Intracellular membrane traffic is thought to be regulated in part by soluble N-ethylmaleimide-sensitive factor-attachment protein receptors (SNAREs) through the formation of complexes between these proteins present on vesicle and target membranes. All known SNARE-mediated fusion events involve members of the syntaxin and vesicle-associated membrane protein families. The diversity of mammalian membrane compartments predicts the existence of a large number of different syntaxin and vesicle-associated membrane protein genes. To further investigate the spectrum of SNAREs and their roles in membrane trafficking we characterized three novel members of the syntaxin and SNAP-25 (synaptosome-associated protein of 25 kDa) subfamilies. The proteins are broadly expressed, suggesting a general role in vesicle trafficking, and localize to distinct membrane compartments. Syntaxin 8 co-localizes with markers of the endoplasmic reticulum. Syntaxin 17, a divergent member of the syntaxin family, partially overlaps with endoplasmic reticulum markers, and SNAP-29 is broadly localized on multiple membranes. SNAP-29 does not contain a predicted membrane anchor characteristic of other SNAREs. In vitro studies established that SNAP-29 is capable of binding to a broad range of syntaxins.

The membrane compartmentalization of cells results in the segregation of biochemical activities through the selective transport and retention of molecules into specific organelles. As part of this process, exo- and endocytosis regulate the availability of secreted chemical messengers and their receptors. The organization and dynamics of the secretory pathway are generated through a series of vesicle budding events from donor compartments and membrane fusion events with acceptor compartments (1). The precise molecular mechanisms responsible for the selective capture of specific cargo into vesicles, the fidelity of vesicle target selection, and the mechanisms of membrane fusion are not fully understood. Contributions to understanding these processes have come from three approaches: yeast genetics, in vitro biochemical reconstitution of intracellular membrane trafficking, and the study of synaptic vesicle proteins and their interactions. These seemingly independent lines of investigation converged, leading to the realization that a common set of molecules appear to mediate membrane trafficking in all eucaryotic cells (2–4).

At the core of the membrane fusion process, six molecules appear to be particularly important. In the nerve terminal, the vesicle and plasma membrane are thought to be brought into close apposition through the formation of a four helix bundle structure (5). A vesicle protein VAMP, contributes one helix, while the plasma membrane proteins SNAP-25 and syntaxin contribute three helices to form a parallel bundle alternatively referred to as the core complex, the fusion complex, or the SNARE complex (6). The structure of this complex and the subcellular localization of the components suggest that its formation may drive the membrane fusion process itself, although this issue remains controversial (7). The core complex is extremely stable and its dissociation is mediated by NSF, an ATPase chaperone, that requires aSNAP to bind the core complex (8). Dissociation of this complex allows the components to recycle for another round of membrane fusion. Since the complex of VAMP, syntaxin, and SNAP-25 binds to aSNAP, these proteins have been referred to as aSNARE receptors or SNAREs. The sixth protein critical for membrane fusion in the nerve terminal is nsec1 (munc-18), a member of the sec1 family (9–11). This protein binds syntaxin to form a complex distinct from the core complex, although the role of nsec1 in membrane fusion is not clearly understood.

The binding of SNAREs to each other from opposite membranes may underlie at least part of the specificity of vesicle trafficking; however, this idea has not yet been experimentally established. A prerequisite for SNARE proteins to impart specificity to the fusion process is that each membrane compartment should have a complement of SNARE proteins sufficient to ensure pairing with appropriate partners. Recently, several SNARE proteins have been characterized based on interactions with known SNAREs or due to sequence homology to known SNARE proteins (12–14). Indeed, it appears that many membrane compartments have a specific and/or unique complement of SNAREs. For example, rsec22b, rbet1, memrin, and syntaxin 5 are localized to the endoplasmic reticulum (ER), intermediate compartment, and the Golgi, where they mediate trafficking at this stage of the secretory pathway (12, 15). In contrast, syntaxin 6 is found in the trans-Golgi network and endosomes while syntaxin 13 localizes to early and recycling endosomes (16, 17). Many other SNARE proteins also appear to distribute uniquely within the secretory pathway even if their precise subcellular localization is not yet known.

A complete understanding of the secretory pathway will require a full definition of the complement of SNARE proteins in mammalian cells. Sequence analysis of the growing family of SNAREs has already provided important insights. In particu...
lar, while all SNARE proteins have been proposed to be members of a large gene family, several subfamilies exist based on higher levels of homology. While many members of the VAMP and syntaxin families have been identified, only two closely related members of the SNAP-25 family (SNAP-25 and SNAP-23) have been characterized (18, 19). In this report, we present a new member of the SNAP-25 subfamily we refer to as SNAP-29. In addition, we characterize two new members of the syntaxin family, syntaxin 8 and syntaxin 17. These proteins are broadly expressed suggesting general roles in membrane trafficking and, like the other SNAREs, appear to localize to specific membrane compartments.

