-nuclear supernatant. In some experiments, the post-nuclear supernatant was incubated with equal volumes of either H_2O, 3 M NaCl, 4 M urea, 0.4 M Na_2CO_3 (pH 11.4), or 4% Triton X-100 for 30 minutes at 4°C. This post-nuclear supernatant was overlaid onto a 17% sucrose, 20 mM Tris (pH 7.4) cushion and centrifuged at 100,000 ***g*** for 30 minutes at 4°C. The supernatant (cytosol) and pellet (membrane) fractions were analyzed by SDS-PAGE and immunoblotting using a syntaxin 11-specific antiserum and horseradish peroxidase-conjugated goat anti-rabbit Ig (Southern Biotechnologies) and enhanced chemiluminescence (Amersham) as described (Anderson and Roche, 1998).

Immunofluorescence microscopy

Transiently transfected HeLa cells were fixed for 1 hour in 4% paraformaldehyde in PBS containing 1 mM EGTA and 2 mM $MgCl_2$. After a brief rinse in PBS containing 50 mM NH_4Cl, the cells were permeabilized for 10 minutes in PBS containing 1% NP40, 0.01% saponin and 0.25% gelatin. The cells were then incubated with primary antibodies in PBS containing 0.01% saponin and 0.25% gelatin for 1 hour at room temperature, washed in the PBS/saponin/gelatin buffer, and stained with the appropriate secondary antibodies (diluted 1:200) for 30 minutes at room temperature in the same buffer. Following extensive washing, the coverslips were mounted to slides using Permafluor (Shandon-Lipshaw) and analyzed using a Zeiss LSM 410 laser scanning confocal microscope. Samples were visualized with a 63×, planapochromat objective (NA 1.4), using 488 nm krypton/argon and 633 nm helium/neon laser excitation. Fluorescence was collected with two photomultiplier tubes using 510-525 nm and 670-810 nm band-pass emission filters for fluorescein and Cy5, respectively. Images were acquired as single optical sections approximately 0.75 μm in thickness focused on the perinuclear region of the cells. For colocalization studies, the fluorophores in double-labeled samples were illuminated both simultaneously and sequentially to verify complete signal separation between the two channels.

RESULTS

cDNA cloning of syntaxin 11

In our effort to identify proteins involved in the regulation of intracellular trafficking of proteins in mammalian cells, we have used the yeast two-hybrid system. In a previous study, we identified SNAP-23 as a ubiquitously expressed syntaxin 4-binding protein which, like its neuronal counterpart SNAP-25, is capable of binding to multiple syntaxin and VAMP isoforms and likely functions to facilitate SNARE complex assembly in vivo (Ravichandran et al., 1996). To identify other SNAP-23 interacting proteins, we have now screened a human B-lymphocyte cDNA library using human SNAP-23 as the 'bait'. In addition to isolating a cDNA encoding syntaxin 4 in this screen, we also isolated a novel cDNA that shared 33% amino acid identity with syntaxin 1. During the preparation of this manuscript, two groups independently described the molecular cloning of syntaxin 11 (Advani et al., 1998; Tang et al., 1998a), and sequence comparison between syntaxin 11 and our clone revealed that the novel SNAP-23 binding protein that we identified in our two-hybrid screen was in fact human syntaxin 11.

We screened a human placenta cDNA library at high stringency using a *syntaxin 11* probe to obtain a full-length clone of *syntaxin 11*. Of five independent and overlapping cDNA clones, one was sequenced completely and we deduced that the open reading frame of the 1.6 kb syntaxin 11 cDNA encodes a 287-amino-acid protein. We have deposited the nucleotide and amino acid sequence of human syntaxin 11 in GenBank (accession number AF071504). The predicted molecular mass of syntaxin 11 is 35 kDa, which is in excellent agreement with results obtained by in vitro translation of the cDNA (data not shown).

Syntaxin 11 is broadly expressed

To examine the expression of syntaxin 11 protein, we probed a blot containing total protein from multiple human tissues using a syntaxin 11-specific antiserum. Pre-incubation of this antiserum with a synthetic syntaxin 11 peptide completely blocked immunoreactivity, confirming the specificity of the serum (data not shown). Fig. 1A demonstrates that this antiserum specifically recognized a 35 kDa protein in each tissue. As observed previously (Advani et al., 1998; Tang et al., 1998a), syntaxin 11 is expressed in many tissues, being especially prominent in placenta, lung, and heart but almost undetectable in brain. To examine the tissue distribution of syntaxin 11 RNA, we probed a blot containing poly(A^+)RNA from these same human tissues with a *syntaxin 11* cDNA probe under high stringency conditions. Fig. 1B demonstrates that this probe hybridized strongly to a 1.8 kb species and more weakly to a 4.3 kb and a 5.6 kb species. It is unclear if these larger transcripts represent alternatively spliced forms of syntaxin 11 or are transcripts that cross-react with the syntaxin 11 probe. The pattern of syntaxin 11 RNA expression mirrored almost exactly that of syntaxin 11 protein, strongly suggesting that our antibody specifically recognized syntaxin 11.

Syntaxin 11 behaves as an integral membrane protein

Most syntaxins associate with membranes by virtue of a carboxy-terminal hydrophobic transmembrane domain (Bennett et al., 1993). Surprisingly, Kyte and Doolittle hydrophobicity analysis revealed that syntaxin 11 does not contain a hydrophobic sequence of amino acids that is sufficiently long to function as a transmembrane anchor. In agreement with this, Tang et al. (1998a) concluded that epitope-tagged syntaxin 11 was present almost exclusively in the cytosol of transfected COS cells, suggesting that syntaxin 11 exists as a soluble syntaxin. On the other hand, Advani et

