Subunit Structure of a Mammalian ER/Golgi SNARE Complex*

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SNARE receptor (SNARE) complexes bridge opposing membranes to promote membrane fusion within the secretory and endosomal pathways. Because only the exocyotic SNARE complexes have been characterized in detail, the structural features shared by SNARE complexes from different fusion steps are not known. We now describe the subunit structure, assembly, and regulation of a quaternary SNARE complex, which appears to mediate an early step in endoplasmic reticulum (ER) to Golgi transport. Purified recombinant syntaxin 5, membrin, and rbet1, three Q-SNAREs, assemble cooperatively to create a high affinity binding site for sec22b, an R-SNARE. The syntaxin 5 amino-terminal domain potently inhibits SNARE complex assembly. The ER/Golgi quaternary complex is remarkably similar to the synaptic complex, suggesting that a common pattern is followed at all transport steps, where three Q-helices assemble to form a high affinity binding site for a fourth R-helix on an opposing membrane. Interestingly, although sec22b binds to the combination of syntaxin 5, membrin, and rbet1, it can only bind if it is present while the others assemble; sec22b cannot bind to a pre-assembled ternary complex of syntaxin 5, membrin, and rbet1. Finally, we demonstrate that the quaternary complex containing sec22b is not an in vitro entity only, but is a bona fide species in living cells.

Complexes of soluble N-ethylmaleimide-sensitive factor attachment protein receptors (SNAREs)1 on opposing membranes are required for membrane fusion within the secretory pathway (1, 2). The atomic structure of a single SNARE complex that is required for synaptic vesicle fusion has been solved and was shown to consist of a highly twisted parallel bundle of four amphipathic helices, one from the vesicle protein VAMP, one from the plasma membrane protein syntaxin 1A and two from the plasma membrane protein SNAP-25 (3, 4). Although the contacting surfaces of the helices are mostly hydrophobic, a layer of residues in the center of the bundle, referred to as the “zero layer,” are comprised of glutamates for the plasma membrane SNAREs (Q-SNAREs) and arginine for the vesicle SNARE (an R-SNARE). Most SNARE proteins appear to possess either a glutamine or arginine at that deduced position, although the functional importance of these residues are not known (5). Although the precise role of SNAREs in the specific docking and fusion of transport vesicles remains controversial, different types of experiments indicate that SNARE complexes are the core machinery for intracellular membrane fusion. For example, purified synaptic SNAREs catalyze the rapid and efficient fusion of synthetic vesicles (6, 7).

Our understanding of SNARE function is limited by a lack of comparisons between SNARE complexes from different fusion events. Without structural knowledge of several SNARE complexes, we cannot understand the functional distinction between Q- and R-SNAREs, nor the general significance of the synaptic four-helix bundle. Although many SNAREs have been discovered and implicated in membrane fusion events, it is only the exocyotic complex for which even a subunit structure is known.

SNARE complexes outside the synapse have been studied primarily using immunoprecipitation experiments in cellular detergent extracts. Five SNAREs involved in yeast vacuolar fusion were shown to coprecipitate, implying the existence of a pentameric cis-SNARE complex (8). However, because at least one of these proteins, Ykt6p, has been shown to operate in multiple transport steps (8, 9), it is possible that the five associated SNAREs are present in two or more distinct complexes with overlapping members. Likewise, in ER/Golgi transport, yeast Sed5p (and mammalian syntaxin 5) coprecipitated with an array of other SNAREs (10, 11), including Gos1p, Bos1p, Sec22p, Ykt6p, and Bet1p (in mammals syntaxin 5 communoprecipitated GOS-28, membrin, sec22b, and rbet1). Further immunoprecipitations in mammals demonstrated that syntaxin 5 and GOS-28 appeared to be in separate complexes from syntaxin 5 and sec22b, membrin, and rbet1 (12). The latter group probably functions in membrane fusion between ER-derived vesicles and vesicular tubular clusters (VTCs) or between homotypically fusing ER-derived vesicles (13). Syntaxin 5, rbet1, membrin, and sec22b have all been localized by immuno electron microscopy to VTCs (12), and all four of their yeast counterparts exist on ER-derived vesicles generated in vitro (14, 15, 16). These four ER/Golgi SNAREs could either form two or more overlapping ternary complexes or a single quaternary complex containing all four members. Immunoprecipitation experiments could not distinguish between these possibilities, whereas functional results from yeast mutants suggested that members of this group of proteins functioned in at least two distinct fusion complexes (17). The precise compositions of ER/Golgi SNARE complexes were not clearly demonstrated using purified yeast ER/Golgi SNAREs, because Sed5p appeared to engage in several potentiated ternary complexes.
including Sed5p, Sec22p, Bos1p, and Sed5p-Bet1p-Bos1p (18, 19). Which one of these ternary complexes, if any, was a complete SNARE complex? The simple possibility that all four proteins form a single quaternary complex has not been experimentally addressed.

