Communication

N-Ethylmaleimide-sensitive Factor (NSF) and α-Soluble NSF Attachment Proteins (SNAP) Mediate Dissociation of GS28-Syntaxin 5 Golgi SNAP Receptors (SNARE) Complex*

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Golgi soluble N-ethylmaleimide-sensitive factor attachment protein receptors (SNAREs) GS28 and syntaxin 5 can be reciprocally communoprecipitated from Golgi extracts, suggesting that they exist in a protein complex. When Golgi extract is preincubated with soluble NSF attachment proteins (α-SNAP) and N-ethylmaleimide-sensitive factor (NSF) under conditions that allow ATP hydrolysis by NSF, GS28 and syntaxin 5 become dissociated. GS28 and syntaxin 5 remain in a protein complex when Golgi extract is preincubated with similar amounts of α-SNAP and NSF under conditions that prevent ATP hydrolysis by NSF, suggesting that ATP hydrolysis by NSF is necessary for dissociating the GS28-syntaxin 5 complex. Since preincubation of Golgi extract with either α-SNAP or NSF alone has no effect on the GS28-syntaxin 5 complex, a concerted action of α-SNAP and NSF therefore mediates the dissociation of the GS28-syntaxin 5 complex. Furthermore, GS28 but not syntaxin 5 is capable of binding to immobilized α-SNAP when the GS28-syntaxin 5 complex is dissociated.

Participation of N-ethylmaleimide-sensitive factor (NSF) and soluble NSF attachment proteins (SNAPs) in diverse transport events (1–4) depends on a family of proteins termed SNAP receptors (SNAREs) associated with the vesicles and target membranes. SNAPs are key determinants for vesicle docking and fusion. The current view based on the SNARE hypothesis is that the specific docking and fusion of vesicles with the target compartment is primarily mediated by interaction between v-SNAREs on vesicles and t-SNAREs on the target membrane (5–11).

Yeast Sed5p is a syntaxin-like protein that functions as a t-SNARE of the cis-Golgi compartment for ER-derived vesicles (12). Syntaxin 5 is a mammalian counterpart of Sed5p and is associated preferentially with the cis-Golgi (13, 14). Syntaxin 5 has been shown to participate in ER-Golgi transport in mammalian cells (15). GS28 (also termed GOS-28) is a recently identified SNAP associated with the cis-Golgi, which participates in ER-Golgi transport (16–18). However, more mechanistic aspects remain to be established for the involvement of GS28 and syntaxin 5 in ER-Golgi transport with regard to their inter-relationship and their interaction with the general factors NSF and SNAP.

In this report, we demonstrate that GS28 and syntaxin 5 exist in a protein complex in Golgi extracts and that this complex can be dissociated by the concerted actions of NSF and α-SNAP. In addition, the dissociated GS28 but not syntaxin 5 remains capable of interacting with α-SNAP.

EXPERIMENTAL PROCEDURES

Expression and Purification of Recombinant Proteins—Expression and purification of His6-tagged NSF and α-SNAP have been described previously (18). For expression of the cytoplasmic domain of syntaxin 5 (C-terminally HisX6 tagged version), primers 1 (5′-CCCACATGGCTATGTCTGCGGGATGCGACC) and 2 (5′-TCGGATCCGTTGGACCATGACCACCGATT) were used to amplify the coding sequence by PCR. The PCR product was digested with Ncol and BamHl, ligated into the corresponding sites of pET28a, and transformed into Escherichia coli BL21(DE3). Recombinant protein was purified as described previously for NSF and α-SNAP (18). For the expression of the cytoplasmic domain of syntaxin 5 fused to the C terminus of GST (GST-syntaxin 5), primer 3 (5′-TCGGATCCGTTGGACCATGACCACCGATT) and primer 4 (5′-GCTCTAGACCTATTTTGACCATGAGCCACCGATT) were used to retrieve the coding sequence by PCR. The PCR product was digested with SmaI and XhoI and ligated into pGEX-KG (19), which had been digested similarly, GST fusion protein was purified as described (20).

