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Preferential association of syntaxin 8 with the early endosome

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SUMMARY

Members of the syntaxin family play a fundamental role in vesicle docking and fusion of diverse transport events. We have molecularly characterized syntaxin 8, a novel member of the syntaxin family. The nucleotide sequence of cloned rat cDNA predicts a polypeptide of 236 residues with a carboxyl-terminal 18-residue hydrophobic domain that may function as a membrane anchor. Characteristic of syntaxins, syntaxin 8 also contain regions that have the potential to form coiled-coil structures. Among the known syntaxins, syntaxin 8 is most homologous to syntaxin 6 which is predominantly associated with the trans-Golgi network (TGN). The syntaxin 8 transcript is detected in all rat tissues examined by northern blot. Antibodies against recombinant syntaxin 8 recognize a 27 kDa protein that is enriched in membrane fractions containing the Golgi apparatus and the endosomal/lysosomal compartments. Syntaxin 8 in membrane extract could be incorporated into a 20S protein complex in a way that is dependent on the soluble *N*-ethylmaleimide-sensitive factor (NSF) and soluble NSF attachment protein (α-SNAP), suggesting that syntaxin 8 is indeed a SNAP receptor (SNARE). Indirect immunofluorescence microscopy reveals that the majority of syntaxin 8 is localized to the early endosome marked by Rab5. This is corroborated by immunogold labeling experiments showing enrichment of syntaxin 8 in the early endosome and its co-labeling with Rab5.

Key words: Syntaxin, SNARE, Vesicular transport, Endosome, Membrane

INTRODUCTION

Protein transport along the secretory and endocytotic pathways is primarily mediated via various types of transport vesicles that bud from a donor compartment and fuse with a target compartment (Hong, 1996; Palade, 1975; Pryer et al., 1992; Rothman, 1994; Rothman and Wieland, 1996; Schekman and Orci, 1996). Soluble *N*-ethylmaleimide-sensitive factor (NSF; or its yeast counterpart, Sec18p) and soluble NSF attachment proteins (SNAPs; or the yeast counterpart, Sec17p) have been shown to participate in many different transport events (Clary et al., 1990; Graham and Emr, 1991; Griff et al., 1992; Whiteheart and Kubalek, 1995). The action of NSF and SNAP is mediated through SNAP receptors (SNAREs) that participate in vesicle docking and fusion events (Rothman, 1994; Whiteheart and Kubalek, 1995). It is generally believed that the specific docking and fusion of vesicles with the target compartment require interaction between v-SNAREs on the vesicles and t-SNAREs on the target membrane (Ferro-Novick and Jahn, 1994; Pfeffer, 1996; Rothman, 1994; Rothman and Warren, 1994; Scheller, 1995; Söllner et al., 1993; Südhof, 1995).

Because of the importance of SNAREs in vesicle docking and fusion, identification and characterization of novel SNAREs is of significance because these studies will not only uncover new proteins participating in various trafficking events but also provide a novel molecular avenue for the detailed molecular and morphological studies of the respective trafficking events. Members of the syntaxin family represent an important class of t-SNAREs involved in diverse trafficking events (Bennett et al., 1993; Bock and Scheller, 1997; Hay et al., 1996, 1997). Syntaxin 1, the first member of this family, was originally identified by its co-immunoprecipitation with synaptotagmin and is enriched in the presynaptic plasma membrane (Bennett et al., 1992). Syntaxin 1 is a substrate for botulinum neurotoxin C (BoNT/C), which is known to act at the motor neuron to inhibit the exocytotic release of acetylcholine from the synaptic vesicles and cause the clinical manifestation of botulism, providing solid evidence for the role of syntaxin 1 in synaptic vesicle docking and fusion (Blasi et al., 1993). Syntaxin 1, SNAP-25 and synaptobrevin/VAMP are known to be the major components involved in the docking and fusion of synaptic vesicles (Pevsner et al., 1994; Söllner et al., 1993; Scheller 1995; Südhof, 1995). Several other members of the syntaxin family have been identified, suggesting that members of this family are key molecules in docking and fusion processes of diverse transport events (Bennett et al., 1993; Bock et al., 1997; Wang et al, 1997; Wong et al., 1998; Tang et al., 1998a,b,c,d).

In our present study, we have molecularly characterized a

novel member (syntaxin 8) of the syntaxin family and have biochemically established that syntaxin 8 does behave like a SNARE. Immunofluorescence microscopy and immunogold labeling establish that syntaxin 8 is enriched in the early endosome.

MATERIALS AND METHODS

Materials

Clone 9 normal rat liver cells (ATCC CRL-1439, NRL) were obtained from American Type Culture Collection (Rockville, MD). Synthetic oligos were either obtained from Oligos Etc. (Wilsonville, OR) or Research Biolabs (Singapore). The rat multiple tissue northern blot filter was purchased from Clontech (Palo Alto, CA). *Pyrococcus furiosus* polymerase and the rat brain cDNA library in λZAP were obtained from Stratagene (La Jolla, CA). Ni^{2+}-NTA beads and the pQE-60 vector were purchased from Qiagen (Hilden, Germany). Local New Zealand White rabbits were obtained from the Sembawang Laboratory Animal Center (Singapore). Brefeldin A (BFA) was from Epicentre Technologies (Madison, WI). Nocodazole, chloroquine and other common reagents were purchased from Sigma (St Louis, MO). EST clones from I.M.A.G.E. consortium were purchased from Research Genetics (Huntsville, AL).

Antibodies

The monoclonal antibody against rat TGN38 was a generous gift from Dr G. Banting (University of Bristol, UK). Monoclonal antibodies against Rab5 were a generous gift from A. Wandinger-Ness (Northwestern University, USA) and antibodies against Rabaptin5 were purchased from Transduction Lab. (USA). Monoclonal antibody against GS28 (Subramaniam et al., 1995), rabbit anti-asialoglycoprotein receptor subunit R2 and R3 and rabbit antibodies against syntaxin 7 (Wong et al., 1998) have been described. FITC-conjugated goat anti-rabbit antibodies were purchased from Boehringer Mannheim and Texas Red-conjugated goat anti-mouse IgG from Jackson Immunoresearch.

cDNA cloning and sequencing

Using the BLAST program (Altschul et al., 1997) to search the database of the expressed sequence tags (EST), several EST clones (GenBank accession nos AA000586, W41301, W63907 and W83363) were revealed whose sequences could encode protein fragments homologous to syntaxin 6 (Bock et al., 1996). Primers 1 (5′-GAACTCCCAACCCTT) and 2 (5′-ACTTTCTGTCCACCA) were used to obtain a fragment of about 280 bp from the EST clone no. W83363 by polymerase chain reaction (PCR) which was ^{32}P-labeled and used to screen a rat brain λZAP cDNA library. Four positive clones were obtained. One clone with an insert size of 1 kb was sequenced completely.

Northern blot analysis

A fragment of about 600 bp retrieved by *Cla*I and *Eco*RI digestion of the rat cDNA was ^{32}P-labeled and used as a probe on a rat multiple tissues blot of poly(A)$^+$ mRNA as described previously (Lowe et al., 1996).

Expression of recombinant proteins in bacteria

Primers 3 (5′-TTTAAAGCCATGGCCCCAGACCCC) and 4 (5′-GTGGATCCCTTTCTGTCCACCAG) were used to amplify the coding sequence for the cytoplasmic domain (residues 1-210) of syntaxin 8 by PCR to express C-terminally His×6-tagged syntaxin 8 (His×6-syntaxin 8). This PCR product was digested with *Nco*I and *Bam*HI, ligated into the corresponding sites of pQE60 vector and then transformed into the *Escherichia coli* bacterial host, M15(pRep4). The recombinant protein was purified on Ni^{2+}-NTA beads as recommended by the supplier (Qiagen).

Preparation and purification of polyclonal antibodies

250 μg of His×6-syntaxin 8 was emulsified in complete Freund's adjuvant and injected subcutaneously into two local New Zealand White rabbits. Booster injections with the same amount of antigen in incomplete Freund's adjuvant were given every two weeks. Ten days after the second and subsequent boosters, the rabbits were bled. The serum was diluted with equal volume of PBS and then incubated with antigen coupled to cyanogen bromide-activated Sepharose overnight at 4°C. The beads were washed and then specific antibodies were eluted with low pH elution buffer as described previously (Lowe et al., 1996).