We used purified mammalian ER/Golgi SNAREs to precisely define the higher-order SNARE complexes they can form in vitro. Here we document the subunit composition, assembly, and regulation of a quaternary SNARE complex that likely mediates the first fusion event in the secretory pathway. The complex demonstrates a remarkable similarity to the synaptic fusion complex, establishing a pattern to be repeated at other transport steps. Syntaxin 5, membrin, and rbet1, three Q-SNAREs, assemble to create a high affinity binding site for sec22b, an R-SNARE. This assembly pattern is consistent with sec22b opposing the other three proteins on opposite membranes, as VAMP opposes SNAP-25 and syntaxin 1 in the synapse. The resulting quaternary complex has a stoichiometry of 1:1:1:1 (Q:Q:R, as in the synapse), perhaps arranged in a four-helix bundle as in the synapse, but where the Q-helices of membrin and rbet1 (one from each) play the role of the two SNAP-25 helices. In addition, we have explored the kinetic assembly mechanism of the ER/Golgi complex and its negative regulation by the syntaxin 5 amino-terminal domain.

**EXPERIMENTAL PROCEDURES**

**Antibodies**—Monoclonal and affinity purified polymerized anti-SNARE antibodies were described previously (11, 12). Anti-VAMP2 polyclonal antibody was obtained from Stressgen (Victoria, British Columbia, Canada).

**Expression and Purification of ER/Golgi SNAREs**—GST fusion proteins were expressed in *Escherichia coli* using vector pGEX-KG (20), whereas hexahistidine-tagged sec22b employed pQE-9 (Qiagen). See the Fig. 1 legend for the specific residues of each protein expressed. Bacteria were grown in Luria Broth at 37 °C to an *A*<sub>600</sub> of 0.4–0.5, when they were either shifted to 15 °C (for GST-syntaxin 5 constructs) for 30 min prior to protein induction at 15 °C for 2–3 h, or induced immediately at 37 °C (all other constructs) for 2–3 h. GST fusion protein production was induced with 0.1 mM isoprropyl-1-thio-β-D-galactopyranoside; His<sub>6</sub>-sec22b with 1 mM isoprropyl-1-thio-β-D-galactopyranoside; His<sub>6</sub>-sec22b with 1 mM isoprropyl-1-thio-β-D-galactopyranoside; His<sub>6</sub>-sec22b with 1 mM isoprropyl-1-thio-β-D-galactopyranoside. After harvesting the cultures, bacteria were resuspended in 50 mM Tris, pH 7.6, 0.1 M potassium chloride, 1 mM EDTA, 0.05% Tween 20, 1 mM DTT, 2 μg/ml aprotinin, 1 μg/ml pepstatin A, and 1 mM phenylmethysulfonyl fluoride; for sec22b, the same buffer lacking DTT) at 20 °C for 20 min, and the supernatant (S1) was recovered. For His<sub>6</sub>-sec22b, the final supernatant was loaded onto a column of Ni<sup>2+</sup> Sepharose G-25 (Amersham Pharmacia Biotech), cleaved in solution with thrombin, passed back over glutathione-Sepharose to deplete GST and uncleaved fusion protein, and then purified on velocity gradients as described above. Ni<sup>2+</sup>-NTA-agarose-purified sec22b was separated from a major degradation product by two consecutive runs on a 100-ml Superose 12 column (Amersham Pharmacia Biotech) run in 20 mM Tris, pH 7.6, 0.1 mM KCl, 1 mM EDTA. The purified His<sub>6</sub>-sec22b was then dialyzed into Buffer A, supplemented with protease inhibitors as above, and stored at −80 °C.

