A New Syntaxin Family Member Implicated in Targeting of Intracellular Transport Vesicles* *(Received for publication, March 5, 1996, and in revised form, May 6, 1996) J ason B. Bock, Richard C. Lin†, and Richard H. Scheller§ From the Howard Hughes Medical Institute, Department of Molecular and Cellular Physiology, Stanford University Medical Center, Stanford, California 94305-5428

Despite the central role vesicular trafficking occupies in protein targeting, the molecular coding of the trafficking signals and the mechanism of vesicle docking and fusion are just beginning to be understood. We report here the cloning and initial characterization of a new member of the syntaxin family of vesicular transport receptors. Syntaxin 6 is a 255-amino acid protein with two domains predicted to form coiled-coils, as well as a carboxyl-terminal membrane anchor. Syntaxin 6 is broadly expressed and localizes in the region of the Golgi apparatus. In vitro binding studies established that syntaxin 6 binds to α-soluble NSF attachment protein (α-SNAP). The sequence homology, topology, localization, and α-SNAP binding suggest that syntaxin 6 is involved in intracellular vesicle trafficking.

Eukaryotic cells contain highly specialized organelles that are defined by their specific protein complements. The mechanism by which cells are able to route proteins along particular pathways to these various organelles has been the focus of intense investigation. While it was theorized since the 1970's that transport vesicles mediated this process (1), it was not until recently that a convergence of genetic, biochemical, and cell biological approaches permitted significant insights into the molecular mechanisms underlying this process (2, 3). The intersection of these approaches took place at the mammalian presynaptic nerve terminal, where a biochemical model for synaptic vesicle docking and fusion has been proposed (4). This model has been put forward as a paradigm for all intracellular vesicle trafficking (5).

Current models suggest that synaptic vesicle proteins (v-SNAREs) interact specifically with plasma membrane-localized molecules (t-SNAREs), and that these complexes act as a scaffold for the assembly of the fusion apparatus (6, 7). At the synapse a promenade of protein-protein interactions mediate the docking, priming, and fusion of synaptic vesicles (8). The first step in defining this mechanism was the identification of the proteins involved. Syntaxin 1a and SNAP-25 are the t-SNAREs present on the plasma membrane, while VAMP or synaptobrevin and synaptotagmin are the v-SNAREs on synaptic vesicles (4). A complex of these four membrane-bound proteins can be isolated in vivo and reconstituted in vitro (7, 9, 10). Concurrent with the dissociation of synaptotagmin, this complex binds the cytosolic protein α-SNAP, which was originally isolated through its requirement in a reconstituted Golgi transport vesicle fusion assay (11). The model predicts that the ATPase NSF then binds α-SNAP and, through the hydrolysis of ATP, primes the complex for the eventual calcium influx leading to vesicle fusion. Thus, it is the interaction of the t-SNAREs with their cognate v-SNAREs, which contributes to the specificity, and it is the binding of α-SNAP to the members of this complex that is essential to the transition from a docking state toward a fusion competent one.

The fundamental question that we begin to address in this study is: can the molecular machinery mediating vesicle fusion at the synapse serve as a model for all intracellular vesicle trafficking? The genetically tractable organism Saccharomyces cerevisiae has played a key role in helping to understand mammalian vesicle trafficking. For example, it has been shown that the yeast Golgi to plasma membrane constitutive secretory and mammalian synaptic transmission pathways are evolutionarily related (12). Syntaxin 1a, SNAP-25, VAMP, α-SNAP, and NSF all have yeast homologues. This molecular consistency appears to hold up in studies of intracellular trafficking pathways aside from Golgi to plasma membrane transport. Mammalian homologues of Bet1 and Sec22, yeast proteins involved in endoplasmic reticulum to Golgi trafficking, have been identified recently (13).