Preparation and Purification of Rabbit Antibodies against Syntaxin 5—100 μg of purified HisX6-tagged syntaxin 5 was emulsified with complete Freund’s adjuvant and injected subcutaneously into New Zealand White rabbits. Booster shots containing the same amount of protein emulsified with incomplete Freund’s adjuvant were administered at intervals. Rabbits were bled 10 days after the third and subsequent injections. Affinity purification of antibodies was performed using GST-syntaxin 5, which had been cross-linked to glutathione-Sepharose beads with dimethylpimelidate (21) using a procedure described previously (20).

Immunoprecipitation—Antibodies cross-linked to Protein A-Sepharose beads with dimethylpimelidate (21) were used in immunoprecipitation experiments. Golgi membranes solubilized in extraction buffer (20 mM Hepes, pH 7.3, 100 mM KCl, 2 mM EDTA, 1 mM dithiothreitol, 1% Triton X-100) were incubated with Protein A-Sepharose beads bearing the respective antibodies for 2 h at 4 °C. Beads were spun down, washed 4 times in the extraction buffer and twice in buffer minus Triton X-100, boiled in SDS-PAGE sample buffer, and resolved by SDS-PAGE followed by immunoblotting.

Indirect Immunofluorescence Microscopy, SDS-PAGE, and Immunoblot Analysis—These were performed as described previously (17, 20). Some immunoblots were analyzed by using the Supersignal chemiluminescent kit (Pierce), which employed goat anti-mouse or goat anti-rabbit antibodies conjugated to horseradish peroxidase, as recommended by the manufacturer.

RESULTS AND DISCUSSION

Characterization of Polyclonal Antibodies against Syntaxin 5—Rabbits were immunized with purified recombinant C-terminal HisX6-tagged cytoplasmic domain of syntaxin 5 (Syn5-HisX6). Antibodies specific for syntaxin 5 were purified on
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**Fig. 1. Characterization of syntaxin 5 antibodies.** A, antibodies against syntaxin 5 detect specifically two polypeptides of about 32 and 38 kDa enriched in the Golgi membrane (GM) as compared with the total membrane (TM) and microsomal membrane (MM). Enrichment of syntaxin 5 in the Golgi membrane is comparable with that of α2,6-sialyltransferase (ST). B, antibodies against syntaxin 5 label the Golgi apparatus (a) marked by GS28 (b). Bar, 10 μm.

beads coupled with the recombinant cytoplasmic domain of syntaxin 5 fused to GST (GST-Syn5). As shown in Fig. 1 (lanes 4–6), the affinity-purified antibodies specifically detected polypeptides of 32 and 38 kDa that were highly enriched in the Golgi membrane as compared with the total membrane and microsomal membrane. The detection of these two polypeptides was abolished by preincubation of the antibodies with either Syn5-HisX6 or GST-Syn5 (data not shown). The enrichment of syntaxin 5 in the Golgi fraction is comparable with that of α2,6-sialyltransferase (lanes 1–3). When the antibodies were used for indirect immunofluorescence microscopy, syntaxin 5 (Fig. 1B, a) was found to be associated with the Golgi apparatus marked by GS28 (b), a cis-Golgi SNARE (16–18). These results establish that the antibodies are specific for syntaxin 5.

**GS28 and Syntaxin 5 Exist in a Protein Complex—**Since GS28 and syntaxin 5 are both associated with the cis-Golgi region and both function as SNAREs in ER–Golgi transport (13–16, 18), we have investigated their possible interaction. When Golgi extract was immunoprecipitated with various antibodies and the immunoprecipitate analyzed by immunoblotting to detect GS28 (Fig. 2, left panel), GS28 was present not only in the immunoprecipitate by antibodies against GS28 (lane 2) but also antibodies against syntaxin 5 (lane 3). The coimmunoprecipitation of GS28 by antibodies against syntaxin 5 suggests that GS28 exists in a syntaxin 5-containing protein complex. This conclusion is further sustained by the observation that syntaxin 5 could be immunoprecipitated from the Golgi extract by not only antibodies against syntaxin 5 but also by antibodies against GS28 (Fig. 2, right panel). The reciprocal co-immunoprecipitation clearly establishes that GS28 and syntaxin 5 exist in the same protein complex.

