Syntactin 5 Regulates Endoplasmic Reticulum to Golgi Transport*

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SYNTAXINS ARE A FAMILY OF VESICULAR TRANSPORT RECEPTORS

That are involved in membrane traffic through both the constitutive and regulated secretory pathways. Syntaxins 1A/B, 2, 3, and 4 are predominantly associated with the plasma membrane. Two of the syntaxins, 1A and 1B, have been suggested to be the docking receptors for synaptic vesicles with the presynaptic membrane. The most distant member of the family, syntaxin 5, has been found in the Golgi region and has significant homology (35% identity) with Sed5p, an essential protein in yeast which is required for vesicular transport from the endoplasmic reticulum (ER) to the Golgi stack. Here we present evidence that syntaxin 5 performs an analogous function in ER to Golgi transport in mammalian cells. Transient expression of an hemagglutinin-tagged full-length clone of syntaxin 5 and a truncated mutant lacking the transmembrane domain inhibited the transport of vesicular stomatitis virus glycoprotein to the Golgi stack. Under these conditions, vesicular stomatitis virus glycoprotein accumulated in pre-Golgi intermediates, which were strongly enriched in syntaxin 5. Our results suggest that syntaxin 5 is the functions mammalian homologue of Sed5p and provides evidence for its role in regulating the potential targeting and/or fusion of carrier vesicles following export from the ER.

Two types of vesicular trafficking mechanisms contribute to the secretory pathway of eukaryotic cells: those involved in constitutive secretion, delivering protein to the cell surface through continuous exocytosis; and those in the regulated pathway, which respond to extracellular signals in order to trigger the release of vesicular content. The biochemical components required for transport in both pathways are now thought to have a common molecular mechanism(s) in which related proteins comprising gene families serve analogous functions in the events dictating vesicle budding, targeting, and fusion between different compartments. Synaptobrevin/VAMP1 and syntaxin 1A, originally identified as components of the machinery mediating neurotransmitter release at the nerve terminal (1–3), are archetypes for two of these gene families (4). Members of the VAMP/synaptotagmin and syntaxin families are believed to form complexes which promote vesicle docking and fusion (1, 5, 6). The VAMP family consists of integral membrane proteins associated with carrier vesicles and includes synaptobrevins 1 and 2 (7), cellubrevin (8), and their putative yeast relatives Bos1p (9), Bet1p (10), Sec22p (10), and Snc1p (11). Members of the syntaxin family include syntaxins 1A, 1B, 2, 3, 4, and 5 in mammalian cells (12) and Pep22p (13), Sso1p (14), and Sed5p (15) in yeast. Syntaxins contain a large cytoplasmic domain, a single hydrophobic membrane anchor, and a short extracellular or luminal carboxy-terminal tail. Syntaxin 1A has been found in the pre-synaptic membrane and, while thought to serve as a component of the docking machinery for synaptic vesicles, is also distributed along the entire surface of the neuron, suggestive of a more general role in protein traffic to the plasma membrane (1, 4). In this study we report on the functional properties of mammalian syntaxin 5, demonstrating that it is found on pre-Golgi intermediates and that it is essential for vesicular traffic between the ER and the Golgi stack in mammalian cells.

EXPERIMENTAL PROCEDURES

Materials—All materials were obtained as described previously (16). Antibodies specific for the hemagglutinin (HA) epitope were obtained from I. Wilson (The Scripps Research Institute, La Jolla, CA). A polyclonal serum recognizing α-1,2-mannosidase II (anti-Man II) was kindly provided by M. Farquhar (University of California, San Diego) (17). Antibodies recognizing the pre-Golgi intermediate marker proteins p63 (18) and p58 (19) were generously provided by H.-P. Hauri (Biozentrum, Basel, Switzerland) and J. Saraste (University of Bergen, Oslo, Norway), respectively. A monoclonal antibody specific for the carboxyl terminus of VSV-G (20) was kindly provided by T. Kreis. We thank K. Scheller (Stanford University, Stanford, CA) for generously providing us with cDNA clones for syntaxins 1A and 5 (12) and C. Der (University of North Carolina, Charlotte, NC) for the pET11d-HA expression vector.