Not only do protein family members span species, but they also stretch across different intracellular trafficking pathways. The best example of this is the syntaxin family, whose members specifically localize to several organelles. For example, syntaxin 5 is present on the cis Golgi (14) and mediates endoplasmic reticulum to Golgi transport (15). Another member of the syntaxin family is yeast Pep12 (also termed VPS6). It was initially identified in a yeast genetic screen for proteins involved in vacuolar function (16) and is believed to mediate Golgi to vacuole transport (17).

In order to determine if the synapse can serve as an accurate model for all intracellular vesicle trafficking, we set out to identify other mammalian members of the syntaxin family present on distinct intracellular organelles. We characterized overlapping cDNAs encoding a 255-amino acid protein with two domains predicted to form coiled-coils and a carboxyl-terminal transmembrane domain. This protein shows homology to Pep12 and the mammalian syntaxin family members. An unexpected result was that this protein also shows significant identity to SNAP-25. Subcellular localization determined that this protein resides in the Golgi region. In vitro binding studies demonstrate its specific interaction with α-SNAP. Due to its structure, sequence homology, and protein-protein interactions...
we classify this protein as member of the syntaxin family. The data presented here are consistent with a role for syntaxin 6 in Golgi vesicle trafficking.

**EXPERIMENTAL PROCEDURES**

dRNA Cloning and Sequencing—The Human Genome Sciences, Inc. data base of expressed sequence tags (ESTs) was searched by comparing its translated contents to the yeast Pep12 primary sequence. One clone was found to be 21% identical to Pep12 over a 112-amino acid domain. This EST clone was initially discovered using established EST methods by Human Genome Sciences, Inc. (18, 19). Custom-designed oligonucleotides were used in the polymerase chain reaction using a skeletal muscle, kidney, and testes were dissected, homogenized, and processed. Total cellular RNA was isolated using the guanidine isothiocyanate-cesium chloride method (22). The integrity and concentration of the RNA were determined by ethidium bromide staining of agarose gels. The RNA was then treated with RNase-free DNase I (Boehringer Mannheim Corp., Indianapolis, IN) according to the manufacturer's protocol. Poly(A) + RNA was purified using a PolyATRribin affinity system (BRL).

**RESULTS**

A New Mammalian Syntaxin Family Member—Genetic studies in yeast have identified a panel of genes coding for proteins involved in intracellular transport (21). These studies have advanced our understanding of mammalian vesicle transport in two significant ways. First, in areas where mammalian studies were further progressed, such as exocytosis, later yeast studies corroborated the role of several proteins implicated in mammalian vesicle transport (12). Second, in areas where yeast studies are more advanced, such as in endoplasmic reticulum to Golgi transport, they have allowed the isolation of several novel mammalian homologues (13). Pep12p is a yeast member of the syntaxin family and plays a role in Golgi to vacuole transport. We exploited the availability of large data bases of DNA sequence information to identify a human EST model 300A). The pixel values from Fig. 5 were as follows (from left to right): Construct 1–235 (50, 76, 115, 155, 130); Construct 1–166 (47, 98, 137, 177, 152); Construct 1–265 (95, 133, 172, 211, 186).

**Immunofluorescence Microscopy—**Indirect immunofluorescence localization was performed on the rat FAO hepatoma cell line as described previously using the affinity-purified anti-syntaxin 6 serum diluted 1/2000 (14). Anti-mannosidase II monoclonal (BABCO, Berkeley, CA) was used at a 1/20000 dilution. Rhodamine-conjugated wheat germ agglutinin (Vector, Burlingame, CA) was used at a 1/20000 dilution. Anti-rbet1 was used at a 1/3000 dilution.

In Vitro Binding Assay—Fusion protein beads, soluble α-SNAP preparation, and binding assays were prepared as described (9, 20), with two modifications: 1) incubation buffer (buffer A) consisted of 20 mM HEPES-KOH, pH 7.4, 150 mM KCl, 0.05% Tween 20 and 2) incubations were at 25°C. Band intensities following Western blotting and ECL were quantified using a computing densitometer (Molecular Dynamics, model 300A). The pixel values from Fig. 5 were as follows (from left to right): Construct 1–235 (50, 76, 115, 155, 130); Construct 1–166 (47, 107, 145, 116, 118); Construct 166–235 (9, 7, 6, 14, 12); Construct 26–235 (95, 188, 210, 238, 218).