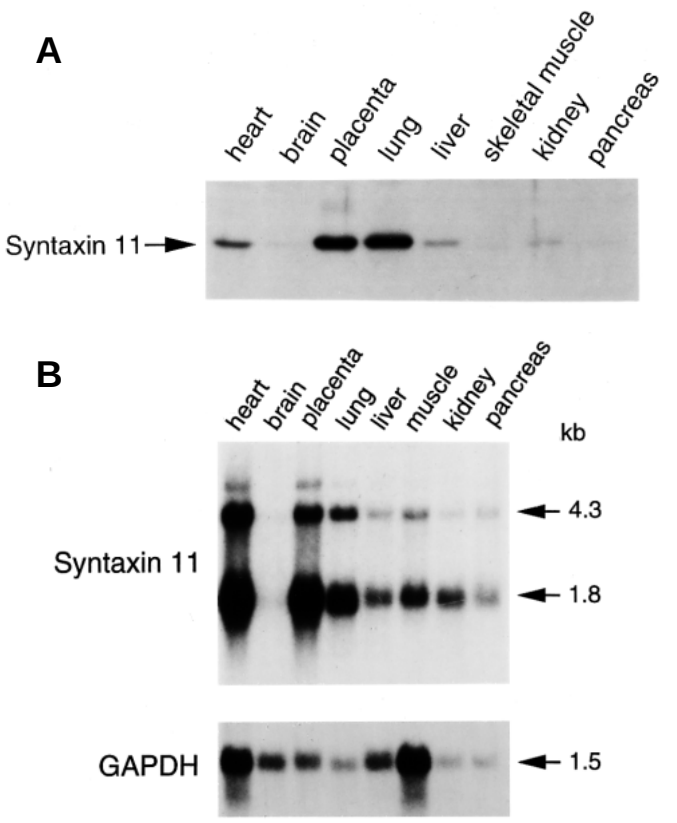

Fig. 1. Expression of human syntaxin 11. (A) Western blot analysis of syntaxin 11 expression. Total protein from eight different human tissues (heart, brain, placenta, lung, liver, skeletal muscle, kidney and pancreas) was separated by SDS-PAGE and analyzed by western blotting using an anti-syntaxin 11 serum at a 1:1000 dilution. The mobility of the 35 kDa syntaxin 11 protein is indicated. Syntaxin 11 protein can be observed in brain, skeletal muscle and pancreas only with long exposures of the western blot. (B) Northern blot analysis of syntaxin 11 expression. Poly(A^+)RNA from the same human tissues listed above was separated on an agarose gel, transferred to a nylon membrane, and probed with ^{32}P-labeled DNA probes derived from *syntaxin 11* (upper panel) or GAPDH (lower panel). The sizes of the major transcripts are indicated.

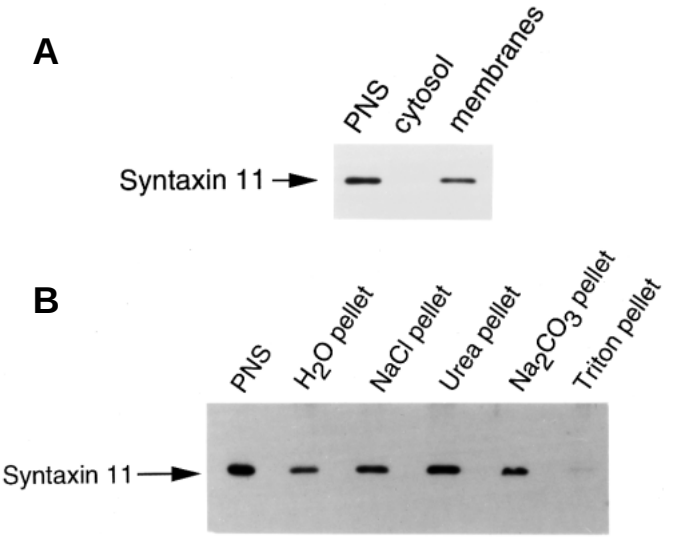

Fig. 2. Syntaxin 11 behaves as an integral membrane protein. (A) A human B lymphocyte post-nuclear supernatant in hypotonic buffer was subjected to centrifugation at 100,000 ***g*** and equivalent fractions of the starting post-nuclear supernatant (PNS), high-speed supernatant (cytosol) and high-speed pellet (membranes) were separated by SDS-PAGE and analyzed by western blotting using anti-syntaxin 11 serum. (B) The human B lymphocyte post-nuclear supernatant was incubated with equal volumes of either H_2O, 3 M NaCl, 4 M urea, 0.4 M Na_2CO_3 (pH 11.4), or 4 % Triton X-100 and cell membranes were pelleted by centrifugation at 100,000 ***g***. Equivalent fractions of the starting post-nuclear supernatant (PNS) and membrane pellet from each condition were analyzed by western blotting using anti-syntaxin 11 serum.

al. (1998) concluded that epitope-tagged syntaxin 11 was predominantly present on intracellular membranes in transfected NRK cells. To examine the subcellular distribution of endogenous syntaxin 11 in untransfected cells, we isolated total cellular membranes and cytosol from human B lymphocytes. Immunoblotting with a syntaxin 11-specific serum revealed that the majority of syntaxin 11 present in a post-nuclear supernatant was membrane associated in these cells (Fig. 2A). Furthermore, despite the lack of a transmembrane domain, syntaxin 11 remained in the 100,000 ***g*** membrane pellet even after the cell membranes were treated with 1.5 M NaCl, 2 M urea, or Na_2CO_3 (pH 11.4) (Fig. 2B). Treatment with Triton X-100 results in solubilization of cellular membranes and in this case the majority of syntaxin 11 was absent from the high-speed pellet. Immunoblotting of the high-speed supernatants demonstrated that only Triton X-100 treatment liberated syntaxin 11 from the membranes (data not shown). These data demonstrate that despite the lack of a traditional transmembrane domain, syntaxin 11 behaves as an integral membrane protein.

Syntaxin 11 binds to SNARE proteins in vitro

To confirm that syntaxin 11 can bind to SNARE proteins using an assay other than the yeast two-hybrid system, we examined the binding of in vitro translated syntaxin 11 to various GST-SNARE fusion proteins and a representative experiment is shown in Fig. 3. Whereas syntaxin 11 never bound to GST alone, it consistently bound well to GST-VAMP 2 and GST-SNAP-23. Furthermore, syntaxin 11 did not bind to GST-syntaxin 2, a t-SNARE protein that would not necessarily be expected to interact with syntaxin 11. Although these experiments should not be taken to imply that syntaxin 11 necessarily binds to VAMP 2 in vivo, they do demonstrate that syntaxin 11 behaves as a 'typical' syntaxin in that it is capable of binding to VAMP and SNAP-23.

Syntaxin 11 binds to SNAP-23 in vivo

To study the interaction of syntaxin 11 and SNAP-23 in greater detail, human B lymphocytes were radiolabeled with [^{35}S]methionine to study the biosynthesis of the syntaxin 11/SNAP-23 complex. Unfortunately, the expression of these proteins was quite low in these cells. Therefore, HeLa cells were transfected with syntaxin 11 alone, SNAP-23 alone, or syntaxin 11 and SNAP-23 together to examine the association of these t-SNAREs in vivo. The cells were metabolically radiolabeled with [^{35}S]methionine and cell lysates were