Immunofluorescence microscopy

NRL cells were grown on coverslips overnight to a confluency of 50-80%. Cells were rinsed twice with cold PBS and processed as described (Neuhaus et al., 1998). Cells were then incubated with the indicated primary antibodies and then with goat anti-rabbit conjugated to FITC and Texas Red-conjugated goat anti-mouse secondary antibodies. Confocal microscopy was performed using a scan head (model MRC1024; Bio-Rad Labs, Carlsbad, CA) connected to an Axiophot microscope (Carl Zeiss) with epifluorescence optics.

Immunogold labeling

NRK cells were allowed to internalize 15 nm BSA-gold for 1 hour in DMEM followed by an overnight chase to label the late endocytotic structures. Cells were then allowed to internalize 5 nm BSA-gold for 6 minutes and were immediately fixed with 8% paraformaldehyde in 200 mM Hepes, pH 7.4. Cryosections were prepared and labeled with antibodies against syntaxin 8 as described (Griffiths, 1993) except that the new method of Liou et al. (1997) was used to pick up frozen sections. The sections were then detected with 10 nm gold-Protein A. The labeling was quantified using systematic sampling and intersection counts as described (Griffiths, 1993). For each structure 20 micrographs were quantified.

For double labeling of syntaxin 8 and Rab5, PC12 cells were rapidly frozen and then processed for freeze-substitution and embeding in Lowicry HM-20 (Steyer et al., 1997; Neuhaus et al., 1998). Ultra-thin sections were double-labeled with syntaxin 8 and Rab5 antibodies and detected with 4 nm gold-Protein A and 9 nm gold-Protein A, respectively.

Preparation of membrane fractions

Membranes were prepared as described (Subramaniam et al., 1992), with some minor modifications. Livers obtained from Sprague-Dawley rats which had been fasted overnight were rinsed once in ice-cold PBS and then in homogenization buffer (0.25 M sucrose, 25 mM Hepes, pH 7.4, 5 mM $MgCl_2$, 1 mM PMSF). Livers were homogenized in 3 volumes (ml/g) of homogenization buffer. The homogenate was centrifuged at 2000 ***g*** to remove nuclei, mitochondria and any unbroken cells. The postnuclear supernatant was then spun at 40000 rpm in a TY45 (Beckman) rotor for 1 hour to pellet the total membrane fraction (TM). This fraction was resuspended in 1.25 M sucrose, overlaid with step gradients of 1.1 M, 1.0 M, 0.8 M and 0.25 M sucrose. The gradients were centrifuged at 27000 rpm for 3 hours in a SW28 (Beckman) rotor. Four major fractions were isolated: Golgi enriched fractions G1 (1.1/1.0 M interphase), G2 (1.0/0.8 M interphase), endosome-enriched membrane fraction EM (0.8/0.25 M sucrose interphase) and the microsomal pellet (M). The G1, G2 and EM membranes were diluted fourfold with 25 mM Hepes, 5mM $MgCl_2$ and 1 mM PMSF and pelleted by a 100,000 ***g*** centrifugation step in a TY45 rotor as above. Membranes were resuspended in homogenization buffer and flash-frozen in aliquots. For some experiments, the gradients were prepared by overlaying the 1.25 M total membrane suspension with step gradients of 1.1 M and 0.5 M sucrose. The interphase between the 0.5 M and 1.1 M steps (GEM) which comprises Golgi, endosomes and lysosomes was pelleted, and resuspended in the appropriate buffer.

A

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ATTCACACAGAAACAGCTATGACCATGATTACGCCAAGCTCGAAATTAACCCTCACTAAAGGGAACAAAAGCTGGAGTCT    80
CCACGCGGTGGTCGGCCGCTCTAGAACTAGTGGATCCCCCGGGCTGCAGGAATTCGGCACGAGCTGCAGGCGGAGACTGC   160
ACCATGGCCCCAGACCCCTGGTTCTCCACGTACGATTCTACTTGTCAGATTGCCCAAGAAATCGCTGAGAAGATTCAAGA   240
    M  A  P  D  P  W  F  S  T  Y  D  S  T  C  Q  I  A  Q  E  I  A  E  K  I  Q  E    26
ACGAAATCAGTGTGAAAGAAGAGGTGAGAAGACACCTAAGCTTACCCTGACAATCAGAACTTTGTTGAAGAATCTGAAGG   320
  R  N  Q  C  E  R  R  G  E  K  T  P  K  L  T  L  T  I  R  T  L  L  K  N  L  K     52
TAAAGATCGACCTCTTGAAGGACTTACTTCTAAGAGCTGTGTCGACGCGCCAGATAACACAACTGGAGGGGGATCGAAGA   400
V  K  I  D  L  L  K  D  L  L  L  R  A  V  S  T  R  Q  I  T  Q  L  E  G  D  R  R     79
CAGAACCTTCTGGATGATCTTGTCACCCGAGAGAGACTGCTCCTGGCATCGTTTAAGAATGAGGGTTCCGAGCCGGATTT   480
 Q  N  L  L  D  D  L  V  T  R  E  R  L  L  L  A  S  F  K  N  E  G  S  E  P  D  L   106
GATCAGGTCCAGCCTGATGAGTGAAGAAGCAAAGCGAGGAACTCCCAACCCTTGGCTCTGTGAGGAGCCGGAGGAGACCA   560
  I  R  S  S  L  M  S  E  E  A  K  R  G  T  P  N  P  W  L  C  E  E  P  E  E  T     132
GAGGCTTGGGTTTCGATGAGATCCGGCAACAGCAGCAGAAAATTATTCAAGAACAGGACGCAGGTCTTGATGCCCTTTCC   640
R  G  L  G  F  D  E  I  R  Q  Q  Q  Q  K  I  I  Q  E  Q  D  A  G  L  D  A  L  S    159
TCTATCATAAGTCGCCAAAAGCAAATGGGCCAGGAGATTGGGAATGAACTGGACGAACAGAACGAGATCATCGATGACCT   720
 S  I  I  S  R  Q  K  Q  M  G  Q  E  I  G  N  E  L  D  E  Q  N  E  I  I  D  D  L   186
TGCCAACCTGGTGGAGAACACAGATGAGAAGCTTCGCACTGAAGCCAGGCGAGTGACCCTGGTGGACAGAAAGTCAGCTT   800
  A  N  L  V  E  N  T  D  E  K  L  R  T  E  A  R  R  V  T  L  V  D  R  K  S  A     212
CCTGTGGGATGATAATGGTGATCTTATTGCTGCTCGTGGCTATTGTGGTGGTTGCAGTGTGGCCAACCAACTGATGGCAA   880
S  C  G  M  I  M  V  I  L  L  L  L  V  A  I  V  V  V  A  V  W  P  T  N  *          236
TAAGGGGACCACCCGCAGTGACACAGCCAACAATGAGTGAAAGCCCAGCACCCTTTTGGTACACAACACCTCCTCTCAAT   960
AAATTCTCCACGCTCCCGGCAAAAAAAAAAAAAAAACTCGTGCCGAATTCGATATCAAGCTTATCGATACCGTCGACCTCG  1040
AGGGGG                                                                             1046
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B

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152 DAGLDALSSIISRQKQMGQEIGNELDEQNEIIDDLANLVENTDEKLRTEARRVTLVDRKS 211 rSyntaxin 8
170 LEQLELVSGSIGVLKNMSQRIGGELEEQAVMLDDFSHELESTQSRLDNVMKKLAKVSHMT 229 rSyntaxin 6
147 DENLEQVSGIIGNLRHMALDMGNEIDTQNRQIDRIMEKADSNKTRIDEANQRATKM-LGS 205 rSNAP-25a
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Fig. 1. (A) Nucleotide and derived amino acid sequences of rat syntaxin 8. The carboxyl-terminal membrane anchor sequence is boxed. The region around the third potential coiled-coil region is underlined. (B) Alignment of the amino acid sequences (corresponding regions) of syntaxin 8, syntaxin 6 and SNAP-25. Identical residues are shaded.

Differential extraction

The GEM fraction (25 μg) was extracted in a total volume of 200 μl with PBS, 1 M KCl, 2 M urea, 150 mM Na_2CO_3 (pH 11.5), 1% Nonidet P-40 and 1% sodium deoxycholate for 1 hour at 4°C. The suspension was centrifuged at 55,000 rpm in a TLA100 rotor (Beckman) for 60 minutes. Supernatants and pellets were resuspended in SDS sample buffer, electrophoresed and immunoblotted to detect syntaxin 8.