**Experimental Procedures**

**Isolation of SNAREs**

**Syntaxin 8**—In our earlier search of the GenBank<sup>TM</sup> EST data base (20), we obtained a human epithelial cell cDNA clone (accession number AA654455). Sequencing of this clone and comparison to another human cDNA clone (accession number AF062077) yielded an open reading frame (ORF) of 7.1 base pairs and we designated syntaxin 8. The clone AA654455 encodes for all but the first 8 amino acids of human syntaxin 8. AA062077 encodes for the complete ORF of human syntaxin 8 plus 150 base pairs of 5′-untranslated region.

**Syntaxin 17**—The complete coding region of human syntaxin 3 (21) was amplified in the polymerase chain reaction (PCR) and inserted in frame with the GAL4 DNA binding domain in pAS2K, a vector derived from pAS1 (22), to create pAS/syn3. Yeast strain Y190 (CLONTECH) was sequentially transformed with first pAS/syn3 and grown up in SD–Trp medium (CLONTECH) to generate Y190/syn3 strain. A combined human lymphocyte/HeLa cell cDNA library was transferred into the Y190/syn3 strain. Eight million transformants were plated on SD–Leu–Trp4–His1–40 α-methyl-1,2,4-triazole plates and incubated at 30 °C for 8 days. 287 blue colonies were selected for further analysis. In addition to αSNAP, SNAP-25A, SNAP-25B, membrin, munc-18B, and Ykt6, partial coding regions of two novel human proteins, with homology to different syntaxins and to SNAP-25, respectively, were identified.

Multiple clones contained the partial sequence of human syntaxin 17. Custom-designed oligonucleotides were used to amplify a 390-base pair fragment using the human clone as a template for the PCR. The PCR product was then <sup>32</sup>P-labeled by a random hexamer priming reaction and used to screen 6 × 10<sup>9</sup> plaques from a rat brain 5′-Stretch PLUS cDNA library (CLONTECH) at 61 °C. Library screening yielded one positive, overlapping clones. λ DNA was obtained by standard methods, and the EcoRI fragments were subcloned into pBluescript KS vector (Stratagene Inc.), and both strands were sequenced using Sequenase (Amersham Pharmacia Biotech) and custom oligonucleotide primers. The largest clone (1.7 kb) contained an 906-base pair ORF which was designated syntaxin 17.

**SNAP-29**—Using syntaxin 3 as a bait in the yeast-two-hybrid system (see above), multiple clones were isolated, which contained partial coding region of SNAP-29. Custom-designed oligonucleotides were designed and used to amplify a 390-base pair fragment using the human clone as a template for the PCR. The PCR product was then <sup>32</sup>P-labeled by a random hexamer priming reaction and used to screen 6 × 10<sup>5</sup> plaques from a rat brain 5′-Stretch PLUS cDNA library (CLONTECH) at 61 °C. Library screening yielded seven positive, overlapping clones. λ DNA was obtained by standard methods, and the EcoRI fragments were subcloned into pBluescript KS vector (Stratagene Inc.), and both strands were sequenced using Sequenase (Amersham Pharmacia Biotech) and custom oligonucleotide primers. The largest clone (1.1 kb) contained an 777-base pair open reading frame, which was designated SNAP-29.

**Bioinformatics**

DNA and protein sequences were analyzed with the Genetics Computer Group software package (23). The basic local alignment search tool (BLAST) algorithm was used to search the GenBank<sup>TM</sup> data base (24). Multiple sequence alignments were performed using the PILEUP program (using default parameters (gap creation penalty = 12 and gap extension penalty = 4), and pairwise sequence comparisons were performed with the BESTFIT program using identical parameters. Statistical significance of the homology of two proteins was also determined with this program by generating z scores, z scores were calculated from the quality scores provided by the BESTFIT program: z = (quality score of alignment in question – mean quality score of 100 alignments using randomized sequences)/S.D. of 100 quality scores from randomized sequences. The phylogenetic tree of the syntaxin/SNAP-25 gene family was generated by first aligning all family members using the PILEUP program (gap creation penalty = 1.5 and gap extension penalty = 1). A table of the pairwise distances was then used by the DISTANCES program (Kimura method), and the GROWTREE program (UPGMA method) was used to reconstruct the actual tree from the distance matrix. Coiled-coil motifs were analyzed with the Coils program, version 2.1, using both the MITKD and the MTK matrix (25).