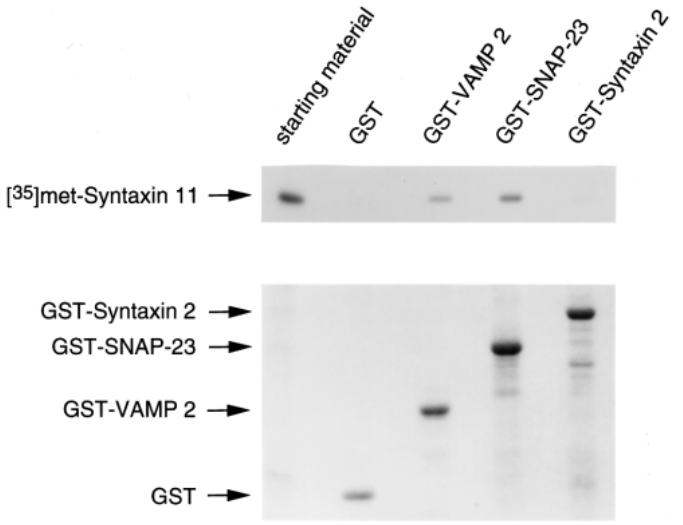

Fig. 3. Binding of syntaxin 11 to GST-SNARE fusion proteins. Bacterially expressed GST, GST-VAMP2, GST-SNAP-23 or GST-syntaxin 2 fusion proteins bound to glutathione-Sepharose beads (approximately 3 μg each protein) were incubated with ^{35}S-labeled in vitro-translated syntaxin 11 at 4°C, washed and analyzed by SDS-PAGE. One-tenth of the total amount of ^{35}S-syntaxin 11 present in each sample was also analyzed to give an indication of the binding efficiency. The gels were subjected to fluorography to detect bound syntaxin 11 (upper panel) and were stained with Coomassie Blue to confirm equal loading of the GST fusion proteins (lower panel). The electrophoretic mobility of each GST fusion protein is indicated.

analyzed by immunoprecipitation using syntaxin 11- or SNAP-23-specific antisera. Fig. 4A demonstrates that the syntaxin 11 antiserum specifically recognized the 35 kDa syntaxin 11 protein in cells transfected with syntaxin 11 alone and the SNAP-23 antiserum specifically recognized the 28 kDa SNAP-23 protein in cells transfected with SNAP-23 alone, confirming that there was no cross-reactivity between the two rabbit antisera. It should be noted that a small amount of the endogenous SNAP-23 protein can be seen in the syntaxin 11 only transfectant. Fig. 4A also demonstrates that complexes containing syntaxin 11 and SNAP-23 were efficiently co-precipitated using either antiserum in cells expressing both syntaxin 11 and SNAP-23. Although the majority of the immunoprecipitable syntaxin 11 was associated with SNAP-23, only a fraction of the SNAP-23 was found in the anti-syntaxin 11 immunoprecipitate. This could reflect either the presence of a large free pool of SNAP-23 in these cells, binding of SNAP-23 to other syntaxin isoforms, partial masking of the anti-syntaxin 11 antibody epitope in the syntaxin 11/SNAP-23 complex, or non-stoichiometric association between syntaxin

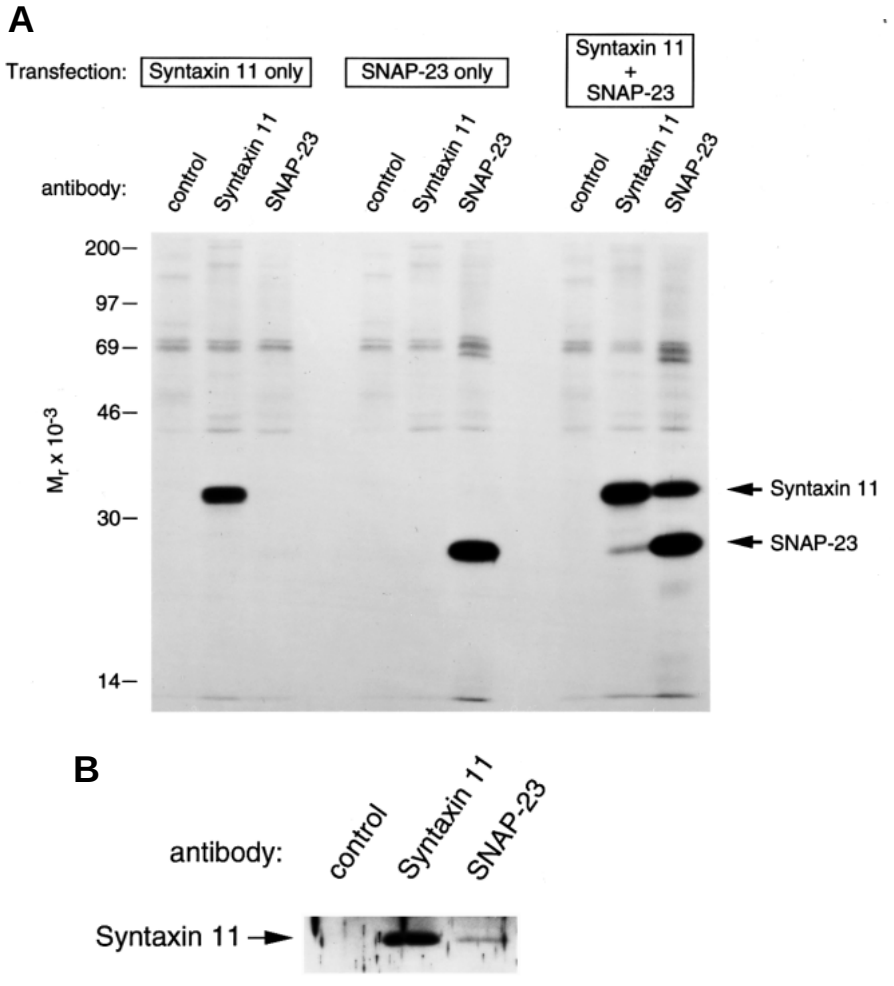

Fig. 4. Binding of syntaxin 11 to SNAP-23 in vivo. (A) HeLa cells overexpressing syntaxin 11 alone, SNAP-23 alone, or syntaxin 11 and SNAP-23 together, were metabolically labeled with [^{35}S]methionine, lysed and equivalent fractions of post-nuclear supernatant were incubated on ice with either pre-immune serum, anti-syntaxin 11 serum or anti-SNAP-23 serum. Immune complexes were bound to protein A-agarose, washed extensively, and analyzed by SDS-PAGE and fluorography. The mobilities of molecular mass markers are indicated on the left of the figure, and the mobilities of syntaxin 11 and SNAP-23 on the right. (B) Human B lymphocytes were lysed in Triton X-100 and immunoprecipitations were performed using a control pre-immune rabbit serum, a syntaxin 11 antiserum, or a SNAP-23 antiserum. The immunoprecipitates were separated by SDS-PAGE and probed by western blotting using the anti-syntaxin 11 serum and horseradish peroxidase-conjugated protein A.