20S SNARE complex formation

Formation of the 20S SNARE complex was performed as described (Subramaniam et al., 1996). GEM fraction was extracted with assembly buffer (20 mM Hepes, pH 7.3, 100 mM KCl, 2 mM EDTA, 2 mM DTT, 0.5 mM ATP) containing 1% Triton X-100 for 45 minutes at 4°C. The extract was centrifuged for 45 minutes in a TLA100.2 rotor (100,000 ***g***). 15 μg of recombinant α-SNAP and 60 μg of recombinant NSF were incubated with 300 μg of membrane extract in the absence (assembly buffer) or presence (disassembly buffer) of 8 mM $MgCl_2$ in a final volume of 500 μl. The complex was allowed to form for 30 minutes on ice and then loaded onto a cushion of 15-40% glycerol in assembly or disassembly buffer. After centrifugation for 18 hours at 40,000 rpm in a SW41 rotor (Beckman), 0.8 ml fractions were collected from the bottom. Samples were electrophoresed on 12% SDS-PAGE and immunoblotted.

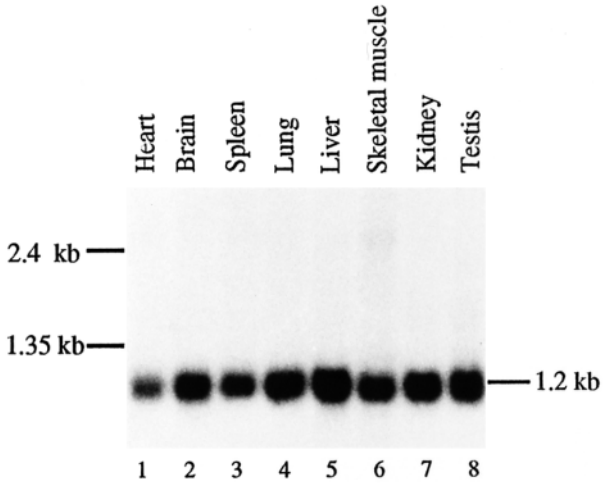

Fig. 2. Syntaxin 8 transcript is ubiquitously expressed. A transcript of 1.2 kb is detected in all rat tissues examined by northern blot analysis.

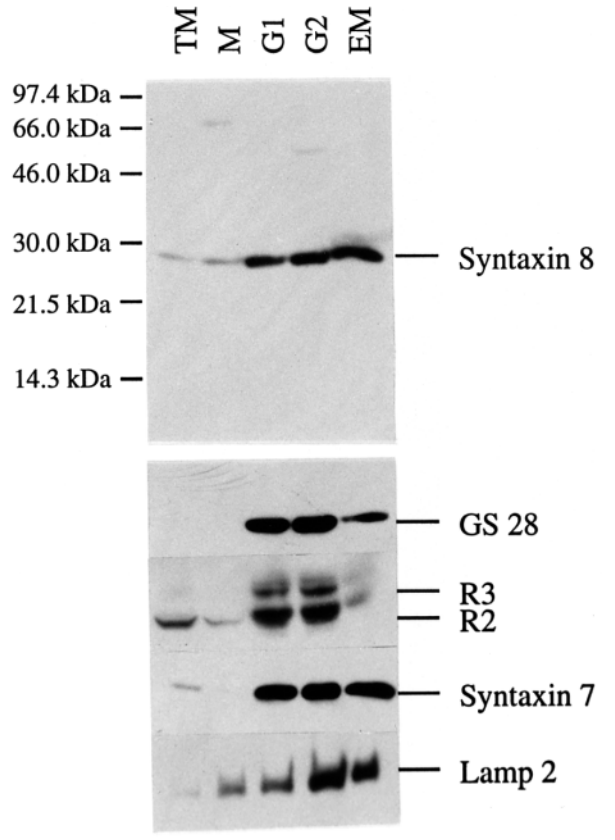

Fig. 3. Syntaxin 8 is a 27 kDa protein enriched in the endosomal fraction. 20 μg of total (TM), microsomal (M), Golgi (G1 and G2), and 5 μg of endosomal (EM) fractions were analyzed by immunoblot to detect syntaxin 8, GS28, asialoglycoprotein receptor subunits R2 and R3, syntaxin 7 and lamp-2.

Immunoprecipitation

20S complex fractions were diluted with assembly buffer containing 0.1% BSA and incubated with control antibodies (rabbit IgG) or affinity purified antibodies against syntaxin 8 bound to Protein A-Sepharose beads. Immunoprecipitates were washed five times with assembly buffer. Beads were divided into 2 equal portions and incubated with assembly or disassembly buffer (assembly buffer supplemented with 4 mM $MgCl_2$, 4 mM $CaCl_2$, and 25 μM GTP) for 60 minutes at RT. The beads and eluates were processed for immunoblotting to detect α-SNAP and NSF. For large-scale immunoprecipitation of syntaxin 8 protein complex, 100 μg of GEM membrane fraction was extracted for 1 hour at 4°C with buffer containing 1% Triton X-100, 20 mM Hepes, pH 7.3, 100 mM KCl, 2 mM EDTA and protease inhibitors. The extract was centrifuged at 150,000 ***g*** for 1 hour at 4°C and the supernatant was cleared for 2 hours with rabbit IgG agarose. The precleared extract was then divided equally and incubated with Protein A-Sepharose CL4B coupled with 200 μg of rabbit IgG or affinity-purified antibodies against syntaxin 8. After 18 hours at 4°C, the suspension was transferred to a disposable column (Bio-Rad) and washed with 50 ml of 20 mM Hepes, pH 7.3, 100 mM KCl, 2 mM EDTA, and 1% Triton X-100. Proteins were eluted with 500 μl of IgG elution buffer (Pierce Chemicals, Rockford, Illinois) three times. The eluates were neutralized with 50 μl of 1 M Tris-HCl, pH 9.0, pooled and precipitated with trichloroacetic acid (final concentration of 10%) for 1 hour at 4°C. The precipitate was collected by centrifugation for 30 minutes in a table-top centrifuge (Eppendorf), washed twice with ice-cold acetone and air-dried. Precipitates were separated on a 12% SDS-PAGE gel, stained with 0.1% Coomassie Blue R-250 in 40% methanol and 10% acetic acid, and destained with 20% methanol and 5% acetic acid, and rinsed with water. Protein bands specific to the immunoprecipitation with antibodies against syntaxin 8 were excised and digested in situ with trypsin. The resulting peptides were fractionated by HPLC and sequenced.

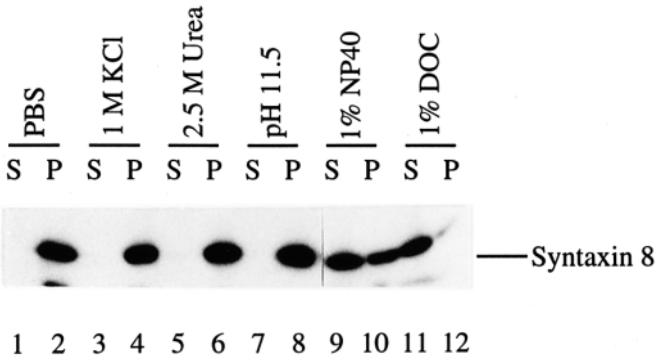

Fig. 4. Syntaxin 8 is an integral membrane protein. 25 μg of Golgi/endosomal-enriched membrane fractions (GEM) were extracted with PBS, 1 M KCl, 2.5 M urea, 0.15 M sodium carbonate pH 11.5, 1% NP-40, or 1% sodium deoxycholate (DOC). After centrifugation, the supernatant (S) and pellets (P) were analyzed by immunoblot to detect syntaxin 8.