**Dissociation of GS28-Syntaxin 5 SNARE Complex by NSF and α-SNAP—**SNAREs play a key role in vesicle docking and fusion. Since NSF and α-SNAP participate in diverse transport events by interacting with SNAREs (4–11), we have investigated whether the GS28-syntaxin 5 complex is regulated by NSF and/or α-SNAP. As shown in Fig. 3, NSF (lanes 2) or α-SNAP (lane 3) alone did not affect the GS28-syntaxin 5 SNARE complex. In the presence of α-SNAP, NSF dissociated the GS28-syntaxin 5 complex in a dose-dependent manner under conditions that allow ATP hydrolysis by NSF (lanes 4–7). However, dissociation of the GS28-syntaxin 5 complex was not significant when similar amounts of α-SNAP and NSF were present in conditions that prevent ATP hydrolysis by NSF (lane 8). These results demonstrate that the GS28-syntaxin 5 complex could be dissociated by the concerted actions of α-SNAP and NSF and that ATP hydrolysis by NSF is essential for this dissociation.

**Dissociated GS28 but Not Syntaxin 5 Interacts with α-SNAP—**Both GS28 and syntaxin 5 in the Golgi extract were retained by immobilized α-SNAP fused to GST (GST-α-SNAP) in either assembly (Fig. 4, lane 1) or disassembly (lane 7) buffers, suggesting GS28 and/or syntaxin 5 could interact with α-SNAP. In the conditions that prevent ATP hydrolysis by NSF (lanes 2–6), the retention of GS28 and syntaxin 5 was not affected by NSF. However, in conditions that allow ATP hydrolysis by NSF and dissociation of the GS28-syntaxin 5 complex (lanes 8–12), NSF, in a dose-dependent manner, abolished the association of syntaxin 5 but not GS28 with the immobilized α-SNAP. Under the disassembly conditions in the presence of NSF, the amounts of GS28 retained are reduced to about 30–50% of those retained in the assembly conditions; this could be explained by the possibility that the affinity of dissociated GS28 for α-SNAP may be lower than that of the GS28-syntaxin 5 complex. This result suggests that a significant amount of dissociated GS28 but not syntaxin 5 could still interact with α-SNAP and that GS28 may be responsible for the retention of the GS28-syntaxin 5 complex by immobilized α-SNAP. This observation suggests that α-SNAP could interact with a single SNARE even when it is not present in a SNARE complex, in contrast to the original proposal that α-SNAP binds only with a paired SNARE complex (10).
Conclusions—GS28 and syntaxin 5 have previously been shown to participate in ER-Golgi transport by functioning as SNAREs (13–16, 18). However, more mechanistic aspects remain to be established. Several novel points in our study have shed more light onto our understanding about the mechanistic involvement of GS28, syntaxin 5, NSF, and α-SNAP in ER-Golgi transport. First, our demonstrations that GS28 and syntaxin 5 exist in a SNARE complex in the Golgi detergent extract suggest that interaction of GS28 with syntaxin 5 is important for ER-Golgi transport. There exist two possible explanations for the observed GS28-syntaxin 5 complex in the Golgi extract. One is that GS28 and syntaxin 5 exist in a protein complex in vivo and this complex is directly involved in ER-Golgi transport. Alternatively, the majority of GS28 and syntaxin 5 do not exist in a stable complex in vivo, and they are incorporated only transiently into a SNARE complex coupled to ER-Golgi transport. Detergent lysis of Golgi membranes may result in the formation of a stable GS28-syntaxin 5 complex in a way that mimics the transient formation of the in vivo complex. Second, our findings that the GS28-syntaxin 5 complex could be dissociated by α-SNAP and NSF in conditions that allow ATP hydrolysis by NSF provide direct evidence that one aspect of the involvement of α-SNAP and NSF in ER-Golgi transport is to dissociate the GS28-syntaxin 5 SNARE complex. In addition, the observation that dissociated GS28 but not syntaxin 5 was retained by immobilized α-SNAP suggests that GS28 is a key molecule responsible for interaction with α-SNAP and that GS28 can interact with α-SNAP even when it is not in a SNARE complex.

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Note Added in Proof—A recent study by Hay et al. (Hay, J. C., Chao, D. S., Kuo, C. S., and Scheller, R. H. (1997) Cell 89, 149–158) has also demonstrated that GS28 and syntaxin 5 exist in a SNARE complex.

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