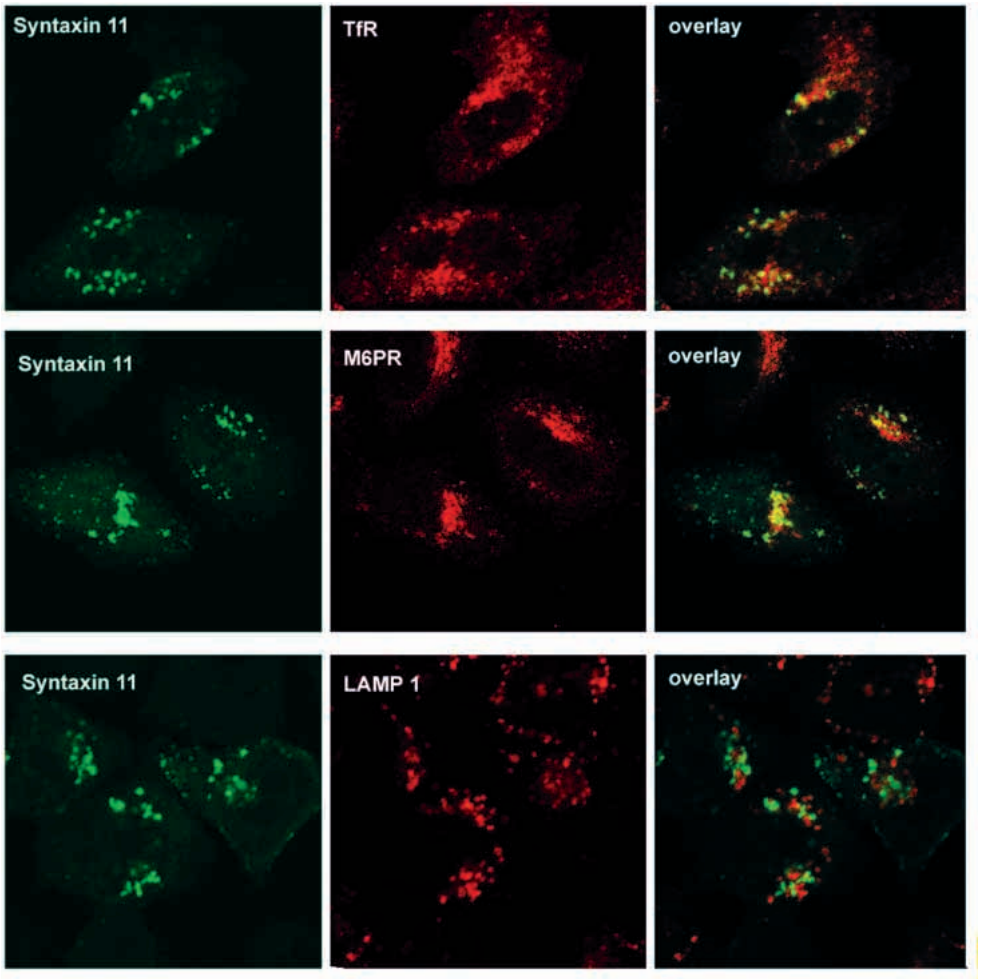

Fig. 5. Syntaxin 11 colocalizes with the mannose 6-phosphate receptor on late endosomes and the TGN. HeLa cells were transiently transfected with a cDNA encoding human syntaxin 11. The cells were fixed, permeabilized and analyzed by confocal immunofluorescence microscopy for expression of syntaxin 11 (green) and endogenous organelle markers (red). Upper panel, syntaxin 11 and transferrin receptor; middle panel, syntaxin 11 and cation-independent mannose 6-phosphate receptor; and lower panel, syntaxin 11 and LAMP 1. The FITC (green) and Cy5 (red) signals were electronically merged and regions of colocalization appear yellow. Note that since the cells were transiently transfected, not all cells express detectable syntaxin 11 protein.

11 and SNAP-23. We are currently attempting to resolve this issue. In any event, these studies conclusively demonstrate that syntaxin 11 is capable of binding to SNAP-23 in vivo.

We next performed experiments to determine if endogenous syntaxin 11 was associated with endogenous SNAP-23 in mammalian cells in vivo. Human B lymphocytes were lysed in Triton X-100 and immunoprecipitations were performed using control serum, anti-syntaxin 11 serum, or anti-SNAP-23 serum. Immunoblotting using a syntaxin 11-specific antibody confirmed that syntaxin 11 was present in both the anti-syntaxin 11 and the anti-SNAP-23 immunoprecipitate (Fig. 4B), demonstrating that a significant fraction of the total pool of syntaxin 11 is associated with SNAP-23 in untransfected mammalian cells.

Syntaxin 11 is associated with late endosomes and the TGN

To provide insight into the possible function of syntaxin 11 in vivo, we have attempted to identify the cellular compartment with which syntaxin 11 is associated. As mentioned above, syntaxin 11 is expressed at too low a level to be efficiently detected in untransfected cells using our syntaxin 11 antisera. We have therefore transfected syntaxin 11 cDNA into HeLa cells and co-stained the cells with antibodies to syntaxin 11 and other known marker proteins to examine the cellular distribution of syntaxin 11. Immunofluorescence microscopy demonstrates that syntaxin 11 is present on perinuclear structures and associated vesicles in HeLa cells (Fig. 5). Essentially identical staining patterns were observed in HeLa cells expressing lower levels of syntaxin 11, suggesting that the observed localization was not a consequence of overexpression of syntaxin 11. Although this staining pattern was reminiscent of the Golgi apparatus, confocal immunofluorescence microscopy using an antibody against galactosyltransferase (a marker of the trans-cisternae of the Golgi apparatus) revealed that syntaxin 11 was present in a compartment adjacent to but distinct from the Golgi apparatus (data not shown).

Given the distribution of syntaxin 11, we attempted to co-localize this protein with endogenous markers of the endosome system in HeLa cells. There was minimal co-localization of syntaxin 11 with the transferrin receptor and the overall distribution of these two proteins was quite different (Fig. 5, upper panel). The transferrin receptor is concentrated on the plasma membrane and on tubulo-reticular early/recycling endosomes, although small but significant amounts are present in pre-lysosomal endosomes (Killisch et al., 1992). In contrast, syntaxin 11 colocalized extensively with the cation independent mannose 6-phosphate receptor and, most importantly, the overall structure of the syntaxin 11 compartment was very similar to that of the mannose 6-phosphate receptor compartment (Fig. 5, middle panel). Immunoelectron microscopy has revealed that the mannose 6-