RESULTS

Syntaxin 8, a novel member of the syntaxin family

Searching the expressed sequence tags (ESTs) database using the Blast program (Altschul et al., 1997) with the amino acid sequence of syntaxin 6 (Bock et al., 1996) revealed the existence of several EST clones (accession numbers AA000586, W41301, W63907 and W83363) that could potentially encode protein fragments homologous to syntaxin 6. A PCR fragment of the EST clone W833633 was used to screen a rat brain cDNA library, resulting in the isolation of full-length cDNA clones for this protein. While our work was in

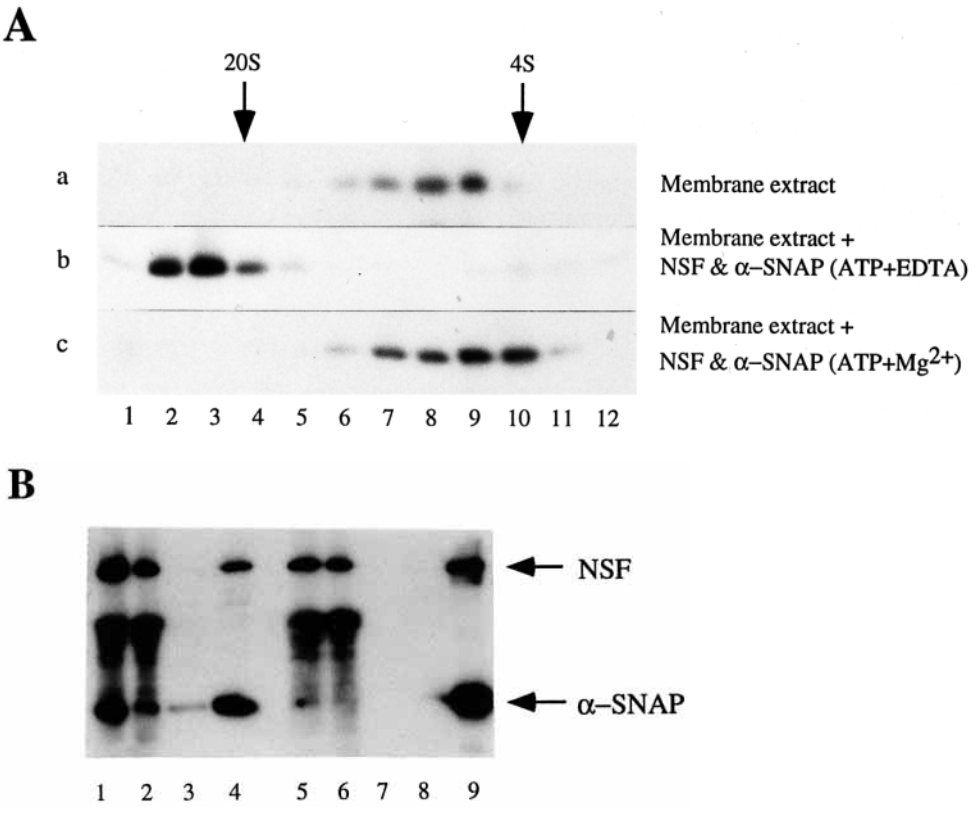

Fig. 5. Syntaxin 8 is a SNARE. (A) GEM Triton X-100 extracts were incubated without (a) or with (b and c) NSF and α-SNAP in assembly (a and b) or disassembly buffer (c) and then separated on a 15-40% glycerol gradient. Gradient fractions were analyzed for the distribution of syntaxin 8 by immunoblotting. (B) The syntaxin 8 containing 20S fractions were immunoprecipitated with antibodies against syntaxin 8 (lanes 1-4) or control antibodies (lanes 5-8) under assembly conditions. The immunoprecipitates were eluted with assembly buffer (lanes 1, 3, 5, and 7) or disassembly buffer (lanes 2, 4, 6, and, 8). The eluates (lanes 3, 4, 7, and 8) and beads (lanes 1, 2, 5 and 6) were resolved by SDS-PAGE along with 100 ng of recombinant NSF and α-SNAP (lane 9) and analyzed for the presence of α-SNAP and NSF by immunoblot analysis.

progress, Scheller and Bock presented a series of EST clones that could potentially encode novel syntaxins (Bock and Scheller, 1997). Since the EST clones revealed during our database search correspond to what they have termed syntaxin 8, we have retained the name syntaxin 8 for this protein. The nucleotide and the derived amino acid sequence of rat syntaxin 8 is shown in Fig. 1A. Syntaxin 8 is a protein of 236 residues with a calculated molecular mass of 26894 dalton. There exists an 18-residue hydrophobic region (boxed in Fig. 1A) at the C terminus that could function as a carboxyl-terminal membrane anchor, a characteristic of the majority of known SNAREs (Pfeffer, 1996; Rothman and Warren, 1994). Similar to other SNAREs, there exist three regions (residues 13-37, 44-68 and 166-204) that have the potential to form coiled-coil structures as predicted using the COILS program with a window of 21. The region around the third potential coiled-coil region (underlined in Fig. 1A) bears the highest amino acid identity with syntaxin 6 and to a lesser extent with SNAP-25. The amino acid sequences of the corresponding regions of syntaxin 8, syntaxin 6 and SNAP-25 are aligned and shown in Fig. 1B.

The transcript of syntaxin 8 is widely expressed

Syntaxin 8 could potentially participate in a common trafficking event that occurs in every cell or in a trafficking event that is unique to specific cells of certain tissues. To distinguish between these two possibilities, we have analyzed the mRNA levels of syntaxin 8 by northern blot (Fig. 2). A single transcript of about 1.2 kb was detected, at comparable levels, in all the tissues examined. The widespread expression of syntaxin 8 transcript is more consistent with the notion that syntaxin 8 is likely to be involved in a common transport event.

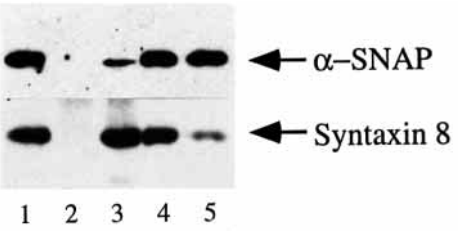

Fig. 6. Co-immunoprecipitation of α-SNAP with syntaxin 8 from total NRK cell lysate. NRK cell lysate was incubated with antibodies against syntaxin 8 (lanes 3 and 5) or control rabbit IgG (lanes 2 and 4). The immunoprecipitates (lanes 2-3) and 10% of the supernatants (lanes 4-5), along with 10% of starting lysates (lane 1) were resolved by SDS-PAGE, transferred to a filter, and probed with antibodies against syntaxin 8 and α-SNAP.

Syntaxin 8 is an integral membrane protein enriched in the endosomal fraction

The predicted cytoplasmic domain (residues 1-210) of syntaxin 8 was expressed in bacteria as a His×6-tagged protein and purified His×6-syntaxin 8 was used to immunize rabbits. Antibodies specific for syntaxin 8 were affinity-purified. The total membrane (TM) derived from rat liver was floated onto step sucrose gradient, resulting in the isolation of four membrane fractions (M fraction at the 1.25/1.1 M sucrose interface, G1 fraction at the 1.1/1.0 M sucrose interface, G2 fraction at the 1.0/0.8 M sucrose interface, and EM fraction at the 0.8/0.25 M sucrose interface). 20 μg of TM, M, G1, and G2 as well as 5 μg of EM fractions were analysed by immunoblot to detect syntaxin 8, GS28 (Subramaniam et al.,

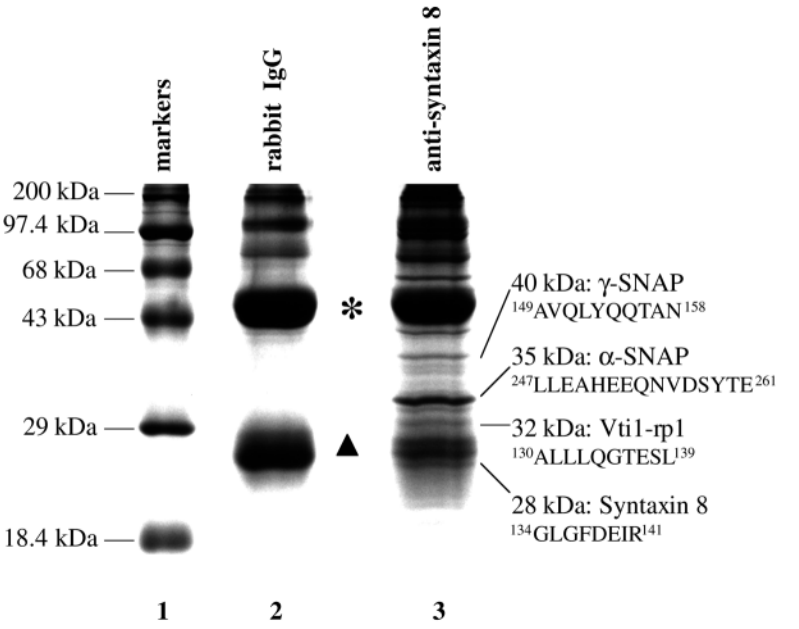

Fig. 7. Co-immunoprecipitation of Vti1-rp1 with syntaxin 8. Detergent extract of Golgi/endosome-enriched membranes were incubated with beads containing control rabbit IgG (lane 2) or affinity-purified antibodies against syntaxin 8 (lane 3). The immunoprecipitates were resolved by SDS-PAGE and specific proteins with sizes of 40 kDa or less were analysed by amino acid microsequencing. The identities and the amino acid sequences of tryptic peptides are indicated.