Generation of Expression Constructs—The pET11d HA-tagged syntaxin 1A and 5 expression constructs were generated as follows. The syntaxin 5 and syntaxin 1A wild-type clones were modified by PCR to possess a 5′-NdeI restriction site encoding the initiating methionine and a 3′-BamHI restriction site immediately after the stop codon. The truncated forms of syntaxin 5, 5-11 (amino acids 4–267) and 5-16 (amino acids 194–267) were generated by PCR using the original plasmid construct as a template. For syntaxin 5-11, a 5′-NdeI restriction site was created with the conversion of cysteine at position 3 to an initiating methionine. At the 3′-end, a stop codon and a BamHI restriction site were encoded immediately following amino acid 267. For syntaxin 5-16, a 5′-NdeI restriction site was generated by PCR which encoded an initiating methionine at position 194; at the 3′-end, a stop codon and BamHI restriction site were encoded immediately following amino acid 267. PCR reactions were carried out using Pfu polymerase (Stratagene) under standard conditions. PCR reaction products were subcloned into the TA cloning vector pCR1 (Invitrogen) and sequenced by the chain termination method. Thesyntaxin 1A and 5 full-length, syntaxin 5-11, and syntaxin 5-16 sequences were then introduced as NdeI/BamHI fragments into a version of the pET11d vector containing an in-frame HA epitope tag immediately upstream of the NdeI restriction site for expression from the T7 promoter as described (16, 21).

Transient Expression and Indirect Immunofluorescence—Transient expression and indirect immunofluorescence in HEK293 and BHK cells were performed as described (16).

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The abbreviations used are: VAMP, vesicle-associated membrane protein; NSF, N-ethylmaleimide-sensitive fusion protein; SNAP, soluble NSF attachment protein; ER, endoplasmic reticulum; HA, hemagglutinin; Man II, α-1,2-mannosidase II; PCR, polymerase chain reaction; BHK, baby hamster kidney, endo H, endoglycosidase H.
RESULTS

The current paradigm for syntaxin function suggests a role in vesicle docking and/or fusion to a specific target membrane (12). While syntaxin 5 shows limited homology with other members of the syntaxin family (12), it has significant homology (35% identity) with Sed5p, a yeast protein that is essential for ER to Golgi transport (15). To determine if syntaxin 5 was functionally homologous to Sed5p, we examined the effects of transient overexpression of a HA-tagged form of syntaxin 5 (HA-syntaxin 5) on the transport of vesicular stomatitis virus glycoprotein (VSV-G) from the ER to the Golgi apparatus in mammalian cells. VSV-G is a type I membrane protein containing two N-linked carbohydrate chains and has served as a marker protein to study the biochemical and molecular basis for transport from the ER in vitro (16, 21–23) and in vitro (22–28). Vectorial transport of VSV-G from the ER to and through sequential cis-, medial, and trans-Golgi compartments can be measured by the processing of its two oligosaccharide chains from the high mannos (Man) endoglycosidase H (endo H)-sensitive form found in the ER and pre-Golgi intermediates to endo H-resistant forms found in the Golgi stack. These processing intermediates can be readily distinguished by their unique electrophoretic mobilities using SDS-polyacrylamide gel electrophoresis (16, 23, 28). The appearance of the first, transient endo H-resistant R₁ form, corresponds to the transient R₂ intermediate forms. Rather, VSV-G that escapes the block was efficiently transported and processed to the R₂ form, diagnostic of efficient delivery to late Golgi compartments (1). From these results, it is apparent that elevated expression of HA-syntaxin 5 inhibits ER to Golgi transport (Fig. 1).

Although transport from the ER to the Golgi stack was strongly inhibited by elevated expression of full-length HA-syntaxin 5, further transport of VSV-G through the Golgi stack was unaffected. For example, cells in which transport was only partially inhibited by low levels of expression of HA-syntaxin 5 (i.e. Fig. 1, 3 h) did not result in the accumulation of VSV-G in the transient R₂ intermediate forms. Rather, VSV-G that escaped the block was efficiently transported and processed to the R₂ form, diagnostic of efficient delivery to late Golgi compartments (1). This result is in contrast to the effects of overexpression of a trans dominant negative mutant of the small GTPase Arfl (Arfl(Q71L)) involved in the assembly of clathrin (COP) coats (31, 32), and which potently inhibits both ER to Golgi and intra-Golgi transport in vivo (16). In cells expressing Arfl(Q71L), VSV-G becomes trapped in both the R₂ and R₃ forms (Fig. 2, compare b (Arfl(Q71L)) to c (HA-syntaxin 5)) (16). In contrast, the inability of elevated expression of HA-syntaxin 5 to inhibit intra-Golgi transport is identical to the effects of a trans dominant negative mutant of Sar1 (Sar1(Q79L)), a small GTPase that is uniquely required for ER to Golgi transport in mammalian cells (23). From these results, it is apparent that elevated expression of HA-syntaxin 5 specifically inhibits the function of carrier vesicles mediating ER to Golgi transport.