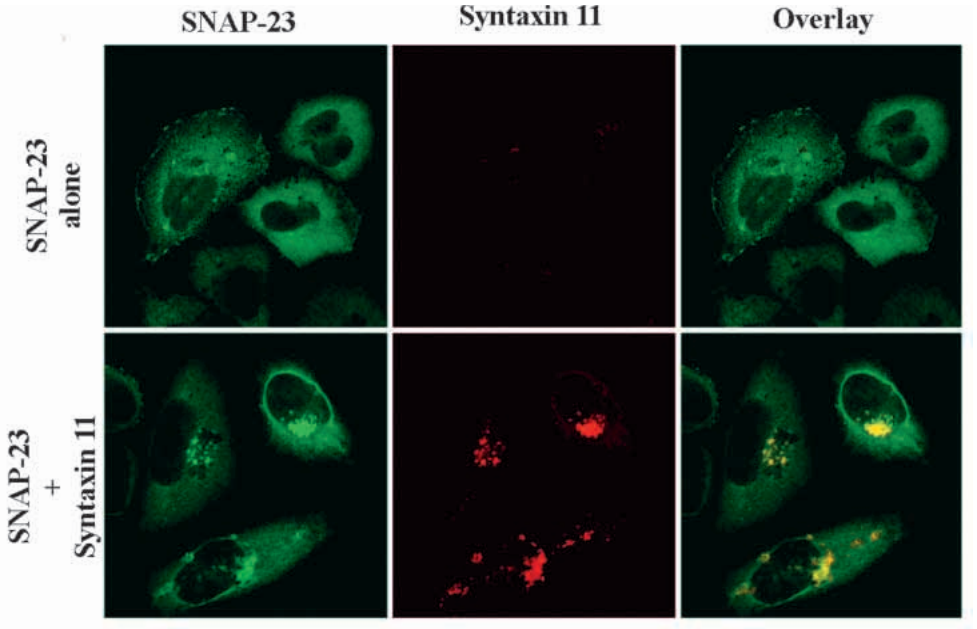

Fig. 6. SNAP-23 colocalizes with syntaxin 11 on intracellular membranes. HeLa cells were transiently transfected with a cDNA encoding human SNAP-23 alone or SNAP-23 together with epitope-tagged syntaxin 11. After 36 hours, the distribution of SNAP-23 (green) and syntaxin 11 (red) was analyzed by confocal immunofluorescence microscopy using an anti-SNAP-23 rabbit serum and an anti-Xpress epitope mouse monoclonal antibody. Areas of colocalization are shown in yellow. Control experiments revealed that the addition of the epitope tag did not alter the distribution of syntaxin 11 in transfected HeLa cells.

phosphate receptor is present predominantly on late endosomes and the TGN, although the precise ratio of mannose 6-phosphate receptor present on these compartments varies between cell types (Griffiths et al., 1988; Geuze et al., 1988; Dintzis et al., 1994). Finally, syntaxin 11 did not co-localize with the lysosomal marker LAMP 1 (Fig. 5, lower panel), demonstrating that syntaxin 11 resides predominantly in the late endosome/TGN system and does not accumulate to an appreciable extent in early endosomes and/or lysosomes.

SNAP-23 colocalizes with syntaxin 11 on intracellular membranes

Since syntaxin 11 was identified as a SNAP-23 binding protein, we examined whether co-expression of syntaxin 11 with SNAP-23 altered the intracellular localization of either protein. Fig. 6 demonstrates that while SNAP-23 is present predominantly on the plasma membrane of HeLa cells when expressed alone, co-expression of syntaxin 11 resulted in the detection of SNAP-23 onto perinuclear membranes that co-localize with syntaxin 11. In agreement with the immunoprecipitation data shown in Fig. 4, Fig. 6 confirms that in addition to the pool of SNAP-23 present on syntaxin 11-positive intracellular membranes, there is a pool of SNAP-23 that fails to associate with syntaxin 11 under these conditions. Only rarely did we find syntaxin 11 present on the plasma membrane of cells expressing SNAP-23, suggesting that the targeting information in syntaxin 11 for the late endosome/TGN compartment predominates over the plasma membrane targeting information in SNAP-23. Taken together, these data suggest that syntaxin 11 and SNAP-23 form a complex on late endosomes and/or the TGN that serves as a t-SNARE for vesicles destined for these compartments.

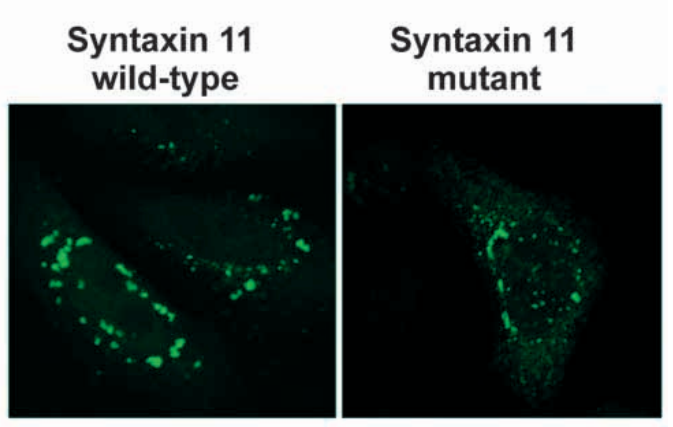

Fig. 7. Syntaxin 11 is not membrane-anchored by the cysteine-rich carboxy-terminal domain. HeLa cells were transiently transfected with a cDNA encoding wild-type human syntaxin 11 or a syntaxin 11 mutant lacking the carboxy-terminal 13 amino acids. The cells were fixed, permeabilized and the intracellular distribution of each form of syntaxin 11 was determined by immunofluorescence microscopy.

Membrane association of syntaxin 11

Although syntaxin 11 does not possess a hydrophobic transmembrane domain, there is a cluster of six cysteine residues at the carboxy terminus of this molecule. As has been documented for SNAP-25 (Veit et al., 1996), palmitoylation of one or more of these cysteines could provide a mechanism of membrane attachment for syntaxin 11. To investigate this possibility, we have removed each of these cysteine residues by truncating 13 amino acids from the carboxy terminus of syntaxin 11 and analyzed the distribution of wild-type and truncated syntaxin 11 in HeLa cells by immunofluorescence microscopy. Fig. 7 reveals that despite the absence of the carboxy-terminal cysteine cluster, truncated syntaxin 11 was still found primarily on punctate intracellular structures that appeared similar to those containing wild-type syntaxin 11. These data therefore demonstrate that the amino acid(s) responsible for the targeting and retention of syntaxin 11 on intracellular membranes do not reside exclusively in the cysteine-rich cytosolic domain.

DISCUSSION

Computerized searches of the EST database recently have resulted in the identification of many novel mammalian SNARE isoforms (Bock et al., 1996; Bock and Scheller, 1997; Weimbs et al., 1997; Wang et al., 1997b; Wong et al., 1998; Tang et al., 1998b,c). In each of these cases, the novel syntaxin was identified by screening the EST database for potential syntaxin homologues and isolating a full-length cDNA clone by hybridization using an EST probe. However, a potential drawback using this approach is that the isolation procedure is not biased towards identifying SNARE homologues based on a biochemical interaction with other SNARE proteins. We have successfully used the yeast two-hybrid system to identify the non-neuronal SNAP-25 homologue, SNAP-23, using syntaxin 4 as the 'bait' in the screen of a human B lymphocyte cDNA library (Ravichandran et al., 1996). We have now used human SNAP-23 as the 'bait' and in addition to re-isolating syntaxin 4, we have also identified syntaxin 11 as a SNAP-23 binding partner. Thus, this system allows us to identify functional SNARE binding proteins in the tissue of interest without DNA sequence bias.