1995, 1996), cell surface asialoglycoprotein receptor subunits R2 and R3 (Spiess and Lodish, 1985), endosomal syntaxin 7 (Wang et al., 1997; Wong et al., 1998) and lysosomal integral membrane protein Lamp2/lgp96/lgpB/LIMP IV/lgp110 (Sandoval and Bakke, 1994). As shown in Fig. 3, GS28 is enriched in G1 and G2 fractions and to a lesser extent in the EM fraction. Asialoglycoprotein receptor subunits R2 and R3 are similarly enriched in the G1 and G2 fractions. Although the endosomal syntaxin 7 is also enriched in G1 and G2, it is about four time more enriched in the EM fraction as compared to the G1 and G2 fractions as four times less proteins of the EM fraction was analysed. A 27 kDa protein was detected with syntaxin 8 antibodies and detection of this protein was abolished by pre-incubation of antibodies with recombinant syntaxin 8 but not with other recombinant proteins (such as syntaxin 7; data not shown). As seen, syntaxin 8 is also about four times more enriched in the EM fraction than the G1 and G2 fractions. The lysosomal Lamp2 is more enriched in the G2 and EM fractions. These results indicate that syntaxin 8 is enriched in fractions that are also enriched for markers of the endosomal pathway.

As predicted from the derived amino acid sequence, syntaxin 8 is most likely an integral membrane protein. When a membrane fraction enriched in the Golgi, endosome and lysosome (GEM fraction in the 1.1/0.5 M sucrose interface) was extracted with various reagents, it was found that syntaxin 8 is not extracted with PBS, 1 M KCl, 2.5 M urea, or 0.15 M sodium bicarbonate (pH 11.5), but was effectively by detergents such as 1% NP-40 or 1% DOC (Fig. 4). These results establish that syntaxin 8 is indeed an integral membrane protein.

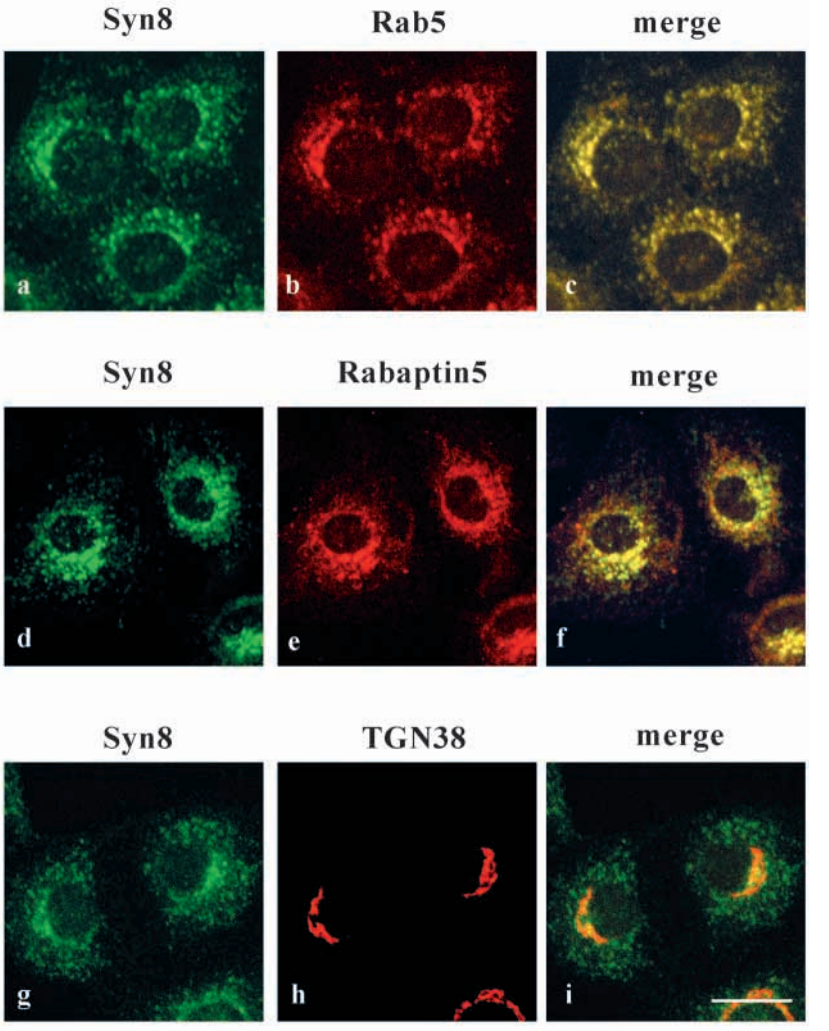

Fig. 8. Colocalization of syntaxin 8 with markers of the early endosome. NRK cells were proceesed for double-labeling using antibodies against syntaxin 8 and Rab 5 (panels a-c), syntaxin 8 and rabaptin 5 (d-f), syntaxin 8 and TGN38 (g-i). Bar, 10 μm.