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Table I
Syntaxin family and SNAP-25 sequence homologies

| Syntaxin 6 | Pep12 | Syntaxin 1a | Syntaxin 3 | SNAP-25 |
|-----------|------|------------|------------|--------|
| Syntaxin 6 | —    | 25         | 20         | 23     |
| Pep12     | 56   | —          | 23         | 22     |
| Syntaxin 1a | 45   | 48         | —          | 24     |
| Syntaxin 3 | 48   | 50         | 77         | —      |
| SNAP-25   | 46   | 51         | 46         | 47     |

searched with the predicted amino acid sequence of syntaxin 6 using the TBLASTN algorithm (23). Mammalian SNAP-25 received the highest relevant score (p = 0.0024) and yeast pep12 received the second highest relevant score (p = 0.024). When the amino acid sequences of these proteins were compared in a pairwise fashion using the bestfit algorithm (24), syntaxin 6 was found to be 25% identical and 56% similar to pep12 (Table I). Syntaxin 6 was found to be 22% identical and 47% similar to SNAP-25.

Syntaxin 6 displays structural characteristics similar to the syntaxin family of vesicle transport proteins. Its 20-most carboxyl-terminal amino acids are hydrophobic and presumably act as a membrane anchor. Using the Coils algorithm (25, 26), we determined that syntaxin 6 has two regions with high probabilities of participating in coiled-coils. The first is a short stretch from amino acids 47–71 (90% probability), and the second directly precedes the hydrophobic tail from amino acids 166–225 (80% probability, see Fig. 5). The protein is also predicted to have a relatively low pi of 4.7. These characteristics are reminiscent of the syntaxin family.

Tissue Expression Patterns—To determine if syntaxin 6 performs a general vesicle trafficking function in all tissues or whether it is restricted to a subset of tissues, we performed Northern and Western blot analysis as shown in Fig. 2. A predominant RNA at 3.0 kb was found in all tissues examined, with relatively higher expression in brain, lung, and kidney. Transcripts of 5.9 and 8.2 kb were also present in most tissues, although in lower abundance. It is unclear whether these larger transcripts represent alternative splice products or distinct, but related, sequences. In addition to the 759 base pairs of coding sequence, our overlapping clones also contained 150 base pairs of 5'-untranslated sequence and 1.1 kb of 3'-untranslated sequence.

To corroborate the RNA distribution we performed Western blot analysis using affinity-purified antisera prepared by immunizing a rabbit with recombinant syntaxin 6. This antisera recognized a single band of 29 kDa in rat brain post-nuclear supernatant, which was not recognized by pre-immune sera. This band was eliminated by preincubating the antisera with soluble recombinant syntaxin 6 (data not shown). Western blot analysis confirmed the Northern blot analysis as shown in Fig. 2. Syntaxin 6 was found to be widely expressed, with relatively more protein in brain, lung, and kidney. This broad tissue expression would be expected of a protein involved in constitutive vesicle trafficking to a ubiquitous organelle, such as the Golgi or lysosome.

Membrane Association—The carboxyl-terminal 20 amino acids of syntaxin 6 are hydrophobic and likely to act as a membrane anchor. Consistent with this syntaxin 6 was found to reside in the high speed pellet fraction of rat brain post-nuclear supernatant. To determine if syntaxin 6 is indeed an integral membrane protein, we extracted rat brain membranes with a series of reagents (Fig. 3). Syntaxin 6 remained associated with the membrane pellet after extraction with NaCl, urea, and high pH; however, it was present in the supernatant following extraction with the nonionic detergent Triton X-100. These results are consistent with the prediction that syntaxin 6 is an integral membrane protein.