**Binding Assays**—All binding incubations were conducted in Buffer A (see above) containing 0.1% Triton X-100. A typical bead-binding reaction consisted of 10 μl of 50% glutathione-Sepharose beads containing 20 μg/ml of immobilized protein (equivalent to 0.1–0.3 pmol of soluble binding partners in a final volume of 200 μl. After 1 h at 4 °C with constant agitation, beads were washed three times with Buffer A plus 0.1% BSA prior to solubilization of the bound proteins in SDS-PAGE sample buffer. All Western blots that were quantified utilized 125I-labeled secondary antibodies and standards quantified relative to BSA. For solution binding assays, which were incubated for varying time periods at 4 °C (see each figure legend), a 300-μl reaction typically contained ~600 pmol (2 μM) of each protein, 250 μl of which was injected onto a 24-ml Superdex 200 gel filtration column (Amersham Pharmacia Biotech) run in Buffer A containing 0.1% Triton X-100 and 30 μg/ml BSA. Individual column fractions were either analyzed directly by SDS-PAGE and Western blotting or were precipitated with acetone prior to gel and Western analysis.

For the experiment in Fig. 8, A and B, typical ternary or quaternary binding incubations were carried out for 1 h, followed by gel filtration as usual. To the purified ternary complex (fraction 17) we added twice the normal concentration of sec22b and incubated for an additional hour. This fraction along with the corresponding purified quaternary complex fraction were then mock-immunodepleted using BSA-blocked 1:1 protein complexes. The G bands and the supernatant were collected and centrifuged at 100,000 × g for 30 min. Supernatant from the 100,000 × g spin was immunoprecipitated using BSA-blocked protein A/G beads for a control or the same beads containing covalently cross-linked (using dimethylimelidimine) monoclonal 4E11 anti-erb1 antibodies. The beads were then washed three times with Buffer A containing 0.1% Triton X-100, and noncovalently bound proteins were solubilized for SDS-PAGE using the immobilized protein 4E11 immunoprecipitation section.

**Stoichiometry Experiments**—E. coli strain NM522 containing the protein construct was streaked on minimal M9 plates and grown 48 h at 37 °C. Fresh colonies were then used without storage to inoculate 100 ml of minimal medium (1X M9 salts, 0.2% dextrose, 0.1 mM CaCl<sub>2</sub> 2.0 mM MgCl<sub>2</sub>, 50 μg/ml ampicillin, and 10 μg/ml thiamine hydrochloride) and grown overnight to high density. One ml of this culture was then used to inoculate fresh 100-ml cultures in the medium containing 0.5 mM of [U-14C]glucose (ICN cat. no. 1104701, 316 mCi/mmol). Protein induction and purification were essentially as described above. Solution-binding reactions were similar to nonradioactive reactions (above). After gel filtration as usual, fractions 17 and 18 were pooled and either directly acetate precipitated or immunoprecipitated with monoclonal 4E11 anti-erb1 antibody covalently cross-linked (using dimethylimelidimine) to a 1:1 mixture of protein A- and protein G-Sepharose (Amersham Pharmacia Biotech). Samples were solubilized in SDS-PAGE sample buffer and electrophoresed using high Tris/urea gels (21) as described. Gels were Coomassie Blue-stained and dried, and bands were first quantitated with a laser densitometer and then excised from Kodak Biomax-MS x-ray film with a Kodak Biomax Transilluminator LE intensifying screen. Dried gels were stained, minced, and incubated overnight at 50 °C with 1 ml of T2-2 soluble solubilizer (Research Products International) and counted in 10 ml 3a20 tolune-based counting mixture (Research Products International). To calculate molar stoichiometries using Coomassie Blue staining, stain intensities were divided by the predicted protein molecular weight and expressed relative to syntaxin 5. To calculate stoichiometries using
RESULTS