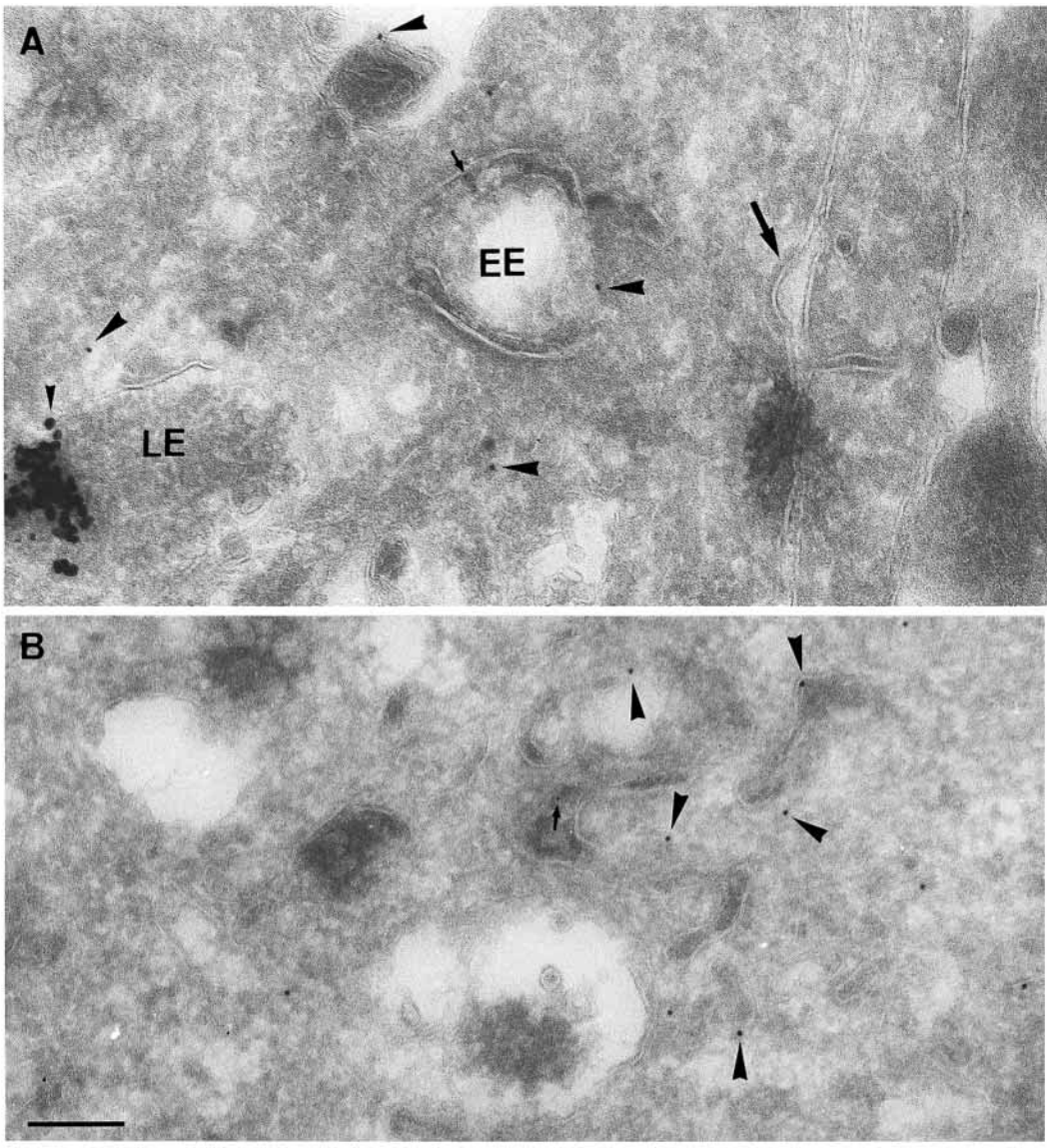

Fig. 9. Cryosections from NRK cells with 15 nm gold-BSA (small arrowheads) internalized into late endocytic structures and 5 nm gold-BSA (small arrows) internalized into the early endosomes were labeled with antibodies against syntaxin 8 followed by 10 nm gold-Protein A (large arrowheads). (A) One gold particle for syntaxin 8 in an early endosome, the remaining syntaxin 8 labelling in A is probably in late endosomes. (B) Syntaxin 8 labelling over extended regions of early endosomes. The cell surface is indicated by the large arrow. Bar, 200 nm.

Syntaxin 8 behaves as a SNARE

The common property of known SNAREs is that they can be incorporated into SNARE complexes with a sedimentation coefficient of 20S in the presence of NSF and α-SNAP under conditions that prevent ATP hydrolysis by NSF (Söllner et al., 1993; Subramaniam et al., 1996). To provide evidence that syntaxin 8 is indeed a SNARE, we have investigated whether syntaxin 8 also possesses this property (Fig. 5A). When the GEM membrane extract was resolved with a glycerol gradient, syntaxin 8 was found to have a sedimentation coefficient of 7-8 S (upper row). In the presence of NSF and α-SNAP, under conditions that prevent ATP hydrolysis by NSF, syntaxin 8 was shifted to positions of about 20 S (middle row). However, in conditions that promote ATP hydrolysis of NSF, NSF and α-SNAP shifted syntaxin 8 to positions of about 5-6 S (lower row). Since NSF and α-SNAP are known to disrupt SNARE complexes (Subramaniam et al., 1997), the shifting of syntaxin 8 from 7-8 S to 5-6 S by NSF and α-SNAP in conditions that faciliate ATP hydrolysis suggest that syntaxin 8 in the membrane extract exists in a protein complex that is disassembled by NSF and α-SNAP. These effects of NSF and α-SNAP on syntaxin 8 are characteristic of known SNAREs and suggest that syntaxin 8 is indeed a SNARE.

To provide additional evidence for the notion that syntaxin 8 is a SNARE, the 20 S fractions were immunoprecipitated with antibodies against syntaxin 8 (lanes 1-4, Fig. 5B) or control rabbit IgG (lanes 5-8). The immunoprecipitates were eluted either in assembly buffer (lanes 1, 3, 5, 7) or disassembly buffer (lanes 2, 4, 6, 8). The beads (lanes 1, 2, 5, 6) and the eluates (lanes 3, 4, 7, 8), together with 100 ng of recombinant NSF and 100 ng of recombinant α-SNAP (lane 9) were resolved by SDS-PAGE and processed for immunoblotting to detect NSF and α-SNAP. Some non-specific association of NSF but not α-SNAP with control rabbit IgG was observed (lanes 5-6) and the associated NSF was not released from the control beads either in assembly (lane 7) or disassembly buffer (lane 8). Both NSF and α-SNAP were seen to be co-immunoprecipitated by antibodies against syntaxin 8 (lanes 1-2). Significantly, both NSF and α-SNAP could be released

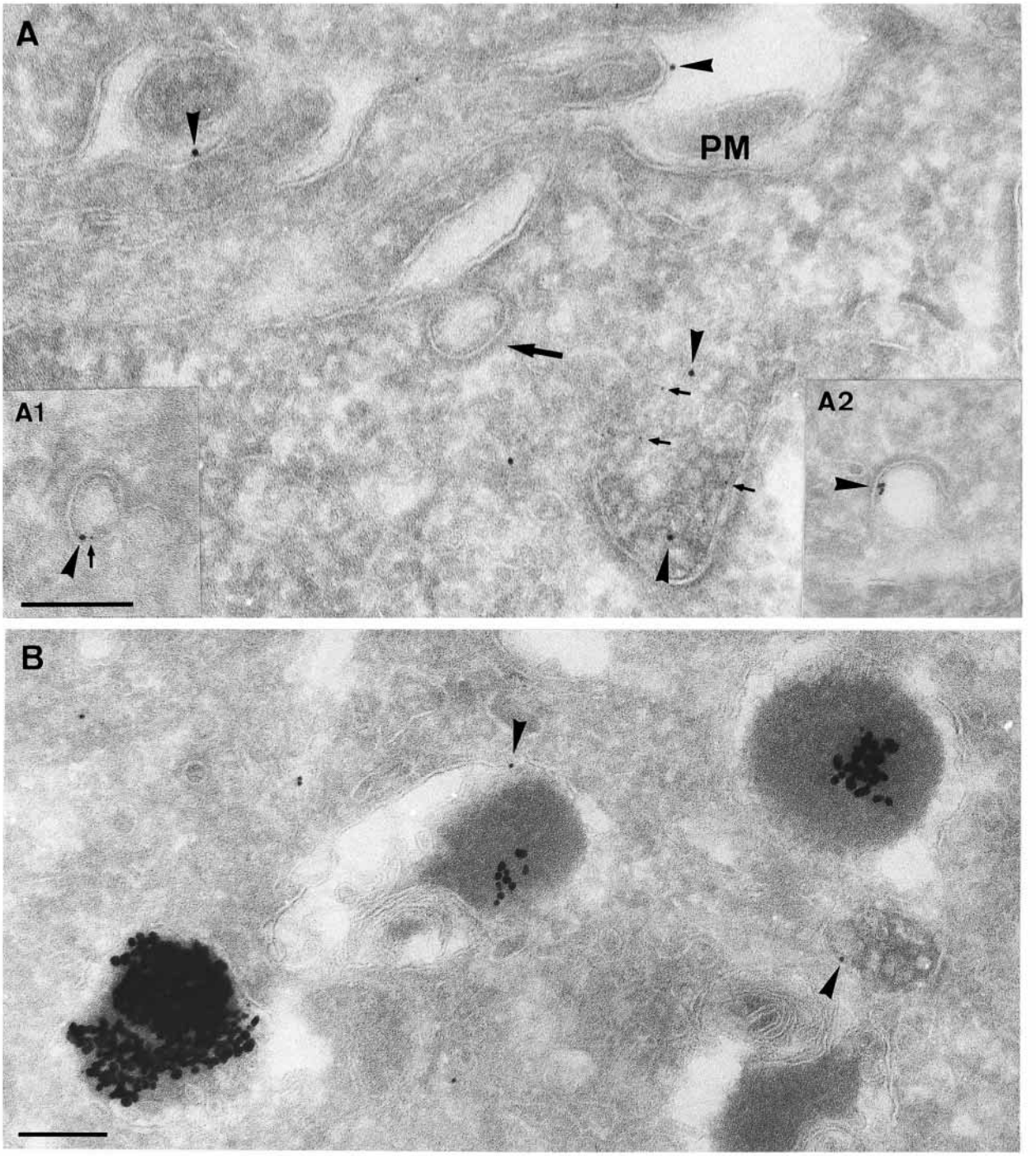

Fig. 10. Cryosections from NRK cells with 15 nm gold-BSA internalized into late endocytic structures and 5 nm gold-BSA (small arrows) internalized into the early endosomes were labeled with antibodies against syntaxin 8 followed by 10 nm gold-Protein A (large arrowheads). (A) Syntaxin 8 labeling on the plasma membrane (PM) and in an early endosome. A1 and A2 show two examples of syntaxin 8 labeling over coated pits. (B) Labeling over late endocytic structures. Bar, 200 nm.

from the immunoprecipitate in disassembly buffer (lane 4) but not in assembly buffer (lane 3). These results suggest that syntaxin 8 does exist in a SNARE complex with NSF and α-SNAP in the 20S fraction and this SNARE complex can be dissociated in conditions that promote ATP hydrolysis by NSF, thus dissociating the SNARE complexes.