Relative to other mammalian syntaxins, syntaxin 11 is most closely related to syntaxins 1-3, displaying approximately 30% amino acid identity overall with these syntaxin isoforms (Advani et al., 1998; Tang et al., 1998a). Despite the limited homology, however, syntaxin 11 appears to be a 'typical' syntaxin in that it is approximately 35 kDa in mass and possesses sequences in the amino and carboxy termini of the molecule which have a high probability of forming coiled-coil domains (Advani et al., 1998; Tang et al., 1998a). These domains are present in all syntaxins, VAMPs and SNAP-23 family members and are thought to be the major driving force leading to SNARE-SNARE interactions in vitro and in vivo (Hayashi et al., 1994; Chapman et al., 1994; Kee et al., 1995; Pevsner et al., 1994; Fasshauer et al., 1997). Like other syntaxin family members, syntaxin 11 is able to bind efficiently to VAMP 2 and SNAP-23 in in vitro GST pull-down assays. Furthermore, syntaxin 11 binds to SNAP-23 very efficiently in transfected HeLa cells and a significant fraction of endogenous syntaxin 11 exists in a complex with SNAP-23 in human B lymphocytes at steady state. The stable association of syntaxin 11 with SNAP-23 is reminiscent of the stable association of syntaxin 3 with SNAP-23 in epithelial cells (Galli et al., 1998) and syntaxin 4 with SNAP-23 in rat adipose cells (St Denis et al., 1999). Taken together, these data are consistent with the hypothesis that syntaxin 11 is a typical SNARE that facilitates vesicular transport by engaging in protein-protein interactions with other SNAREs.

Every syntaxin isoform identified thus far exists predominantly as an integral membrane protein that is anchored to membranes by a carboxy terminal hydrophobic transmembrane domain (Bennett et al., 1993; Bock et al., 1996; Wang et al., 1997b; Wong et al., 1998; Tang et al., 1998b,c). Syntaxin 11 does not possess a significant carboxy-terminal hydrophobic domain, yet the protein behaves as an integral membrane protein in that it remains associated with membranes even in high salt or high pH conditions, and is soluble when the membranes are treated with Triton X-100. The stable association of syntaxin 11 with membranes is in excellent agreement with the results obtained by Advani et al. (1998), although in this previous report syntaxin 11 was not extracted from membranes by Triton X-100. However, these investigators examined myc epitope-tagged syntaxin 11 in NRK cells, and it is possible that the overexpression of this altered protein rendered it insoluble. We did not observe additional proteins co-precipitating with overexpressed syntaxin 11 in HeLa cells, suggesting that the association of syntaxin 11 with membranes is not a consequence of syntaxin 11 binding to another integral membrane protein. Like SNAP-23 and SNAP-25, syntaxin 11 does contain a number of cysteine residues, leading to the speculation that, in a manner analogous to SNAP-25 (Veit et al., 1996), palmitoylation of one or more carboxy-terminal cysteine residues is responsible for the association of syntaxin 11 with membranes (Advani et al., 1998). However, we have truncated the carboxy-terminal cysteine cluster from syntaxin 11 and the resulting construct is still membrane-associated when expressed in HeLa cells. Although these studies do not rule out the possibility that the carboxy-terminal region of syntaxin 11 is palmitoylated, they do demonstrate that there are regions outside of the cysteine-rich carboxy terminus that are able to mediate the association of syntaxin 11 with membranes.

Immunofluorescence microscopy of syntaxin 11 in transfected HeLa cells reveals that a significant fraction of syntaxin 11 co-localizes with the mannose 6-phosphate receptor, demonstrating that syntaxin 11 is present on late endosomes and the TGN. This is in excellent agreement with the recent localization of epitope-tagged syntaxin 11 to a post-Golgi compartment (Advani et al., 1998), although in this study the precise location of syntaxin 11 was not defined. The mannose 6-phosphate receptor functions by cycling between the TGN and late endosomes to deliver proteolytic enzymes to lysosomes. Given the ubiquitous expression of syntaxin 11 (with the notable exception being the brain) and the importance of the TGN and late endosomes in lysosome biogenesis, it is likely that syntaxin 11 performs a 'housekeeping' function by regulating vesicular traffic into or out of these compartments. Although syntaxin 6 (Bock et al., 1996, 1997) and syntaxin 10 (Tang et al., 1998c) have been localized to the TGN and syntaxin 7 has been reported to reside on early endosomes (Wong et al., 1998), syntaxin 11 is the first syntaxin isoform localized to late endosomes. Based on the colocalization data with the mannose 6-phosphate receptor, therefore, it is likely that syntaxin 11 regulates transport between the TGN and late endosomes. With the original description of syntaxins as t-SNAREs, syntaxin 11 would be expected to regulate vesicle docking and fusion into late endosomes and/or the TGN. However, recent evidence has blurred the distinction between v- and t-SNAREs, as organelles and highly purified vesicles have been shown to possess both types of SNAREs (Tagaya et al., 1995; Walch-Solimena et al., 1995; Bock et al., 1997; Rowe et al., 1998) and these SNAREs can bind to each other on the same cellular membrane (Otto et al., 1997; Ungermann et al., 1998). For this reason, it is equally possible that syntaxin 11 regulates membrane traffic either into or out of late endosomes or the TGN.

SNAP-23 has been localized predominantly to the plasma membrane in polarized epithelial cells (Low et al., 1998b; Gaisano et al., 1997; Leung et al., 1998; Galli et al., 1998), adipocytes (Wang et al., 1997a; Araki et al., 1997; Wong et al., 1997), HeLa cells and human B lymphocytes (A. C. V., M. J.

B. and P. A. R., unpublished observations). However, in most cases a small amount of internal membrane staining can be observed, leading to the hypothesis that SNAP-23 is associated with a syntaxin family member present on intracellular membranes. Our co-precipitation data using transfected HeLa cells and untransfected B lymphocytes confirms that a fraction of SNAP-23 is associated with syntaxin 11 at steady state, and the redistribution of SNAP-23 from the plasma membrane to syntaxin 11-positive intracellular compartments in transfected HeLa cells is in excellent agreement with the hypothesis that a fraction of the total pool of SNAP-23 exists in a complex with many different syntaxins on distinct intracellular membranes. Although the localization studies were obtained in a system in which both syntaxin 11 and SNAP-23 were overexpressed, we found identical results over a broad range of expression levels of each protein. We feel it is likely, therefore, that syntaxin 11 is associated with SNAP-23 in the late endosomes and/or the TGN at steady state. Furthermore, we and others have found that SNAP-23 binds to a variety of syntaxin isoforms in vivo (Ravichandran et al., 1996; Araki et al., 1997), leading us to propose that the prevalence of SNAP-23 on the plasma membrane reflects not only the accumulation of SNAP-23 monomers at the plasma membrane, but is also due to the fact that the total mass of syntaxin isoforms present on the plasma membrane greatly exceeds the mass of syntaxin isoforms present on intracellular membranes. This hypothesis can now be tested given the identification of multiple syntaxin isoforms on internal compartments.