**RNA Blot Hybridizations**

Human and rat multiple tissue Northern blots were purchased from CLONTECH. Human blots were used for syntaxin 8 and SNAP-29, and a rat blot was used for syntaxin 17. A random-primed <sup>32</sup>P probe was generated using full-length coding region for each gene. Hybridization and washing were performed as described in the manufacturer’s protocol.

**Expression Constructs and Transfections**

The complete coding regions of syntaxin 8, syntaxin 17, and SNAP-29 were amplified by PCR using custom-designed oligonucleotide primers with appropriate restriction sites. The PCR products were subcloned into a derivative of the pcDNA3 mammalian expression vector (Invitrogen Corp.) that contained an oligonucleotide encoding the myc epitope so that the epitope was at the amino-terminal end of the protein.

NRK and COS cells (passage 3–20) were maintained using standard techniques. For transfections with the expression constructs, one barely confluent 10-cm dish of NRK cells (1 × 10<sup>6</sup> cells) was harvested with trypsin-EDTA, washed once with phosphate-buffered saline, resuspended in 600 μl of phosphate-buffered saline, and incubated for 10 min on ice with 10 μg of plasmid DNA in a 0.4-cm gap electroporation cuvette (Bio-Rad). The cells were then electroporated using 960 microfarads and 0.25 kV in a Bio-Rad Gene Pulser with capacitance extender, seeded in appropriate density into each well of an eight-chamber microscope slide (Nunc), and fixed for microscopy 24 h after transfection.

**COS cells were transiently transfected using the LipofectAMINE Plus system (Life Technologies Inc.). For extraction studies, transfected cells were harvested 48 h after transfection.**

**Immunofluorescence Microscopy**

Indirect immunofluorescence localization was performed on NRK and COS cells as described previously (26).

**Antibodies**

Monoclonal anti-myc (9E10) and rabbit anti-myc polyconal IgG were purchased from Santa Cruz Biotechnology and utilized for immunofluorescence at a dilution of 1:1000 and 1:500, respectively. For immunoblotting, the 9E10 monoclonal antibody was used at a 1:2000 dilution. For immunofluorescence, affinity-purified anti-syntaxin 5 rabbit antibody was used at a 1:300 dilution (26), mouse anti-transferrin receptor at a dilution of 1:1000 (Zymed Laboratories Inc.), polyclonal rabbit anti-BIP/GRP78 antibody at a dilution of 1:1000 (Affinity Bioreagents, Inc.), and anti-calcxin polyclonal antiserum (27) at a dilution of 1:1500. Monoclonal antibodies against NSF were obtained from the corresponding hybridoma cell lines generated by fusion of NS-1 mouse myeloma cells with spleen cells from BALB/c mice immunized with His-tagged bovine NSF fusion protein (28). Ascites fluid was produced by Josman Laboratories (Napa Valley, CA). For immunofluorescence, ascites fluid of the anti-NSF hybridoma cell line 6A11–3 was used at a dilution of 1:1000. For Western blotting, ascites fluid of the anti-NSF hybridoma cell line 9E10 was used at a dilution of 1:2000.

**In Vitro Transcription and Translation**

Using the forward primers T7-sSNAP-29 (5′-TATAAGACCTACACACCCGAAAAACTGGGATGATACTCATCAGAAAG-3′) and T7-mSNAP-29 (5′-TATAAGACCTACTATATAGGAGACACAGCCGATTGGTAGCCGAAAGCCGATCGG-3′) as well as the reverse primer GEX3′ (5′-GGCGGCGCTAGTCATGCTAGG-3′), the full-length coding region of human SNAP-29 and mouse SNAP-29 was sequenced by PCR by using the bacterial expression constructs pGEX/SNAP-29 and pGEX/mSNAP-29 as templates (18, 19). Full-length cDNA for SNAP-29 was linearized from the expression construct pcDNA3 (Invitrogen). mRNA was generated by using the T7-mMESSAGE mMACHINE kit (Ambion Inc.), and in vitro translations were performed in rabbit reticulocyte lysate (Promega) supplemented with [35<sup>S</sup>]methionine (NEF Life Science Products).
Three Novel Mammalian SNAREs

In Vitro Binding Assay

Glutathione S-transferase (GST) fusion proteins incorporating the full cytoplasmic domains of either rat or human synthins 1a, 3, 4, 7, and 13 were prepared as described (17, 29–31). The full-length cytoplasmic domain of syntaxin 17 was prepared by PCR using custom-designed oligonucleotide primers with appropriate restriction sites. The fragment was subcloned into pGEX-4T-1 (Qiagen), transformed into the Escherichia coli strain XL1Blue-MRF, and induced and purified as described (29). Fusion proteins were eluted off the glutathione-agarose beads and 5 μl of reduced glutathione, 150 mM NaCl, 50 mM Tris/HCl, pH 8.0, and excessively dialyzed against buffer A (20 mM Hepes/NaOH, pH 7.4, 150 mM NaCl, 0.05% Tween 20).