Further evidence for syntaxin 8 as a SNARE came from our observation that a significant fraction of α-SNAP could be co-immunoprecipitated with syntaxin 8 from total NRK cell lysate (Fig. 6). Total NRK cell lysate was immunoprecipitated with control rabbit IgG (lanes 2, 4) and antibodies against syntaxin 8 (lanes 3, 5). The beads (lanes 2-3) and 10% of the supernatants (lanes 4-5), together with 10% of starting lysate (lane 1) were analysed by immunoblot to detect syntaxin 8 and α-SNAP. Syntaxin 8 is efficiently immunoprecipitated by its antibodies (lanes 3, 5) but not by control rabbit IgG (lanes 2,

4). Furthermore, a significant amount of α-SNAP was specifically co-immunoprecipitated by antibodies against syntaxin 8 (lane 3) but not by control IgG (lane 2). Given the fact that α-SNAP is involved essentially in all the transport events and is associated with all the SNARE complexes in the cells, the clear co-immunoprecipitation of a significant amount of α-SNAP with syntaxin 8 from total cell lysate further supports that syntaxin 8 does function as a SNARE.

Co-immunoprecipitation of Vti1-rp1 with syntaxin 8

To investigate which SNAREs could potentially interact with syntaxin 8, we have performed a large-scale immunoprecipitation of detergent extract derived from rat liver Golgi/endosome-enriched membranes (Fig. 7). As shown, control rabbit IgG did not immunoprecipitate any specific proteins (lane 2), while several distinct polypeptides were specifically immunoprecipitated with antibodies against syntaxin 8 (lane 3). Since SNAREs typically have sizes below 40 kDa and the region below 40 kDa was resolved better than higher molecular mass region (lane 3), polypeptides with sizes of 40 kDa or less were individually excised out from the gel and subjected to trypsin digestion. Tryptic peptides were resolved by HPLC and purified peptides were subjected to protein microsequencing. It was revealed that the 40, 35, 32 and 28 kDa proteins represent γ-SNAP, α-SNAP, Vit1-rp1, and syntaxin 8, respectively. The co-immunoprecipitation of γ-SNAP, α-SNAP with syntaxin 8 in this experiment further supports the notion that syntaxin 8 is a SNARE. Vti1-rp1 is one of the two mammalian homologues (Xu et al., 1998) of yeast Vti1p, which functions in both retrograde intra-Golgi transport as well as several events in the endosomal pathway (von Mollard et al., 1999). The other mammalian homologue of Vti1p (Vit1-rp2) is associated with the Golgi apparatus and is apparently important for protein transport along the secretory pathway (Xu et al., 1998). Although detailed studies of Vit1-rp1 have not been reported, it was speculated that it may participate in the endosomal pathway (Xu et al., 1998). Consistent with this view, Our preliminary studies suggest that Vti1-rp1 is associated both with the Golgi apparatus (particularly the TGN) as well as endosomal compartments (Y. Xu et al., unpublished observations).

Immunofluorescence microscopy showing colocalization of syntaxin 8 with early endosomal markers Rab5 and Rabaptin5

Using the affinity-purified antibodies against syntaxin 8, we investigated its subcellular localization by indirect immunofluorescence microscopy. Double-labeling of syntaxin 8 with several markers of the secretory and endocytotic pathways showed that syntaxin 8 (Fig. 8, a and d) colocalized well with Rab5 (b) and Rabaptin5 (e), two markers of the early endosome (Stenmark et al., 1995; Simonsen et al., 1998; Christoforidis et al., 1999). However, syntaxin 8 (g) did not colocalize with TGN38 (h), a marker for the Golgi apparatus and the trans-Golgi network (Luzio et al., 1990).

Immunogold labeling showing preferential localization of syntaxin 8 in the early endosome

The early and late endosomes of NRK cells were marked by internalized 5 nm and 15 nm gold-conjugated BSA,

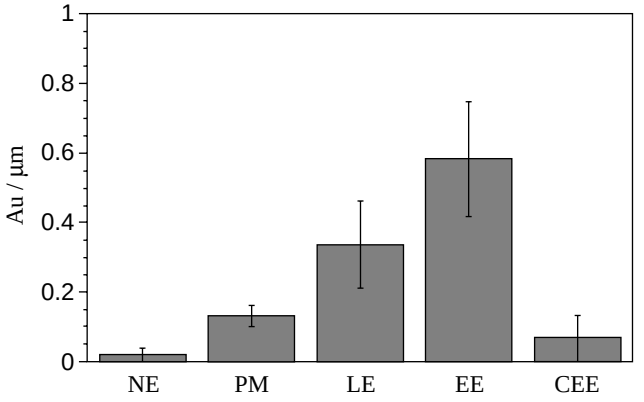

Fig. 11. Quantification of immunogold labeling of syntaxin 8 over nuclear envelope (NE), plasma membrane (PM), late endosomes (LE), early endosomes (EE). Labeling in early endosome in the absence of syntaxin 8 antibodies was used as negative control (CEE). Au/μm = number of gold particle/μm.

respectively. Cryosections of these cells were processed for immunogold labeling for syntaxin 8 and then examined by electron microscopy as described (Griffiths, 1993; Liou et al., 1996). Syntaxin 8 labeling was found in both the multivesicular (Fig. 9A and Fig. 10A, large arrowheads) as well as vesicular-tubular structures (Fig. 9B) of the early endosome (EE, small arrows). To a lesser extent, syntaxin 8 labeling was also found in the late endosome (Fig. 10B), the cell surface and the coated pits (Fig. 10A). The immunogold labeling of syntaxin 8 was quantified (Fig. 11) and the highest density of syntaxin 8 labeling was found in the early endosome. Significant densities were found also in the late endosome and the plasma membrane. These results suggest that syntaxin 8 is preferentially enriched in the early endosome with some also present in structures preceding it (the plasma membrane and the coated pits) and its downstream structure (the late endosome).

Colocalization of syntaxin 8 and Rab5 in the early endosome as revealed by double immunogold labeling

To gain additional support for the notion that syntaxin 8 is enriched in the early endosome, double immuno-gold labeling of syntaxin 8 and Rab5 was performed on sections derived from rapidly-frozen PC12 cells that have been processed for freeze-substitution and embeding in Lowicry HM-20 (Neuhaus et al., 1998). Ultra-thin sections were double-labeled with syntaxin 8 and Rab5 antibodies. The majority of syntaxin 8 labeling (large arrowheads) is found in the endosomal structures marked by Rab5 labeling (small arrowheads; Fig. 12). These results, taken together, establish that syntaxin 8 is indeed enriched in the early endosome.

DISCUSSION

We have cloned full-length rat cDNAs encoding a novel member of the syntaxin family. It is a member of the syntaxin family based on the homology of its amino acid sequence with known members of the family, the presence of regions with the