Expression and Purification of ER/Golgi SNAREs—We expressed in bacteria the following protein constructs: 1) GST fused to the full cytoplasmic domain of the shorter isoform of rat syntaxin 5 (amino acids 55–333 where amino acid 1 would be the N terminus of the longer syntaxin 5 isoform, Ref. 24), 2) GST fused to full-length rat membrin (amino acids 2–212), 3) hexahistidine-tagged mouse sec22b cytoplasmic domain (amino acids 2–196), and 4) GST fused to rat bet1 cytoplasmic domain (amino acids 1–95). Although we were unable to express the cytoplasmic domain of membrin in bacteria, we found that the full-length protein expressed well and was partially soluble. In addition, we found that partially soluble GST-membrin and GST-syntaxin 5 fusion proteins could be completely solubilized using the ionic detergent sarkosyl (25), which was then replaced with Triton X-100 during the purification on glutathione-Sepharose (see “Experimental Procedures” for details). The resulting GST-syntaxin 5 and GST-membrin were fully soluble and functional in binding reactions. All expressed proteins were first purified by glutathione-Sepharose or nickel-affinity chromatography, the GST moiety was cleaved from GST fusion proteins and subsequent purification steps are described under “Experimental Procedures.” Approximately 200 ng of each protein was loaded in each gel lane. The His6-sec22b and rbet1 constructs encoded essentially the entire cytoplasmic domains of the proteins (2–196 and 1–95, respectively). Rbet1 appears as a doublet of bands that behave nearly identically in binding assays; the lower band is a carbonyl-terminal degradation/truncation product (amino acids 1 to ~90). Syntaxin 5 encoded the entire cytoplasmic domain of the smaller 34-kDa syntaxin 5 isoform (residues 55–333 of the entire syntaxin 5 open reading frame, Ref. 24). The upper syntaxin 5 band contains residues 55–333, the second band is an amino-terminal degradation product (amino acids 70–333), and the third band is a carbonyl-terminal degradation product (amino acids 55–251) produced during thrombin digestion (see the legend to Fig. 7 for details). The upper of the two syntaxin 5 degradation products was active in some binding reactions, the lower was not. The membrin construct encoded essentially the entire protein including the transmembrane domain (residues 2–212). These four purified protein constructs were used throughout our studies except where specifically stated otherwise.

Binary Interactions of ER/Golgi SNAREs—Although all of the four VTC-localized ER/Golgi SNAREs coprecipitated in detergent extracts (12), we wanted to characterize the sets of direct protein interactions responsible for their association. We therefore immobilized GST-rbet1, GST-membrin, and GST-syntaxin 5 onto glutathione beads and incubated them separately with soluble rbet1, membrin, syntaxin 5, or sec22b. After washing with buffer, the amount of each soluble protein bound was determined by quantitative Western blotting. As shown in Fig. 2, B–D, we detected strong direct, binary interactions between syntaxin 5 and rbet1 and syntaxin 5 and membrin. We also found that rbet1 and membrin bound each other with high affinity. Interestingly, sec22b did not interact with any of these proteins with comparable affinity. Thus, it would appear that the tight association of sec22b with the other SNAREs observed in cellular extracts (12) results either from indirect binding or else cooperative binding where sec22b binds only to an assemblage of these blazages.

Note that soluble syntaxin 5 binds well to immobilized rbet1 (Fig. 2A) whereas soluble rbet1 did not bind to immobilized syntaxin 5 (Fig. 2C). This asymmetry in binding could be attributed to steric hindrance caused by the GST moiety in the latter binding reaction and points out the inherent limitations of solid-phase binding assays.

Formation of a Highly Cooperative Ternary Complex Composed of Rbet1, Syntaxin 5, and Membrin—Because syntaxin 5, rbet1, and membrin all bound strongly to each other in binary binding reactions (Fig. 2), we wondered whether they could form a higher-order complex involving all three proteins. To test this possibility, we continued with bead-binding assays using immobilized GST-membrin and soluble syntaxin 5 and rbet1. As shown in Fig. 3A, the specific binding of soluble syntaxin 5 to immobilized membrin was substantially increased in the presence of soluble rbet1, indicative of potentiated binding between the three proteins. Fig. 3B demonstrates that soluble rbet1 binding to immobilized membrin is increased by soluble syntaxin 5. Thus, the two soluble proteins mutually potentiate binding to form a ternary complex composed of membrin, rbet1, and syntaxin 5.

We examined the formation of the ternary complex in solution using gel filtration to resolve complexes containing syntaxin 5, rbet1, and membrin. Rbet1 gel filtered at a low molecular weight by itself or in pairwise combinations with membrin or syntaxin 5 (Fig. 4, panels 1–3 from top). These fractions (30–34) are consistent with a monomer or possibly dimer of rbet1. Only when all three proteins were combined did rbet1 shift to a high molecular weight peak (Fig. 4, panel 4 from top). The peak fractions also contained membrin (Fig. 4, panels 5

Radioactivity, 14C counts in each band were divided by the predicted number of carbon atoms in each protein construct (calculated from the sequence), and the resulting quotient was expressed relative to that of syntaxin 5.