Intracellular targeting of a protein to a particular organelle often provides valuable insight into its specific role in the cell. To this end, the full-length cDNA of syntaxin 8 was tagged at its amino terminus with the c-myc epitope and transiently expressed in NRK cells and COS cells. The second predicted coiled-coil domain directly precedes the hydrophobic tail. The amino-terminal predicted coil does not have the characteristics of a sequence that functions in forming a core SNARE complex. For example, the heptad repeat is not centered on a glutamine or arginine residue that appears in all well characterized domains that associate to form SNARE complexes. In contrast, the carboxy-terminal repeat of syntaxin 8 aligns well with the predicted coiled-coil of syntaxin 6 and SNAP 25, and includes a conserved hydrophobic amino acids. Syntaxin 8 is also predicted to contain two coiled-coil domains. The first helical domain resides in the amino-terminal part of the protein, whereas the second predicted coiled-coil domain directly precedes the hydrophobic tail. The amino-terminal predicted coil does not have the characteristics of a sequence that functions in forming a core SNARE complex.

Sequence analysis reveals several potential structural motifs. The carboxy-terminal 26 residues of syntaxin 8 probably function as a membrane anchor because they are not charged and contain a central stretch of hydrophobic amino acids. Syntaxin 8 is also predicted to contain two coiled-coil domains. The first helical domain resides in the amino-terminal part of the protein, whereas the second predicted coiled-coil domain directly precedes the hydrophobic tail. The amino-terminal predicted coil does not have the characteristics of a sequence that functions in forming a core SNARE complex.

RESULTS

Syntaxin 8 Is a Novel Member of the Mammalian Syntaxin/SNAP-25 Family—Searches of the data bases of expressed sequence tags (ESTs) using the amino acid sequence of syntaxin 6 revealed the presence of several mouse and human EST clones (AA111025, W41301, AA654455) predicted to encode a homologous protein (20). The EST clone AA654455 was fully sequenced, and found to contain an ORF of 228 amino acids. A BLAST search performed with this sequence identified another human cDNA clone (AF062077), which completely overlapped with the sequence of the clone AA654455. In addition, AF062077 provided the nucleotide sequence of 174 nucleotides preceding the sequence of AA654455. By assembling both sequences, a putative start Met (at nucleotides 151–153), an open reading frame predicted to encode a protein of 236 amino acids and a molecular mass of 26,879 Da, and a stop codon at nucleotides 858–860 were identified (Fig. 1). When the GenBank™ nucleotide data base was translated and searched with the predicted amino acid sequence of syntaxin 8 using the TBLASTN algorithm, significant homology to mammalian syntaxin 6 and to mammalian SNAP-25 was revealed (see Fig. 1) (13, 18). When the amino acid sequences of these proteins were compared in a pairwise fashion using the BESTFIT algorithm, syntaxin 8 was found to be 32% identical and 45% similar to syntaxin 6 (z-score = 23.7), when considering the most conserved carboxy-terminal coiled-coil domain (amino acids 180–230). By using the same algorithm, syntaxin 8 was also found to be 30% identical and 44% similar to SNAP-25 (z-score = 7.8; amino acids 150–203). These results indicate an ancestral relationship between these three proteins.

Sequence analysis reveals several potential structural motifs. The carboxy-terminal 26 residues of syntaxin 8 probably function as a membrane anchor because they are not charged and contain a central stretch of hydrophobic amino acids. Syntaxin 8 is also predicted to contain two coiled-coil domains. The first helical domain resides in the amino-terminal part of the protein, whereas the second predicted coiled-coil domain directly precedes the hydrophobic tail. The amino-terminal predicted coil does not have the characteristics of a sequence that functions in forming a core SNARE complex. For example, the heptad repeat is not centered on a glutamine or arginine residue that appears in all well characterized domains that associate to form SNARE complexes. In contrast, the carboxy-terminal repeat of syntaxin 8 aligns well with the predicted coiled-coil of syntaxin 6 and SNAP 25, and includes a conserved glutamine residue at the center of the domain. From this analysis, we conclude that syntaxin 8 will contribute one helical domain to the formation of a four-stranded SNARE complex.