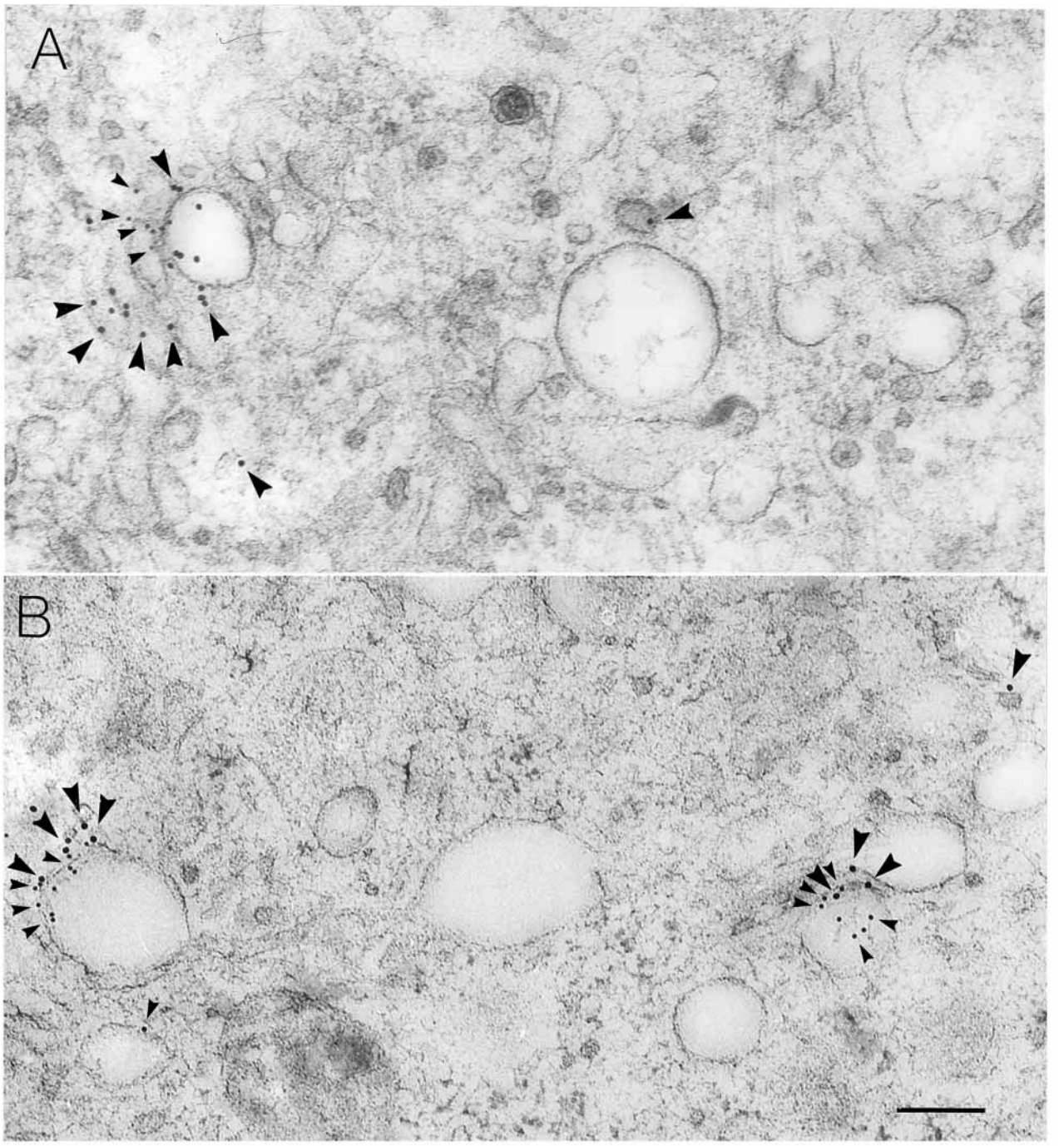

Fig. 12. Double immunogold labeling of syntaxin 8 and Rab5 in the early endosome. Ultra-thin sections derived from rapidly-frozen PC12 cells were double-labeled with syntaxin 8 (followed by 14 nm gold-Protein A) and Rab5 antibodies (followed by 9 nm gold Protein A). The majority of syntaxin 8 labeling (large arrowheads) is found in the endosomal strucutres marked by Rab5 labeling (small arrowheads). Bar, 200 μm.

potential to form coiled-coil domains, and the existence of a carboxyl-terminal hydrophobic tail anchor. Its identity as a member of the syntaxin family is further supported by our demonstration that it behaves like a SNARE because it could be incorporated into a 20S SNARE complex by NSF and α-SNAP in conditions that promote formation of SNARE complexes and its complex in membrane extract is disrupted by NSF and α-SNAP under conditions that facilitate disassembly of SNARE complexes. The co-immunoprecipitation of a significant amount of α-SNAP by antibodies against syntaxin 8 from total NRK cell lysates further supports our findings.

The endosomal pathway is generally believed to be composed of the early endosome, the late endosome and the lysosome, with the early endosome possibly containing distinct subcompartments (sorting endosome and recycling endosome; Gruenberg and Maxfield, 1995; Mellman, 1996). Although it was proposed that trafficking through the endocytic pathway is

achieved through compartment maturation, recent studies favor the idea that early endosome and late endosome are distinct compartments. Endocytotic vesicles derived from the plasma membrane will fuse selectively with the early endosome and protein movement from the early to the late endosome is mediated by transport intermediates referred to as endosomal carrier vesicle (ECV) or multivesicular bodies (MVB). The late endosome and the lysosome are believed to be in a dynamic equilibrium and fuse to form hybrid organelles, which will regenerate to form the late endosome and the lysosome by fission (Bright et al., 1997). Several lines of evidence establish that syntaxin 8 is preferentially associated with the early endosome. First, immunoblot analysis of subcellular membrane fractions derived from rat liver showed that syntaxin 8 is present at highest levels in membrane fractions enriched for endosomal syntaxin 7 (Wang et al., 1997; Wong et al., 1998). Secondly, double-labeling of syntaxin 8 with several markers of the secretory and endocytotic pathways by indirect immunofluorescence microscopy revealed that syntaxin 8 colocalizes well with Rab5 and Rabaptin5, two well established proteins of the early endosomes (Stenmark et al., 1995; Simonsen et al., 1998; Christoforidis et al., 1999). Thirdly, immunogold labelling at the electron microscopy level revealed that highest levels of syntaxin 8 are present in the early endosome. To lesser extents, syntaxin 8 is also present in late endosome, the plasma membrane and coated pits. This observation is further supported by colocalization of syntaxin 8 in early endosomal structures marked by Rab5 by immunogold electron microscopy.

Currently, two other syntaxins are known to exist in the endosomal pathway. Although syntaxin 7 is present in early endosome in A431 cells (Wong et al., 1998), it is also present in late compartments of the endosomal pathway in NRK cells (Wong et al., unpublished results). Syntaxin 12/13 is another endosomal syntaxin (Advani et al., 1998; Tang et al., 1998d) and a recent study suggests that it may participate in recycling of plasma membrane proteins via tubulovesicular recycling endosome (Prekeris et al., 1998). The establishment of syntaxin 8 as an additional syntaxin associated with the endosomal pathway suggests that there exist many different docking/fusion events in the endosomal pathway and these three syntaxins may differentially participate in various docking and fusion events. Future studies aiming to establish the interacting partners for these endosomal syntaxins and to explore the various functional aspects in the endosomal pathway will enhance our understanding of the molecular mechanisms of membrane traffic in the endosomal pathway.

During the preparation of this manuscript, Steegmaier et al. (1998) and Thoreau et al. (1999) have also reported the cloning of human syntaxin 8. Based on the localization studies using epitope-tagged syntaxin 8 transiently expressed in transfected cells, Steegmaier et al. (1998) have suggested that syntaxin 8 is localized to the ER. Interestingly, syntaxin 8 was identified by Thoreau et al. using the regulator domain of the cystic fibrosis transmembrane conductance regulator in the yeast two-hybrid system (Thoreau et al., 1999). In our present study, we have investigated the subcellular localization of endogenous syntaxin 8 using both immunofluorescence microscopy as well as immunogold labeling. Our results suggest that syntaxin 8 is preferentially localized to the early endosome. Significantly, we have revealed that synstaxin 8 exists in a SNARE complex with Vti1-rp1. Vti1-rp1 and Vti1-rp2 are two homologous proteins (Advani et al., 1998; Lupashin et al., 1998; von Mollard and Stevens, 1998; Xu et al., 1998) related to yeast Vti1p, which is essential for intra-Golgi retrograde transport (von Mollard et al., 1997). In addition, Vti1p participate in several events in the endocytotic pathway in yeast, including transport from the trans-Golgi to the endosome, direct transport from the trans-Golgi to the vacuole, and other transport events linked to the vacuole (von Mollard and Stevens, 1999). Vti1-rp2 is associated with the Golgi apparatus and participates in the biosynthetic pathway (Xu et al., 1998), while Vti1-rp1 is mainly associated with the TGN and the endosome (Y. Xu et al., unpublished observations). The coexistence of syntaxin 8 and Vti1-rp1 in the same SNARE complex indicates that TGN-derived vesicles containing Vti1-rp1 may dock and fuse with syntaxin 8-containing endosomal compartment through their interaction. Since the recycling compartment of the early endosome is the major endosomal site linked to the TGN, syntaxin 8 may have a function in the recycling early endosome. More investigations are needed to address this possibility further.

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Note added in proof

A recent study (Prekeris et al., 1999) also suggests that syntaxin 8 functions in the endocytotic pathway.

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