Mammalian Sly1 Regulates Syntaxin 5 Function in Endoplasmic Reticulum to Golgi Transport*

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Members of the syntaxin gene family are components of protein complexes which regulate vesicle docking and/or fusion during transport of cargo through the secretory pathway of eukaryotic cells. We have previously demonstrated that syntaxin 5 is specifically required for endoplasmic reticulum to Golgi transport (Dascher, C., Matteson, J., and Balch, W. E. (1994) J. Biol. Chem. 269, 29363–29366). To extend these observations we have now cloned a protein from rat liver membranes which forms a native complex with syntaxin 5. We demonstrate that this protein is the mammalian homologue to yeast Sly1p, previously identified as a protein which genetically and biochemically interacts with the small GTPase Ypt1p and Sed5p, proteins involved in docking/fusion in the early secretory pathway of yeast. Using transient expression we find that overexpression of rat liver Sly1 (rSly1) can neutralize the dominant negative effects of excess syntaxin 5 on endoplasmic reticulum to Golgi transport. These results suggest that rSly1 functions to positively regulate syntaxin 5 function.

Transport through the exocytic pathway is mediated by vesicular carriers (1). While a number of coat proteins, including clathrin, COP11, and COPI, have been shown to mediate vesicle budding from different cellular compartments (2–4), the biochemical machinery essential to promote vesicle docking and fusion is less well understood. One of the proteins participating in these events is the yeast protein Sly1p which was identified as a single gene suppressor of the loss of YPT1 function in yeast when carrying a point mutation (5). Sly1p is a member of a family of related proteins which includes the neuronal specific isoforms of Munc-18/n-Sec1/rb-Sec1 (6–9) as well as ubiquitously expressed Munc-18 isoforms in mammalian cells (10, 11). Sly1p-like homologues in Drosophila (Rop) (12) and Caenorhabditis elegans (Unc18) (15) for a recent review see Ref. 16. Yeast Sly1p and mammalian Munc-18/n-Sec1/rb-Sec1 have been shown to form complexes with different members of the syntaxin gene family including yeast Sed5 (17, 18) and the mammalian syntaxins 1–4 homologues (8–10), respectively, and in the case of Sly1p, the small GTPase Ypt1 (5, 17–25).

We have previously demonstrated that the Sed5 mammalian homologue, syntaxin 5, is essential for ER to Golgi transport (21). To extend our analysis of syntaxin 5 function in transport in mammalian cells, we have immunopurified a protein from rat liver membranes which forms a complex with syntaxin 5. Cloning of the protein based on peptide sequences led to the identification of a mammalian homologue to yeast Sly1p. We provide functional evidence that this protein is required for ER to Golgi transport in vivo.

EXPERIMENTAL PROCEDURES

Antibody Production and Purification—Soluble Syn 5-11 protein (21, 22) was used to generate polyclonal antibodies as described (23). Syntaxin 5-specific antibodies were affinity-purified using His6-syntaxin 5-11 recombinant protein covalently coupled to an Aminolink column (Pierce).

Preparation of Rat Liver Detergent Extracts and Immunoprecipitation—160 mg of rat liver crude microsomes (24) were pelleted by centrifugation at 100,000 × g for 30 min and then resuspended in 4.5 ml of solubilization buffer (20 mM HEPES-KOH (pH 7.2), 400 mM KCl, 1 mM DTT, 0.5 mM ATP, 1 mM MgCl2, 2 mM EDTA, 1 mM PMSF, and 2% CHAPS). After incubation on ice for 10 min, the lysate was diluted with an equal volume of dilution buffer (20 mM HEPES-KOH (pH 7.2), 1 mM DTT, 0.5 mM ATP, 1 mM MgCl2, 2 mM EDTA, and 1 mM PMSF), and insoluble material was removed by centrifugation at 100,000 × g for 30 min at 4 °C. The extract was then loaded on a 450-ml Sephacyl S-300 HR gel filtration column (Pharmacia Biotech) equilibrated in 20 mM HEPES-KOH (pH 7.2), 200 mM KCl, 1 mM DTT, 1 mM MgCl2, 2 mM EDTA, and 1% CHAPS. Aliquots of the fractions were tested for the presence of syntaxin 5 protein by SDS-PAGE and Western blotting. Syntaxin 5 was immunopurified with syntaxin 5 affinity-purified antibodies covalently coupled to protein A beads (17).