We would like to thank David Winkler for oligonucleotide synthesis, peptide synthesis, and DNA sequence analysis, Suzanne Pfeffer for the anti-mannose 6-phosphate receptor monoclonal antibody, and Dinah Singer and members of the Singer and Roche laboratories for valuable discussions. We also are indebted to Julie Donaldson and Harish Radhakrishna for expert advice on the co-localization studies.

REFERENCES

Advani, R. J., Bae, H. R., Bock, J. B., Chao, D. S., Doung, Y. C., Prekeris, R., Yoo, J. S. and Scheller, R. H. (1998). Seven novel mammalian SNARE proteins localize to distinct membrane compartments. *J. Biol. Chem.* **273**, 10317-10324.

Anderson, H. A. and Roche, P. A. (1998). Phosphorylation regulates the delivery of MHC class II invariant chain complexes to antigen processing compartments. *J. Immunol.* **160**, 4850-4858.

Araki, S., Tamori, Y., Kawanishi, M., Shinoda, H., Masugi, J., Mori, H., Niki, T., Okazawa, H., Kubota, T. and Kasuga, M. (1997). Inhibition of the binding of SNAP-23 to syntaxin 4 by Munc18c. *Biochem. Biophys. Res. Commun.* **234**, 257-262.

Bennett, M. K., Garcia-Arraras, J. E., Elferink, L. A., Peterson, K., Fleming, A. M., Hazuka, C. D. and Scheller, R. H. (1993). The syntaxin family of vesicular transport receptors. *Cell* **74**, 863-873.

Bennett, M. K. and Scheller, R. H. (1994). A molecular description of synaptic vesicle membrane trafficking. *Annu. Rev. Biochem.* **63**, 63-100.

Bock, J. B., Lin, R. C. and Scheller, R. H. (1996). A new syntaxin family member implicated in targeting of intracellular transport vesicles. *J. Biol. Chem.* **271**, 17961-17965.

Bock, J. B., Klumperman, J., Davanger, S. and Scheller, R. H. (1997). Syntaxin 6 functions in trans-Golgi network vesicle trafficking. *Mol. Biol. Cell* **8**, 1261-1271.

Bock, J. B. and Scheller, R. H. (1997). Protein transport. A fusion of new ideas. *Nature* **387**, 133-135.

Chapman, E. R., An, S., Barton, N. and Jahn, R. (1994). SNAP-25, a t-SNARE which binds to both syntaxin and synaptobrevin via domains that may form coiled coils. *J. Biol. Chem.* **269**, 27427-27432.

Dintzis, S. M., Velculescu, V. E. and Pfeffer, S. R. (1994). Receptor extracellular domains may contain trafficking information. Studies of the 300-kDa mannose 6-phosphate receptor. *J. Biol. Chem.* **269**, 12159-12166.

Fasshauer, D., Bruns, D., Shen, B., Jahn, R. and Brunger, A. T. (1997). A structural change occurs upon binding of syntaxin to SNAP-25. *J. Biol. Chem.* **272**, 4582-4590.

Ferro-Novick, S. and Jahn, R. (1994). Vesicle fusion from yeast to man. *Nature* **370**, 191-193.

Gaisano, H. Y., Sheu, L., Wong, P. P., Klip, A. and Trimble, W. S. (1997). SNAP-23 is located in the basolateral plasma membrane of rat pancreatic acinar cells. *FEBS Lett.* **414**, 298-302.

Galli, T., Zahraoui, A., Vaidyanathan, V. V., Raposo, G., Tian, J. M., Karin, M., Niemann, H. and Louvard, D. (1998). A novel tetanus neurotoxin-insensitive vesicle-associated membrane protein in SNARE complexes of the apical plasma membrane of epithelial cells. *Mol. Biol. Cell* **9**, 1437-1448.

Geuze, H. J., Stoorvogel, W., Strous, G. J., Slot, J. W., Bleekemolen, J. E. and Mellman, I. (1988). Sorting of mannose 6-phosphate receptors and lysosomal membrane proteins in endocytic vesicles. *J. Cell Biol.* **107**, 2491-2501.

Griffiths, G., Hoflack, B., Simons, K., Mellman, I. and Kornfeld, S. (1988). The mannose 6-phosphate receptor and the biogenesis of lysosomes. *Cell* **52**, 329-341.

Hanson, P. I., Otto, H., Barton, N. and Jahn, R. (1995). The N-ethylmaleimide-sensitive fusion protein and alpha-SNAP induce a conformational change in syntaxin. *J. Biol. Chem.* **270**, 16955-16961.

Hayashi, T., McMahon, H., Yamasaki, S., Binz, T., Hata, Y., Südhof, T. C. and Niemann, H. (1994). Synaptic vesicle membrane fusion complex: action of clostridial neurotoxins on assembly. *EMBO J.* **13**, 5051-5061.

Hayashi, T., Yamasaki, S., Nauenburg, S., Binz, T. and Niemann, H. (1995). Disassembly of the reconstituted synaptic vesicle membrane fusion complex in vitro. *EMBO J.* **14**, 2317-2325.

Kee, Y., Lin, R. C., Hsu, S. C. and Scheller, R. H. (1995). Distinct domains of syntaxin are required for synaptic vesicle fusion complex formation and dissociation. *Neuron* **14**, 991-998.

Killisch, I., Steinlein, P., Romisch, K., Hollinshead, R., Beug, H. and Griffiths, G. (1992). Characterization of early and late endocytic compartments of the transferrin cycle. Transferrin receptor antibody blocks erythroid differentiation by trapping the receptor in the early endosome. *J. Cell Sci.* **103**, 211-232.

Leung, S. M., Chen, D., DasGupta, B. R., Whiteheart, S. W. and Apodaca, G. (1998). SNAP-23 Requirement for Transferrin Recycling in Streptolysin O-permeabilized Madin-Darby Canine Kidney Cells. *J. Biol. Chem.* **273**, 17732-17741.

Low, S. H., Chapin, S. J., Weimbs, T., Komuves, L. G., Bennett, M. K. and Mostov, K. E. (1996). Differential localization of syntaxin isoforms in polarized Madin-Darby canine kidney cells. *Mol. Biol. Cell* **7**, 2007-2018.