Immunoprecipitation Experiments Using Cellular Extracts—NRK cells were transfected as before (22) with a HA-syntaxin 5 full-length construct in pCMV4 (23) or Myc-tagged full-length sec22b in pCDNA3 (11). Forty-eight hours after transfection, cells were depleted of intracellular ATP by rinsing them twice with glucose-free buffer (25 mM Hepes, pH 7.3, 70 mM sucrose, 130 mM NaCl, 4.8 mM KCl, 1.3 mM CaCl2, 1.2 mM MgSO4) and they were incubated for 10 min at 37 °C with the same buffer containing 0.2% iodoacetate acid (sodium salt) and 0.1 μM antimycin A. Cells were then solubilized in KCl buffer (20 mM Hepes, pH 7.2, 0.1 mM KCl, 2 mM EGTA, 2 mM EDTA) containing 1% Triton X-100, 1 mM DTT, 2 μg/ml leupeptin, 4 μg/ml aprotinin, 1 μg/ml pepstatin A, and 1 mM phenylmethylsulfonyl fluoride and centrifuged at 100,000 × g for 30 min. The clarified supernatant was subjected to immunoprecipitation using 1:1 protein A; protein G beads loaded with anti-Myc (9E10 from our tissue culture medium) antibodies covalently cross-linked with dimethylpimelimidate. 0.4 ml of extract was incubated with 10 μl of 50% antibody beads for 2 h. The beads were washed three times with KCl buffer containing 0.1% Triton X-100, and noncovalently bound proteins were solubilized by incubation for 5 min at room temperature with nonreducing SDS-PAGE sample buffer. After pelleting the beads, the bead-free supernatant was adjusted to ~5% β-mercaptoethanol and subjected to SDS-PAGE and Western blotting. The immunoprecipitations with wild-type NRK and PC12 cells shown in Fig. 10, B and C followed essentially the same protocol except that we used rabbit preimmune IgG or affinity-purified anti-syntaxin 5 antibody covalently cross-linked to protein A-Sepharose.

FIG. 1. Purified recombinant ER/Golgi SNAREs. Proteins were expressed as GST fusions (membrin, rbet1, and syntaxin 5) or a hexahistidine-tagged construct (His6-sec22b) in E. coli and purified by glutathione- or nickel-affinity chromatography. Thrombin cleavage of fusion proteins and subsequent purification steps are described under “Experimental Procedures.” Approximately 200 ng of each protein was loaded in each gel lane. The His6-sec22b and rbet1 constructs encoded essentially the entire cytoplasmic domains of the proteins (2–196 and 1–95, respectively). Rbet1 appears as a doublet of bands that behave nearly identically in binding assays; the lower band is a carbonyl-terminal degradation/truncation product (amino acids 1 to ~90). Syntaxin 5 encoded the entire cytoplasmic domain of the smaller 34-kDa syntaxin 5 isoform (residues 55–333 of the entire syntaxin 5 open reading frame, Ref. 24). The upper syntaxin 5 band contains residues 55–333, the second band is an amino-terminal degradation product (amino acids 70–333), and the third band is a carbonyl-terminal degradation product (amino acids 55–251) produced during thrombin digestion (see the legend to Fig. 7 for details). The upper of the two syntaxin 5 degradation products was active in some binding reactions, the lower was not. The membrin construct encoded essentially the entire protein including the transmembrane domain (residues 2–212). These four purified protein constructs were used throughout our studies except where specifically stated otherwise.

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and 6) and syntaxin 5 (Fig. 4, panels 7 and 8), confirming that a ternary complex was present. The estimated molecular weight of the ternary complex was ~250 kDa, which is larger than anticipated; however, we do not know whether the complex itself oligomerizes. Moreover, membrin with its transmembrane domain contains an unknown amount of bound Triton X-100, which has a micelle size of ~90 kDa. Thus, gel filtration under these conditions does not provide an accurate estimate of the protein mass in the complex.

Note that the potentiation observed with all three proteins in bead-binding studies (Fig. 3) was amplified many fold in the solution-binding studies where none of the proteins are concentrated on a surface (Fig. 4). Apparently only the very high affinity cooperative interactions of the ternary complex are able to persist during gel filtration in dilute solution, and thus binary combinations do not produce viable complexes. In some experiments, however, we did observe a binary interaction of rbet1 and membrin by gel filtration (not shown). The appearance of this complex depended on the concentrations and activity of particular preparations of proteins.