Peptide Sequencing—1.2 g of rat liver crude microsomes were used for immunoprecipitations. SDS-PAGE resolved a 42-kDa band (p42), corresponding to syntaxin 5 antigen, and a 66-kDa co-immunoprecipitating polypeptide band (p66) were excised and digested with trypsin. Tryptic peptides were separated, and selected peptides were subjected to chemical microsequencing. One peptide of the 42-kDa band (PVSALPGAVVQGPEFV) perfectly matched the sequence of syntaxin 5 (amino acids 161–174) confirming the identity of the syntaxin 5 antigen. Four tryptic peptide sequences were obtained from the 66-kDa band: (I) MLNFNVPHKNS, (II) VPAYVYPFTEE, (III) GTAAEMVAVK, and (IV) SNPETDDY. A search using the Blast WWW Server of the National Center for Biotechnology Information (NCBI) of GenBankTM and EMBL data bases (25) with peptide (II) identified p66 as the potential rat homologue of the Sly1p of Saccharomyces cerevisiae (5) and its homologue in C. elegans (26); GenBank™ accession number Z35640.

cDNA Cloning and Sequencing—Data bases of expressed sequence tags (EST) and nonredundant PDB, GenBank™ and EMBL sequence searches were used to identify homologues by Blast program provided by NCBI. Two partially sequenced human cDNAs, a human fetal heart EST cDNA (GenBank™ accession number Z35640).

DNA Cloning and Sequencing—Data bases of expressed sequence tags (EST) and nonredundant PDB, GenBank™ and EMBL sequence searches were used to identify homologues by Blast program provided by NCBI. Two partially sequenced human cDNAs, a human fetal heart EST cDNA (GenBank™ accession number Z35640).
Bank accession number R57959) and a human HepG2 3′ region cDNA (GenBank accession number D16890), were found that encoded predicted amino acid sequences 90% identical with peptides I and II of p66. The deduced polypeptides showed 38 to 60% identity with the yeast and C. elegans Sly1 proteins over 73 to 88 amino acid overlaps. For the amplification of rat Sly1 (rSly1) sequences by the polymerase chain reaction (PCR), two oligonucleotide primers were synthesized according to the human cDNA sequences. Oligonucleotide P1 was generated corresponding to nucleotides 62–93 (5′-CGATGTTGAATTCTCAATGTGCCCATATTAA-3′; sense primer) of the human HepG2 3′ region cDNA. Rat first strand cDNA was generated from total RNA of NRK cells using the Superscript Pre-amplification System for first strand cDNA synthesis (Life Technologies, Inc.). A 1.8-kilobase fragment was amplified using the NRK cDNA as a template and the Expand Long Template PCR system (Boehringer Mannheim) with the set of primers described above. The PCR product was subcloned into the TA cloning vector pCR1 (Invitrogen) and sequenced by the chain termination method (27). Based on the partial rSly1 cDNA sequence information, a second set of oligonucleotide primers was designed to be used in the amplification of the missing 5′- and 3′-cDNA ends (5′- and 3′-RACE; 3′-RACE primer P3, 5′-CGATCTCCATACAAATGTCGCCACTGCTG-3′; antisense primer P4, 5′-AGGGTCAGATGGAGGGACACG-3′; RACE reactions were performed using rat liver poly(A)+ RNA (Clontech) and the Marathon cDNA Amplification kit (Clontech). PCR products were subcloned into pCR1 and sequenced. In total, 6 clones were fully sequenced. The first methionine of the clone with the longest open reading frame is immediately preceded by a stop codon and a Kozak (28) consensus sequence making it likely to be the initiation codon. Sequence alignments were performed with the multiple alignment program Clustal W 1.5 (29) using the default parameters.

**FIG. 1. Identification of a p66-rSly1 complex with syntaxin 5.** Sepharose beads coupled to preimmune IgG (lane b) or two different affinity-purified syntaxin antibodies (0612 and 0613) (lanes c and d, respectively) were incubated overnight with a detergent extract of rat liver microsomes as described under “Experimental Procedures.” The bound protein was released as described (17), the eluate was precipitated with trichloroacetic acid, separated on SDS-PAGE, and silver-stained. p42/syntaxin 5 (Syn5) and p66/rSly1 are indicated by arrows. Molecular mass marker proteins are shown in lane a. Asterisks mark heavy and light chains of IgGs.