Subcellular Localization—To determine which membrane compartment syntaxin 6 associated with, immunofluorescence was used to visualize its location. Fig. 4 shows the subcellular localization of syntaxin 6 in FAO tissue culture cells. Syntaxin 6 was detected primarily in a juxtanuclear pattern, suggestive of the Golgi apparatus. The Golgi region was not labeled when the antibodies were preincubated with recombinant syntaxin 6 protein (data not shown). To confirm the Golgi region localization, we double-labeled cells with antibodies against the known Golgi markers mannosidase II (27) and rBet1 (13). Syntaxin 6 appeared to substantially colocalize with these two markers (Fig. 4).

Although the Golgi region-specific labeling was predominant, a subset of cells had an additional weak, broadly perinuclear, membranous pattern, which may represent a fraction of syntaxin 6 on vesicles shuttling to another intracellular organelle.

Syntaxin 6 Binds α-SNAP—We used an in vitro binding assay to characterize the interaction between syntaxin 6 and α-SNAP. Syntaxin 6 was expressed from the pGEX-KG vector in E. coli as a glutathione S-transferase fusion protein, with glutathione S-transferase constituting the amino terminus; the fusion protein was then bound to glutathione-agarose beads. These beads were incubated with increasing amounts of soluble histidine-tagged α-SNAP. Following three washes, the amount of bound α-SNAP was determined by SDS-PAGE and Western blot.

As shown in Fig. 5, this titration study demonstrated that α-SNAP binds syntaxin 6 with an EC₅₀ between 1 and 5 μM. This affinity is consistent with syntaxin 6's homology to both the syntaxin family as well as SNAP-25; the estimated EC₅₀ for α-SNAP's binding to rat syntaxin 1a is 0.5–3 μM, while the
Syntxin 6 behaves as an integral membrane protein. Post-nuclear membrane pellet fractions were extracted with various disruptive agents, centrifuged at 100,000 x g, and the resulting supernatants (S) and pellets (P) were analyzed by SDS-PAGE and anti-syntxin 6 (1/1500) Western blot analysis. All membranes were resuspended in homogenization buffer (50 mM Tris, pH 8.0, 150 mM NaCl, 1 mM dithiothreitol) and then an equal volume of the extraction solution was added to each sample to yield the following final concentrations of extracting agents: first and second lanes, only water added; third and fourth lanes, 1.5 mM NaCl; fifth and sixth lanes, 2 mM Na2CO3, pH 11.4, seventh and eighth lanes, 2 m urea; ninth and tenth lanes, 2% Triton X-100.

Fig. 3. Syntaxin 6 localizes to the Golgi region. Double labeling immunofluorescence microscopy illustrating the colocalization of syntxin 6 labeled with fluorescein secondary antibody (A and C) and the Golgi markers mannosidase II (B) and rBet1 labeled with rhodamine secondary (D). Bar: A and B, 20 μm; C and D, 12 μm.

Fig. 4. Syntaxin 6 localizes to the Golgi region. Double labeling immunofluorescence microscopy illustrating the colocalization of syntxin 6 labeled with fluorescein secondary antibody (A and C) and the Golgi markers mannosidase II (B) and rBet1 labeled with rhodamine secondary (D). Bar: A and B, 20 μm; C and D, 12 μm.

The simplest conclusion that can be drawn from the data presented here is that syntaxin 6 is a member of the syntaxin family of vesicular transport receptors. Syntaxin 6 shows significant homology to syntaxin 3, Pep12, and SNAP-25. Syntaxin 6 has a carboxyl-terminal membrane anchor and behaves as an integral membrane protein. The predominant immunofluorescent pattern observed is juxtanuclear and colocalizes with Golgi markers. Syntaxin 6 binds to α-SNAP, which is believed to be a general mediator of vesicle fusion. Thus several lines of evidence implicate syntaxin 6 in vesicle trafficking.