Northern blot analysis revealed one syntaxin 8 transcript of 1.3 kb that displayed similar expression levels across all tissues tested (see Fig. 2A), with elevated expression in heart. These data are consistent with a general role of syntaxin 8 in the secretory pathway.

Intracellular targeting of a protein to a particular organelle often provides valuable insight into its specific role in the cell. To this end, the full-length cDNA of syntaxin 8 was tagged at its amino terminus with the c-myc epitope and transiently expressed in NRK cells and COS cells. The second predicted coiled-coil domain directly precedes the hydrophobic tail. The amino-terminal predicted coil does not have the characteristics of a sequence that functions in forming a core SNARE complex. As illustrated in Fig. 3 (A, C, E, and G), myc-syntaxin 8 was localized to intracellular membranes displaying a tubular/reticular pattern. This staining pattern is characteristic of the ER and overlapped significantly with that of the ER protein calnexin (Fig. 3, E versus F and G versus H).
On the other hand, myc-syntaxin 8 did not overlap with the intermediate compartment and Golgi marker syntaxin 5 (Fig. 3, A versus B), and the endosomal recycling pathway marker transferrin receptor (Fig. 3, C versus D) (21, 32). In summary, syntaxin 8 appears to be a SNARE protein important in membrane trafficking in many different cell types, perhaps functioning within the early secretory pathway.

Syntaxin 17 Is a Novel, Divergent Member of the Syntaxin Family—Using the yeast two-hybrid system (33), we isolated multiple overlapping cDNAs, which encoded a protein that interacted with syntaxin 3. Sequencing of these cDNAs revealed a putative protein that displayed significant homology to various syntaxin family members. A nucleic acid probe was generated from one of these clones and used to screen a rat brain cDNA library. We obtained rat cDNA clones containing an ORF encoding a protein of 301 amino acids and a predicted mass of 33,182 Da (Fig. 4). Preceding the putative start Met, the longest of the isolated cDNA clones contained 129 base pairs of 5'-untranslated region sequence. Syntaxin 7 (p = 0.0095) and syntaxin 13 (p = 0.007) are the highest scoring mammalian proteins that are found when searching the data base with the BLAST program using the full-length amino acid sequence as a query (14, 31). The homology between syntaxin 7, syntaxin 13, and syntaxin 17 is illustrated in Fig. 4. The BESTFIT algorithm revealed a 35% identity and 57% similarity between syntaxin 17 and syntaxin 7 (z score = 17), which resides in the conserved membrane-proximal coiled-coil domain that defines the syntaxin family of proteins (amino acids 174–227). Likewise, the BESTFIT algorithm revealed a 36% identity and 55% similarity between syntaxin 17 and syntaxin 13 (z score = 16.1; amino acids 172–227). Syntaxin 17 also displays high homology to the yeast syntaxins Sso1 (p = 0.0041) and Sso2 (p = 0.0075) (34), as well as to the syntaxin-related protein KNOLLE in Arabidopsis thaliana (p = 0.0080) and a proposed A. thaliana syntaxin T1F9.22 (AC004255) (p = 0.0037) (35).
Unlike other members of the syntaxin family, the predicted transmembrane domain encoded by this sequence is not at the carboxyl terminus of the ORF. Rather, it is followed by a stretch of 52 amino acids that show no obvious homology to known proteins or motifs. A predicted coiled-coil sequence just amino-terminal to the hydrophobic domain fits the consensus for other syntaxins including a glutamine residue at the center of the heptad motif.

To determine if syntaxin 17 performs a general vesicle trafficking function in all tissues or whether it is restricted to a subset of tissues, we determined its expression by Northern blot analysis. As shown in Fig. 2B, syntaxin 17 is expressed in a series of six transcripts of different sizes (2.7, 3.1, 4.0, 4.4, 6.0, and 6.4 kb). There are three major transcripts (3.1, 6.0, and 6.4 kb) in all tissues examined; however, they are particularly abundant in liver. Liver is well known for its prominent secretory role and is especially enriched in the ER compared with all other cell types (36). In testis, the 2.7- and 3.1-kb transcripts are highly expressed. It is unclear whether the larger transcripts represent alternative splice products or distinct, but related, sequences.