To define the potential role of the transmembrane domain in HA-syntaxin 5 function, we examined the effects of elevated expression of two soluble fragments of the protein. One fragment included the entire cytoplasmic domain, lacking only the transmembrane anchor (HA-syntaxin 5–11, amino acids 4–267) (Fig. 2A). The second fragment consisted of the region adjacent to the transmembrane domain that displays significant sequence conservation among known members of the syntaxin family (HA-syntaxin 5–16, amino acids 194–267) (Fig. 2A). The HA-syntaxin 5–11 construct was stably expressed only at a reduced level when compared to the wild-type protein, a level comparable to that observed with full-length syntaxin 5 at 3 h post-
transfection (data not shown). However, even at low levels of expression, significant inhibition (35%) of transport could be detected (Fig. 2B, d), similar to that observed after 3 h of expression of the full-length molecule. This result is consistent with the ability of a similar truncation of syntaxin 1A to prevent synaptic vesicle fusion when microinjected into PC12 cells (12). Interestingly, elevated expression of the truncated form also led to a detectable accumulation of the R, intermediate (Fig. 2B, compare d to c), suggesting a weak effect on intra-Golgi transport.

The absence of the transmembrane domain may lead to mislocalization and the partial interaction of syntaxin 5 with the biochemical machinery involved in transport through the Golgi stack. The small conserved fragment (HA-syntaxin 5-16) was found to be unstable during transient expression (data not shown) and thus had no effect on transport (Fig. 2B, e).

As a control for the effects of overexpression of full-length syntaxin 5 on transport, we examined the ability of syntaxin 1A to inhibit ER to Golgi transport. Syntaxin 1A function should be restricted to vesicle targeting to the cell surface (1, 12). As shown in Fig. 2, only at a high level of overexpression of HA-syntaxin 1A (a level comparable to that of syntaxin 5 at 6 h post-infection (Fig. 1, inset)) did we detect a partial (30%) inhibition of the transport and processing of VSV-G to the R, form (Fig. 2B, f). These results raise the possibility that the overexpression of a syntaxin protein may upset the balance of more general transport factors such as NSF, SNAPs, and SNAP receptors, components that are likely to be involved in function of all members of the syntaxin family (4–6).

The inability of VSV-G to be processed to Golgi forms in the presence of elevated levels of the full-length HA-syntaxin 5 could be explained either by inhibition of export from the ER or by its accumulation in pre-Golgi intermediates. To differentiate between these two possibilities, we examined the transport of VSV-G using indirect immunofluorescence. For these experiments, several different cell lines were transfected with an expression vector carrying a temperature-sensitive form of VSV-G (strain ts045). Strain ts045 VSV-G is retained in the permissive network of the ER when cells are transfected at the restrictive temperature (39.5 °C) as described (16). Coverslips were subsequently incubated at the permissive temperature (32 °C) for 90 min. The distributions of VSV-G, HA-syntaxin 5, and p58 were visualized using indirect immunofluorescence as described (16). A and B, distribution of VSV-G (A) and p58 (B). C and D, distribution of HA-syntaxin 5 (C) and p58 (D). Arrows indicate p58-containing intermediates; arrows indicate the juxtanuclear Golgi region.

When BHK cells were cotransfected with ts045 VSV-G and the full-length HA-syntaxin 5 for 5 h at the restrictive temperature, VSV-G was retained in the ER (data not shown). This result indicates that elevated levels of HA-syntaxin 5 do not interfere with the normal temperature-sensitive phenotype of ts045 VSV-G. HA-syntaxin 5, on the other hand, showed a typical steady state distribution to small punctate elements, which were distributed throughout the peripheral cytoplasm (Fig. 3C, arrows), and to the region encompassing the juxtanuclear Golgi stack (Fig. 3C, arrow). Similar results were observed in HeLa cells (data not shown) and were consistent with the reported distribution of syntaxin 5 in COS cells at 37 °C, where the protein was found to colocalize with β-COP (12), a marker for pre-Golgi intermediates and the cis elements of the Golgi stack (27, 33–35). In contrast to the distribution of HA-syntaxin 5, the distribution of HA-syntaxin 1A was largely restricted to the cell surface as described previously (12) (data not shown).