The tryptic peptides were used to search data bases of EST cDNAs and nonredundant sequences (see “Experimental Procedures”). Two potential human homologues were found. The deduced amino acid sequences were between 35 and 60% identical with the yeast and C. elegans Sly1 protein. Based on the sequence of the two human cDNAs, oligonucleotide primers were designed for the amplification of the rSly1 expression sequence using NRK cDNA as a template (see “Experimental Procedures”). The 5′ and 3′ cDNA ends missing in the original clone were amplified using rat liver cDNA as a template to yield the complete clone. The deduced sequence for rSly1 is shown in Fig. 2. The rSly1 open reading frame predicts a 72-kDa hydrophilic protein, which is in agreement with the size of the precipitated polypeptide of 66 kDa on SDS-PAGE. Rsl1 displays 30% identity with S. cerevisiae Sly1p and 42% identity with the Sly1 homologue of C. elegans when optimally aligned (Fig. 3). Identities were distributed over the entire length of the protein with divergent sequences evident principally in the amino- and carboxy-terminal portions of the three proteins (Fig. 3). The sequence shows only weak (~18%) identity with Sec1-related proteins functioning at the plasma membrane, emphasizing a potential role for rSly1 in ER to Golgi transport. This is consistent with previous studies in yeast which have established a strong interaction between the syntaxin 5 homologue Sed5p and Sly1p in ER to Golgi transport (37).

To explore the potential role of rSly1 in syntaxin 5 function, we describe the advantage of a transient expression system which we have used previously to examine the function of syntaxin 5 and the small GTPases Rab1, ARF1, and Sar1 in vesicle-mediated transport from the ER to the Golgi apparatus in mammalian cells in vivo (21, 30–32). For this purpose, we used a recombinant T7 vaccinia virus system to transiently co-express the specific cDNA of interest with the vesicular stomatitis virus glycoprotein (VSV-G). VSV-G is a type I transmembrane protein containing two N-linked carbohydrate chains. Vectorial transport of VSV-G from the ER to and through sequential cis-, medial-, and trans-Golgi compartments can be monitored by the processing of its two oligosaccharide chains from the high mannosomal (Man9) endoglycosidase H (endo H)-sensitive form found in the ER and pre-Golgi intermediates (R0 in Fig. 4) to endo H-resistant forms found in the Golgi stack (R4 and R5 in Fig. 4). The transient endo H-resistant (R4) form (Fig. 4) corresponds to the transport of VSV-G to the early cis-medial-Golgi compartment where one or both of the oligosaccharide chains becomes processed by the action of resident α1,2-mannosidases and glycosyltransferases. Subsequent transport of VSV-G to trans-Golgi compartments results in the appearance of the fully processed R5 form containing two complex, sialic acid-containing endo H-resistant oligosaccharides (Fig. 4). The sequential

**REFERENCES AND DISCUSSION**

To identify proteins which interact with syntaxin 5, we prepared a crude microsomal fraction containing smooth and rough microsomes as well as Golgi membranes as a source for potential syntaxin 5 complexes. Detergent extracts of crude membranes partially purified using gel filtration (see “Experimental Procedures”) were incubated with protein A beads covalently coupled to affinity-purified antibodies to syntaxin 5. The beads were washed extensively with increasing concentrations of salt prior to elution at low pH. Fig. 1 presents a silver-stained gel of eluates from beads attached to total IgG prepared from preimmune serum (Fig. 1b) and beads coupled to two different affinity-purified antibodies specific for syntaxin 5 (Fig. 1, c and d). Western blot analysis of extracts with a polyclonal antibody containing two complex, sialic acid-containing endo H-resistant oligosaccharides (Fig. 4).
appearance of each of these processing intermediates allows us to assess the differential requirement for factors regulating ER to Golgi and/or intra-Golgi transport.

BHK-21 cells infected with the recombinant vaccinia virus were co-transfected with VSV-G and various combinations of expression vectors carrying the Myc-tagged rSly1, syntaxin 5 or syntaxin 5–11, a truncated, cytosolic form of syntaxin 5 lacking the transmembrane domain. After incubation for 3–6 h to allow time for protein expression, transfected cells were incubated with [S\textsuperscript{35}]methionine for 10 min to label VSV-G in the ER, followed by a chase in the presence of unlabeled methionine for 60 min to promote the migration of VSV-G to the Golgi where it becomes processed to endo H-resistant R\textsubscript{1} and RT forms. Expression of recombinant Myc-rSly1 or Myc-syntaxin 5 was followed by Western blot analysis using the anti-Myc monoclonal antibody or a specific syntaxin 5 antibody, respectively. Overexpression of Myc-rSly1 had no effect on the processing of VSV-G to the RT form (Fig. 4\textsuperscript{A}), suggesting that elevated levels of the protein do not have a dominant negative effect on carrier vesicle function throughout the early secretory pathway. In contrast, expression of the Myc-tagged full-length syntaxin 5 strongly inhibited ER to Golgi transport, but not intra-Golgi transport as indicated by the efficient processing of VSV-G to the RT form (Fig. 4\textsuperscript{B}). This result is in agreement with previous studies where hemagglutinin (HA)-tagged syntaxin 5 was found to potently inhibit ER to Golgi transport (21).