To determine the subcellular localization of syntaxin 17, we transfected NRK cells with an epitope-tagged syntaxin 17 construct. In immunofluorescence studies, the protein appears membrane-bound and is localized much like syntaxin 8 to tubular/reticular structures (Fig. 5, A and C). The enrichment of myc-syntaxin 17 in tubular/reticular structures is comparable with that of BiP (Fig. 5, A versus B) and calnexin (Fig. 5, C versus D) (37, 38). Whereas antibodies against BiP preferentially stain regions of the ER surrounding the nuclear membrane, calnexin staining overlaps with that of the BiP staining but also extends into the more peripheral subcompartmen of the ER. Syntaxin 17 staining also extends to peripheral regions of the cells, but its staining pattern does not precisely overlap with that of calnexin. In comparison to calnexin, the syntaxin 17 staining appears to localize to tubules, which are somewhat larger in size but less dense in number than the classic reticular ER (Fig. 5, E versus F). Thus, while the syntaxin 17 appears to be associated with a membrane compartment, perhaps a subset of the ER such as exit or entrance sites, higher FIG. 4. Syntaxin 17 is homologous to syntaxins 13 and 7. Sequences were aligned using the Pileup program. Identical amino acids are darkly shaded, and conserved amino acids are lightly shaded by the Boxshade program. The Q below the sequence indicates the central residue of the predicted coiled-coil. The dark bar indicates the COOH-terminal hydrophobic region predicted to act as a membrane anchor. Syntaxin 17 is 16% identical and 27% similar to syntaxin 7 and 15% identical and 30% similar to syntaxin 13 when compared along the whole length of the protein.

FIG. 5. Syntaxin 17 partially co-localizes with ER markers. NRK cells were transfected with myc-tagged syntaxin 17 and then fixed, permeabilized, and stained with the following antibodies: anti-myc (A, C, and E), anti-BiP (ER) (B), anti-calnexin (ER) (D and F). Bar = 50 μm (A–D) and 20 μm (E and F).
resolution studies are needed to better understand the local-
ization of this divergent syntaxin.

SNAP-29 Is a Novel Member of the SNAP-25 Subfamily—In
our yeast two-hybrid system approach to identify novel pro-
teins interacting with syntaxin 3, we isolated the cDNA encoding
a second novel protein, which displayed homology to neu-
ronal SNAP-25 and the ubiquitously expressed SNAP-23 (18, 19). A complete cDNA was isolated by screening a human brain
cDNA library with the cDNA insert of the two-hybrid clone.
Sequencing revealed an ORF of 258 amino acids with a deduced
molecular mass of 28,970 Da; therefore, we termed this clone
SNAP-29. The predicted amino acid sequence of SNAP-29 as
well as an alignment of SNAP-29 with SNAP-23 and SNAP-25
protein sequences are shown in Fig. 6. The BESTFIT algorithm
revealed a 26% identity and 35% similarity between SNAP-29
and SNAP-23 (z score = 15.4; amino acids 45–242). Likewise,
the BESTFIT algorithm revealed a 32% identity and 39% simi-
larity between SNAP-29 and SNAP-25 (z score = 12.6, amino
acids 53–142). The regions of highest homology of SNAP-29 to
SNAP-23 and SNAP-25 reside in the amino- and carboxy-
terminal thirds of the SNAP-29 protein, coinciding with the
potential regions that may form coiled-coil structures. Unlike
SNAP-23 and SNAP-25, one or more of these cysteines is palmitoylated, allowing a stable membrane association of these proteins (39).

Fig. 6. A new SNAP SNARE: SNAP-29. Sequences were aligned using the Pileup program. Identical amino acids are darkly shaded, and conserved amino acids are lightly shaded by the Boxshade program. The two Qs below the sequences (residues 48 and 180 in SNAP23) indicate the central residues of the two predicted coiled-coils. The asterisks (*) indicate the cysteine residues in SNAP-23 and/or SNAP-25 that are absent in SNAP-29. SNAP-29 is 17% identical and 31% similar to SNAP-29 and 17% identical and 33% similar to SNAP-25 when compared along the whole length of the protein.