Low, S. H., Chapin, S. J., Wimmer, C., Whiteheart, S. W., Komuves, L. G., Mostov, K. E. and Weimbs, T. (1998a). The SNARE machinery is involved in apical plasma membrane trafficking in MDCK cells. *J. Cell Biol.* **141**, 1503-1513.

Low, S. H., Roche, P. A., Anderson, H. A., van Ijzendoorn, S. C. D., Zhang, M., Mostov, K. E. and Weimbs, T. (1998b). Targeting of SNAP-23 and SNAP-25 in polarized epithelial cells. *J. Biol. Chem.* **273**, 3422-3430.

Niemann, H., Blasi, J. and Jahn, R. (1994). Clostridial neurotoxins: new tools for dissecting exocytosis. *Trends Cell Biol.* **4**, 179-185.

Novick, P. and Zerial, M. (1997). The diversity of Rab proteins in vesicle transport. *Curr. Opin. Cell Biol.* **9**, 496-504.

Otto, H., Hanson, P. I. and Jahn, R. (1997). Assembly and disassembly of a ternary complex of synaptobrevin, syntaxin, and SNAP-25 in the membrane of synaptic vesicles. *Proc. Natl. Acad. Sci. USA* **94**, 6197-6201.

Pevsner, J., Hsu, S. C., Braun, J. E., Calakos, N., Ting, A. E., Bennett, M. K. and Scheller, R. H. (1994). Specificity and regulation of a synaptic vesicle docking complex. *Neuron.* **13**, 353-361.

Ravichandran, V., Chawla, A. and Roche, P. A. (1996). Identification of a novel syntaxin-and synaptobrevin/VAMP-binding protein, SNAP-23, expressed in non-neuronal tissues. *J. Biol. Chem.* **271**, 13300-13303.

Rothman, J. E. (1994). Mechanisms of intracellular protein transport. *Nature* **372**, 55-63.

Rowe, T., Dascher, C., Bannykh, S., Plutner, H. and Balch, W. E. (1998). Role of vesicle-associated syntaxin 5 in the assembly of pre-Golgi intermediates. *Science* **279**, 696-700.

Sadoul, K., Berger, A., Niemann, H., Weller, U., Roche, P. A., Klip, A., Trimble, W. S., Regazzi, R., Catsicas, S. and Halban, P. A. (1997). SNAP-23 is not cleaved by botulinum neurotoxin E and can replace SNAP-25 in the process of insulin secretion. *J. Biol. Chem.* **272**, 33023-33027.

Söllner, T., Bennett, M. K., Whiteheart, S. W., Scheller, R. H. and Rothman, J. E. (1993a). A protein assembly-disassembly pathway in vitro that may correspond to sequential steps of synaptic vesicle docking, activation, and fusion. *Cell* **75**, 409-418.

Söllner, T., Whiteheart, S. W., Brunner, M., Erdjument-Bromage, H., Geromanos, S., Tempst, P. and Rothman, J. E. (1993b). SNAP receptors implicated in vesicle targeting and fusion. *Nature* **362**, 318-324.

St Denis, J.-F., Cabaniols, J.-P., Cushman, S. W. and Roche, P. A. (1999). SNAP-23 participates in SNARE complex assembly in rat adipose cells. *Biochem. J.* (in press).

Südhof, T. C. (1995). The synaptic vesicle cycle: a cascade of protein-protein interactions. *Nature* **375**, 645-653.

Tagaya, M., Toyonaga, S., Takahashi, M., Yamamoto, A., Fujiwara, T., Akagawa, K., Moriyama, Y. and Mizushima, S. (1995). Syntaxin 1 (HPC-1) is associated with chromaffin granules. *J. Biol. Chem.* **270**, 15930-15933.

Tang, B. L., Low, D. Y. and Hong, W. (1998a). Syntaxin 11: a member of the syntaxin family without a carboxyl terminal transmembrane domain. *Biochem. Biophys. Res. Commun.* **245**, 627-632.

Tang, B. L., Low, D. Y., Lee, S. S., Tan, A. E. and Hong, W. (1998b). Molecular cloning and localization of human syntaxin 16, a member of the syntaxin family of SNARE proteins. *Biochem. Biophys. Res. Commun.* **242**, 673-679.

Tang, B. L., Low, D. Y., Tan, A. E. and Hong, W. (1998c). Syntaxin 10: a member of the syntaxin family localized to the trans-Golgi network. *Biochem. Biophys. Res. Commun.* **242**, 345-350.

Ungermann, C., Nichols, B. J., Pelham, H. R. B. and Wickner, W. (1998). A vacuolar v-t-SNARE complex, the predominant form in vivo and on isolated vacuoles, is disassembled and activated for docking and fusion. *J. Cell Biol.* **140**, 61-69.

Veit, M., Söllner, T. H. and Rothman, J. E. (1996). Multiple palmitoylation of synaptotagmin and the t-SNARE SNAP-25. *FEBS Lett.* **385**, 119-123.

Walch-Solimena, C., Blasi, J., Edelmann, L., Chapman, E. R., von Mollard, G. F. and Jahn, R. (1995). The t-SNAREs syntaxin 1 and SNAP-25 are present on organelles that participate in synaptic vesicle recycling. *J. Cell Biol.* **128**, 637-645.

Wang, G., Witkin, J. W., Hao, G., Bankaitis, V. A., Scherer, P. E. and Baldini, G. (1997a). Syndet is a novel SNAP-25 related protein expressed in many tissues. *J. Cell Sci.* **110**, 505-513.

Wang, H., Frelin, L. and Pevsner, J. (1997b). Human syntaxin 7: a Pep12p/Vps6p homologue implicated in vesicle trafficking to lysosomes. *Gene* **199**, 39-48.

Weber, T., Zemelman, B. V., McNew, J. A., Westermann, B., Gmachl, M., Parlati, F., Sollner, T. H. and Rothman, J. E. (1998). SNAREpins: minimal machinery for membrane fusion. *Cell* **92**, 759-772.

Weimbs, T., Low, S. H., Chapin, S. J., Mostov, K. E., Bucher, P. and Hofmann, K. (1997). A conserved domain is present in different families of vesicular fusion proteins: a new superfamily. *Proc. Natl. Acad. Sci. USA* **94**, 3046-3051.

Wong, P. P., Daneman, N., Volchuk, A., Lassam, N., Wilson, M. C., Klip, A. and Trimble, W. S. (1997). Tissue distribution of SNAP-23 and its subcellular localization in 3T3-L1 cells. *Biochem. Biophys. Res. Commun.* **230**, 64-68.

Wong, S. H., Xu, Y., Zhang, T. and Hong, W. (1998). Syntaxin 7, a novel syntaxin member associated with the early endosomal compartment. *J. Biol. Chem.* **273**, 375-380.