The fact that yeast Sly1p shows biochemical interactions with Sed5p (17) and our ability to detect a prominent syntaxin 5–11 complex in rat liver membranes (Fig. 1) led us to examine if rSly1 will interact with syntaxin 5 to suppress the dominant negative effects of the protein during overexpression. Co-expression revealed that Myc-rSly1 and syntaxin 5 (or the soluble, truncated syntaxin 5–11) could be co-immunoprecipitated from lysates using either anti-Myc or anti-syntaxin 5 antibodies (data not shown), demonstrating that the identified clone expressed a protein capable of forming the specific complex observed in vivo. Furthermore, as shown in Fig. 4, co-expression of Myc-rSly1 with a level of syntaxin 5 sufficient to block transport (Fig. 4\textsuperscript{B}), significantly suppressed inhibition (Fig. 4\textsuperscript{C}). In this case, the level of VSV-G processing to the R\textsubscript{1} form was at least 2-fold higher than that observed in the absence of rSly1 (compare the value of R\textsubscript{1} at 5 h (16%) to the value of R\textsubscript{1} at 6 h (35%)). Even more potent suppression was observed when the truncated form of syntaxin 5, syntaxin 5–11, was co-expressed with rSly1. As shown in Fig. 4\textsuperscript{D}, in agreement with previous results (21), this cytosolic form of syntaxin interferes with transport between the ER and the Golgi as well as between compartments of the Golgi stack. This more general effect is characterized by the accumulation of VSV-G in both the R\textsubscript{1} and RT forms (Fig. 4\textsuperscript{D}). In the presence of rSly1, the partial inhibition of transport by syntaxin 5–11 between all compartments was completely suppressed, with VSV-G accumulating exclusively in the RT form (compare R\textsubscript{1} and R\textsubscript{1} in D at 5 h to R\textsubscript{1} and R\textsubscript{1} in E at 6 h). These results suggest that rSly1 is likely to interact with syntaxin 5 through its cytosolic domain in vivo.

In this study, we have purified to homogeneity, cloned, and sequenced the rat homologue to yeast Sly1p. This protein, originally isolated as a suppressor to the loss of Ypt1p function (5), is recognized to play an essential, but as yet undefined role in ER to Golgi transport in yeast (5, 20). Our biochemical data argue strongly for a direct role of rSly1 in syntaxin 5 function in mammalian cells. Not only was the protein purified as a native complex with syntaxin 5 in detergent extracts of rat liver membranes, but the sequence of the cloned fragment of rSly1 is identical to the published sequence of rat liver Sly1p (20).

The nucleotide and deduced amino acid sequences of rat Sly1 (rSly1) are shown in Fig. 2. Tryptic peptides of p66/rSly1 that were identified by microsequencing are underlined. Oligonucleotide primers used for the amplification of rSly1 cDNA sequences are indicated by dashed arrows

FIG. 2. Nucleotide and deduced amino acid sequences of rat Sly1 (rSly1). Tryptic peptides of p66/rSly1 that were identified by microsequencing are underlined. Oligonucleotide primers used for the amplification of rSly1 cDNA sequences are indicated by dashed arrows.

FIG. 3. Comparison of the amino acid sequence of rSly1 with its \textit{S. cerevisiae} (S.c.) and \textit{C. elegans} (C.e.) homologues. Identical amino acid residues between at least two of the proteins are indicated by black boxes. Shaded boxes highlight conservative amino acid substitutions.
Fig. 4. Overexpression of rSly1 suppresses the inhibitory effect of syntaxin 5. BHK-21 cells were co-transfected with VSV-G alone (control) or either with Myc-rSly1 (A), Myc-syntaxin 5 (Syn5) (B), Myc-syntaxin 5 and Myc-rSly1 (C), Myc-syntaxin 5–11 (Syn5–11) (D), or Myc-syntaxin 5–11 and myc-rSly1 (E). Cells were labeled for 10 min with [35S]methionine at the indicated time (3, 5, or 6 h post-transfection) and chased for 60 min in the presence of radioactive free medium, transferred to ice, lysed, and digested with endoglycosidase H (endo H) as described (33). The endo H-sensitive ER and pre-Golgi (R₁) form and the endo H-resistant Golgi-processed R₁ and R₂ forms were separated using SDS-PAGE, and autoradiographs were quantitated using a PhosphorImager (Molecular Dynamics, Sunnyvale, CA) as described (33). The level of expression of the recombinant proteins shown in the lower panel was determined by Western blotting using the Myc-specific antibody 9E10 for Myc-rSly1 or a specific antibody for syntaxin 5. The amount of bound antibody was detected using the enhanced chemiluminescence (ECL) system (Amersham) as described (21).

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