First, epitope-tagged SNAP-29 was transiently expressed in NRK cells and its subcellular localization was visualized by indirect immunofluorescence studies. myc-SNAP-29 appears mostly membrane-bound, but a significant portion of the protein also seems to be of cytosolic nature (Fig. 7, A, C, E, and G). Most of the immunoreactivity appears to be surrounding the nuclear membrane; however, punctate staining extending outward from juxtanuclear regions as well as plasma membrane staining can be observed. myc-SNAP-29 immunoreactivity partially overlaps with syntaxin 5 (Golgi), transferrin receptor (endosomes), and lysosomal marker lgp120 (see Fig. 7, A versus B, C versus D, and E versus F, respectively) (21, 32, 40). Interestingly, the staining of SNAP-29 and the general vesicle trafficking protein NSF coincide almost precisely (Fig. 7, G versus H) (41). This localization indicates that SNAP-29 is involved in multiple membrane trafficking steps (see below). Second, we performed cell fractionation studies on COS cells transfected with epitope-tagged SNAP-29 to investigate the nature of the membrane association observed by indirect immunofluorescence studies. As illustrated in Fig. 8A, a significant portion of the protein is present in the membrane pellet of a 100,000 × g spin, which can be partially extracted by either high salt or high pH, and even more completely by Triton X-100. Like
SNAP-29, NSF is partially extracted by high salt or high pH, but is more efficiently extracted by detergent (Fig. 8B). Since
SNAP-29 does not contain palmitoylation sites or a stretch of
hydrophobic amino acids, the membrane association must be
mediated by other membrane-bound structures, most likely
through the interaction with one or more syntaxins (see below).

We next set out to determine if SNAP-29 recognizes syntaxin
3 exclusively or if it is capable of interacting with multiple
syntaxins. Furthermore, we asked the question if SNAP-29 displays a different binding specificity than SNAP-23 and
SNAP-25. To answer these questions, in vitro translated
SNAP-23, SNAP-25, or SNAP-29 were used to detect binding to
GST-syntaxin fusion proteins. While neither protein bound to
the control protein GST, both SNAP-23 and SNAP-25 bound to
GST-syntaxin 1a, GST-syntaxin 3, and GST-syntaxin 4 (Fig. 9,
A–C). SNAP-29, on the other hand, also bound with comparable
affinities as SNAP-23 and SNAP-25 to GST-syntaxin 1a, 3, and
4, confirming our two-hybrid results, but, in addition, SNAP-29
also bound to GST-syntaxins 7, 13, and 17 (Fig. 9D). These data
support the hypothesis that SNAP-29 is bound to membrane
structures via its interaction with multiple syntaxins. In contrast to syntaxins 1a, 3, and 4, which are localized to the plasma membrane (21), syntaxins 7, 13, and 17 are localized to intracellular membrane structures (14, 42). The association of epitope-tagged SNAP-29 with various intracellular membrane structures, and the plasma membrane observed by indirect immunofluorescence microscopy, reflects its ability to bind multiple syntaxins as observed by our in vitro binding studies.

In summary, our data suggest that SNAP-29 is a cytoplasmic and ubiquitous SNARE protein that is likely important in multiple membrane trafficking steps.

DISCUSSION

The sophisticated intracellular architecture and multiple differentiated and specialized tissues of mammals makes it likely that a large number of mammalian v- and t-SNAREs are required for membrane trafficking pathways. Defining the subcellular localization and tissue distribution of novel SNAREs provides valuable insights necessary for understanding vesicle trafficking in mammalian cells. Indeed, definition of the number, localization, and functions of the SNAREs may define new trafficking pathways not previously appreciated. In this report, we characterize three novel proteins of the syntaxin/SNAP-25 gene family and study their expression patterns, subcellular localization, and protein-protein interactions. All three proteins are ubiquitously expressed indicating a general role in mammalian vesicle trafficking, and each of them localizes to distinct subcellular compartments.

Syntaxin 8 is a previously uncharacterized t-SNARE, which is most closely related to syntaxin 6, a protein implicated in trans-Golgi network vesicle trafficking (16). Like syntaxin 6, syntaxin 8 displays significant homology to both the syntaxin and SNAP-25 subfamilies, confirming our earlier hypothesis that these two families are themselves related (13). Fig. 10 illustrates the evolutionary relationships among the members of this large gene family. Like a typical syntaxin, syntaxins 6 and 8 are anchored to membranes by a carboxyl-terminal
Three Novel Mammalian SNAREs

Evolutionary relationships of the syntaxin/SNAP-25 gene family. A phylogenetic tree was created as described under “Experimental Procedures.” The horizontal distance between two proteins is proportional to their evolutionary divergence. Asterisks (*) denote proteins identified in the paper. The proposed mode of membrane anchoring is given in the left column: P, protein has palmitoylation sites; TM, protein has a transmembrane anchor at its carboxy terminus; N, protein has neither palmitoylation sites nor a transmembrane region. The proposed subcellular localization is given in the right column: ER, endoplasmic reticulum; IC, intermediate compartment; G, Golgi; TGN, trans-Golgi network; E, endosomal compartment; SG, secretory granules; PM, plasma membrane. All the members of the syntaxin subfamily have a conserved glutamine residue at the center of the predicted carboxy-terminal coiled-coil domain. In contrast, the members of the SNAP-25 subfamily have two predicted coiled-coil domains, a predicted carboxyl-terminal coiled-coil domain. In contrast, the syntaxin subfamily have a conserved glutamine residue at the center of the predicted carboxy-terminal coiled-coil domain.