It has been proposed that many of the protein-protein interactions involved in vesicle docking and fusion are mediated by coiled-coils. This idea has been motivated by studies coupling sequence analysis with deletion mapping experiments. In the case of syntaxin 1a, the carboxyl-terminal H3 helix, which is predicted to form coiled-coils, is necessary and sufficient for α-SNAP binding as well as 20 S complex formation. Similar studies have mapped binding sites on SNAP-25; syntaxin and α-SNAP bind to an amino-terminal heptad repeat region, while VAMP binds to a carboxyl-terminal one.

Syntaxin 6 has two regions with a high probability of forming coiled coils. The first one spans residues 47–71 (90% probabil-
ity); the second one, comprising residues 166–225 (80% probability), is reminiscent of syntaxin 1a's H3 helix in terms of its length and position before the transmembrane anchor. In this study we have shown that while α-SNAP specifically binds to syntaxin 6 with an EC<sub>50</sub> of 1–5 µM, the carboxyl-terminal heptad repeat of syntaxin 6 is neither necessary nor sufficient for this binding. These data suggest that, unlike syntaxin 1a, the more amino-terminal heptad repeat region is involved in α-SNAP binding. While this domain is shorter than syntaxin 1a's H3 domain, this length is sufficient for forming coiled coils based on other studies (29). First, syntaxin 1a's H3 domain can be paired to 4.5 repeats and still bind SNAP-25 (9). Second, peptides of only three heptad repeats are able to participate in coiled coils (30, 31). These lines of evidence argue that syntaxin 6's amino-terminal heptad repeat is capable of forming coiled coils and is responsible for binding α-SNAP. As noted above, SNAP-25 also utilizes an amino-terminal coiled-coil domain to interact with α-SNAP. The ability of a protein with syntaxin-like structural features to have SNAP-25-like binding properties supports the intriguing idea that the syntaxins and SNAP-25 are evolutionarily related (see below).

While syntaxin 6 is related to yeast Pep12, it is still unclear if it is the functional homologue or a related protein. There may be other uncharacterized yeast genes more similar to syntaxin 6. For example a 45,000-base pair yeast cosmid (a part of a yeast chromosome sequenced as part of the yeast genome mapping project, accession number gb U41528) contains a sequence which, when translated, is 23% identical to syntaxin 6 and has a carboxyl-terminal hydrophobic domain. Although Golgi region localization would not be predicted of a functional Pep12 homologue, it is not implausible; because subcellular localization within yeast is technically challenging, Pep12p has not been definitively localized.

Syntaxin 6 is distinct from previously characterized proteins implicated in vesicle trafficking, due to its similarity to both the syntaxin and SNAP-25 families. It is striking that when the data base containing thousands of sequences is searched, the two most related protein sequences are proposed t-SNARE's involved in vesicle sorting. The existence of a single peptide sequence showing significant homology to two previously distinct families suggests that the syntaxin and SNAP-25 families are related, indicating they evolved from a common ancestor. It is possible that the transport step involving syntaxin 6 is not as tightly regulated as exocytosis. Thus syntaxin 6 may serve a function which requires both SNAP-25 and syntaxin 1a at the Golgi to plasma membrane step.

The genetic and biochemical studies of the presynaptic nerve terminal have revolutionized our understanding of synaptic vesicle docking and fusion and have allowed the creation of a testable hypothesis for the mechanism of all intracellular vesicle trafficking. The obligatory first step in testing this model is to identify proteins involved in other vesicle trafficking steps. While other proteins, such as syntaxin 5, have been implicated in intracellular vesicle trafficking steps other than Golgi to plasma membrane, little is known about these transport steps in mammalian systems. Future studies will concentrate on defining the specific trafficking step syntaxin 6 participates in and determining the molecular mechanism underlying this step. Syntaxin 6 should be a valuable tool in answering fundamental questions regarding intracellular vesicle trafficking.

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