To identify the morphological step in transport inhibited by overexpression of syntaxin 5, BHK cells cotransfected with ts045 VSV-G and syntaxin 5 for 4 h at 39.5 °C were shifted to the permissive temperature and incubated for 90 min. In the presence of HA-syntaxin 5, ts045-VSV-G did not accumulate in
the compact Golgi stack as observed in control cells. In contrast, in nearly 80–90% of the cells examined, tsO45-VSV-G protein accumulated in small punctate structures scattered throughout the peripheral cytoplasm (Fig. 3A) and within and around the compact, juxtanuclear Golgi stack (Fig. 3A, arrow). All of the compartments containing VSV-G strongly overlapped with the distribution of p58 (Fig. 3, compare A to B, arrowheads), a protein preferentially enriched at steady state in pre-Golgi intermediates composed of clusters of vesicles and small tubular elements (25, 27, 36, 37) and in the cis elements of the Golgi stack. No effect of overexpression of HA-syntaxin 5 was observed on the distribution of the compact, perinuclear localization of the Golgi stack as determined by the distribution of the cis/medial marker enzyme α-1,2-mannosidase II (17) (data not shown). Strikingly, HA-syntaxin 5 was also found to precisely overlap with the punctate distribution of p58 (Fig. 3, compare C to D, arrowheads) at both low and high levels of expression. As expected, given the striking colocalization of VSV-G to p58 containing elements in the presence of elevated levels of HA-syntaxin 5, pre-Golgi intermediate accumulating VSV-G completely overlapped with the distribution of HA-syntaxin 5 (data not shown). Partial overlap of p58 and HA-syntaxin 5 was also observed with the distribution of Man II, consistent with the partial localization of p58 to cis elements of the Golgi stack (data not shown). Identical results were observed in HeLa cells during transient expression of VSV-G and HA-syntaxin 5 (data not shown). These results reinforce the interpretation from the biochemical analyses that syntaxin 5 plays a specific role in ER to Golgi transport and suggest a site of action in the docking and/or fusion of pre-Golgi intermediates.

**DISCUSSION**

We have demonstrated that syntaxin 5 is a functional component of the transport machinery involved in the delivery of pre-Golgi intermediates to the Golgi stack in mammalian cells. Given the morphological phenotype associated with overexpression of the protein, it is apparent that the full-length syntaxin 5 has a potent trans dominant negative effect on the function of ER to Golgi carrier vesicles. In contrast, full-length HA-syntaxin 1A had only a weak effect at high levels of expression, indicating the functional specificity of syntaxin 5. The combined results suggest that syntaxin 5 is likely to be the mammalian homologue of yeast Sed5p (15), a protein originally isolated as a suppressor for loss of Erd2p (the yeast homologue to the mammalian KDEL receptor) (38). Indeed, overexpression of Sed5p also leads to the accumulation of pre-Golgi intermediates in yeast (15). While the biochemical mechanism underlying the trans dominant effects of overexpression of syntaxin 5 is presently unknown, one possibility, among others, is that an excess of the protein restricts the availability of a component(s) involved in a late step in vesicle targeting and/or fusion.

Consistent with a role for syntaxin 5 in ER to Golgi transport, we found the protein was distributed to pre-Golgi transport intermediates composed of 60–80-nm carrier vesicles and small tubular elements present in the peripheral cytoplasm, and to the juxtanuclear Golgi region based on the distribution of Man II. The localization in this region with p58 and p56, which partially overlap with Man II, is suggestive of distribution to the cis elements of the Golgi stack (data not shown).

The striking distribution of HA-syntaxin 5 to pre-Golgi vesicular-tubular intermediates is difficult to reconcile with the concept that members of the syntaxin family function exclusively as docking receptors, such as has been suggested for the role of syntaxin 1A at the pre-synaptic membrane (1). This distribution was observed at both low and high levels of expression and is therefore unlikely to be an artifact of the transient expression system being used. In the proposed model for syntaxin function (1, 5), we would not expect a protein involved in the docking and/or fusion to the target compartment to be a component of the intermediate carrier vesicles en route. One possibility to explain this conundrum is that syntaxin 5 present on ER to Golgi intermediates may be an "inactive" recycling form. If so, then its activation, after encountering a component on the target compartment, would be the critical event directing vesicle docking and fusion. A second possibility is that the small tubular elements associated with pre-Golgi intermediates may comprise a stable "compartment" to which ER-derived vesicles fuse, although there is no evidence to date to support this interpretation (25). A third possibility is that syntaxin 5 is also involved in the formation or maturation of pre-Golgi intermediates. Consistent with this possibility, the early effect of depletion of Sed5p in yeast leads to the extensive elaboration of the ER, rather than accumulation of transport vesicles (15).