Epitope-tagged syntaxin 17 partially coincides with the ER markers BiP and calnexin, but the immunoreactivity is clearly distinct from the typical tubular-recticular pattern. Higher resolution studies will be necessary to precisely determine the compartment to which syntaxin 17 is targeted. The unusual sequence, luminal domain, and localization pattern suggest that syntaxin 17 may function in a different type of membrane trafficking pathway from other syntaxin proteins. Perhaps like KNOLLE, syntaxin 17 is involved in processes related to cell division. For example, syntaxin 17 could be important in membrane organelle breakdown and reassembly during mitosis. Further studies are necessary to determine the role of this very unique syntaxin family member.

In contrast to the large “classical” syntaxin subfamily, the mammalian SNAP-25 subfamily has only three members identified so far, including the newly identified SNAP-29 gene. SNAP-29 is a unique SNARE with regard to its membrane interaction. Most syntaxins and VAMPs are anchored to a distinct membrane compartment by means of their carboxy-terminal stretch of hydrophobic amino acid residues; for example, syntaxin 8 and syntaxin 17 fall into this group. A second group of SNARE proteins have a cysteine-rich palmitoylated domain either in the central part of the protein or at the carboxyl terminus; SNAP-25, SNAP-23, and syntaxin 11 as well as a splice variant of syntaxin 2 belong to this group (14, 18, 19, 21). SNAP-29 contains neither of these membrane attachment properties. The mode of membrane anchoring of the different family members is illustrated in Fig. 10. Whereas SNAP-25 and SNAP-23 are primarily localized to the plasma membrane or upon stimulation to mast cell granules (18, 45), epitope-tagged SNAP-29 localized predominantly to intracellular membrane structures, which partially overlap with endosomal, lysosomal, and Golgi markers and significantly overlap with membrane-bound NSF. Furthermore, we found that in vitro translated SNAP-29 but not in vitro translated SNAP-23 or SNAP-25 binds to a broad range of syntaxin fusion proteins. We propose that SNAP-29, unlike SNAP-25 and SNAP-23, does not have palmitoylation sites and is therefore not anchored to a specific compartment; thus, it is capable of participating in various intracellular transport steps, interacting with different syntaxins and VAMPs, which are themselves specifically localized to a distinct membrane compartment. Considering these findings, it is likely that SNAP-29 is involved in multiple transport steps, being able to contribute two helical domains to the formation of different four-stranded SNARE complexes. Having a “general” SNAP protein participating in multiple transport steps would explain why there are only so few SNAP-25 homologs as compared with the much larger number of syntaxin and VAMP homologs.

While SNARE proteins are certainly critical mediators of membrane trafficking and fusion, their precise function is not yet defined. These proteins may mediate late steps in vesicle docking or priming, they may mediate the membrane fusion itself, or they may be involved in a combination of these processes. It is also still not clear if each vesicle trafficking
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step requires a unique combination of VAMP, syntaxin, and SNAP-25 proteins.

The number of coiled-coil domains that contribute to a SNARE complex seems to be fixed. In the case of the ternary synaptic complex, the syntaxin contributes one helical domain, which is centered on a glutamate residue, the VAMP molecule contributes one helical domain, which is centered on an arginine residue, and SNAP-25 contributes two coiled-coil domains, both of them centered on a glutamate residue. In contrast, the helical domains of four molecules (syntaxin 5, membrin, rsec22b, and rbet1) make up the ER to Golgi SNARE complex (15). In mammalian species, there are now approximately 10 SNAREs with a carboxyl-terminal helical domain centered on an arginine residue, 19 SNAREs with a carboxyl-terminal helical domain centered on a glutamate residue, and three SNAPs (SNAP-23, -25, and -29) that can each provide two helical domains with conserved glutamine residues to the formation of a SNARE complex. Thus, we expect that the approximate total number of these proteins will be less than a factor of 2 more than those that are now known. This number along with patterns of differential expression in various tissues may indeed be sufficient to account for the diversity of membrane compartments in mammalian cells. The evolutionary relationships, the proposed mode of membrane attachment, and the proposed subcellular localization of some of the members of the syntaxin/SNAP-25 gene family are depicted in Fig. 10. What remains to be determined are the binding interactions between this growing number of SNAREs and only then will it be possible to discern if these interaction patterns are consistent with a role in determining the specificity of vesicle trafficking.

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