**Formation of a Highly Cooperative Quaternary Complex Composed of Rbet1, Syntaxin 5, Membrin, and Sec22b**—Because none of the ER/Golgi SNAREs contains two obvious coiled coil domains, as does SNAP-25, we considered the possibility that four ER/Golgi SNAREs could assemble into a single complex that could structurally resemble the synaptic ternary complex. Because sec22b did not bind with high affinity to any of the individual SNAREs (Fig. 2), it seemed possible that the assemblage of membrin, syntaxin 5, and rbet1 could create the binding site for sec22b, much as syntaxin 1 and the two SNAP-25 helices form a high affinity site for VAMP (3, 26, 27). This was tested using bead-binding assays where GST-membrin was immobilized on beads, and syntaxin 5, rbet1, and sec22b were added in soluble form. As shown in Fig. 5, top panels, sec22b bound specifically to the GST-membrin beads but bound strongly only when soluble rbet1 and syntaxin 5 were supplied, demonstrating the formation of a cooperative quaternary complex. Some binding was also observed to the combination of membrin and rbet1. But because equal amounts of rbet1 were bound irrespective of syntaxin 5 (Fig. 5, middle panels) and the membrin-syntaxin 5 complex itself elicited insignificant sec22b binding, the best interpretation of the data is that sec22b binds with highest affinity to the combination of membrin, rbet1, and syntaxin 5.

As with the ternary complex (Fig. 4), quaternary complex formation appeared much more cooperative when studied using a solution binding assay. As shown in Fig. 6, sec22b gel filters at a very low molecular weight in every combination except when all four proteins are present, when it incorporates into a high molecular weight complex. The peak fractions of high molecular weight complex sec22b (fractions 15–18) also contained elevated levels of syntaxin 5, membrin, and rbet1 (data not shown).

**The Syntaxin 5 Amino-terminal Domain Inhibits the Formation of ER/Golgi SNARE Complexes**—The exocytic syntaxin 1A and Sso1p amino-terminal helical domains appear to negatively regulate the entry of the coil domain into SNARE complexes (28, 29) and suppress the rate of liposome fusion stimulated by the purified synaptic SNAREs (6). To test whether the amino-terminal helical domains of syntaxin 5 played any role in ER/Golgi SNARE complex formation, we expressed and purified a construct consisting of only the membrane-proximal coiled coil domain (amino acids 251–333). We then used the solution-binding assay to test the relative abilities of the coil domain construct, or an equimolar amount of the usual full synaptic SNAREs to form a quaternary complex with soluble membrin, rbet1, and sec22b. As shown in Fig. 7B, syntaxin 5 residues 251–333 were sufficient for quaternary complex formation and is efficiently incorporated. In fact, this construct produced significantly more quaternary complex than did the full cytoplasmic domain (Fig. 7A), indicating that the amino-terminal domain hindered SNARE complex formation. However, we were not comfortable directly comparing the efficacy of these two proteins because syntaxin 5 (residues 55–333) is largely insoluble when expressed in bacteria and must be partially denatured and renatured in vitro before use (see “Experimental Procedures”), whereas syntaxin 5 (residues 251–333) is abundantly expressed as a soluble protein. We therefore took advantage of a naturally occurring thrombin cleavage site in syntaxin 5 to separate the amino-terminal helices from the coil domain after protein expression and purification. As shown in Fig. 7C, cleavage of purified syntaxin 5 (55–333) with thrombin results in the production of fragments containing residues 55–251 and 252–333. We were thus able to test the same preparation of syntaxin 5 cytoplasmic domain with or without thrombin cleavage to liberate the coiled coil. As shown in Fig. 7D, thrombin cleavage of syntaxin 5 resulted in remarkable activation of SNARE complex formation. This unambiguously demonstrates that the amino-terminal region of syntaxin 5 acts as a potent negative regulator of
A maximum of 6.6% of the added syntaxin 5 bound to the beads with a molar ratio of 0.044 (syntaxin 5/GST-membrin). In part B, staining of blots indicated that equal amounts of GST or GST-membrin were loaded for each condition (not shown). At the doses used in part A, a 1-h incubation at 4 °C, beads were washed three times and analyzed by quantitative immunoblotting for syntaxin 5 (A). The seventh and top four panels were immunoblotted for rbet1 ( ), membrin ( ), and gel-filtered on Superdex 200. Individual column fractions were mixed in solution for combinations (right) of three or four proteins. After a 2-h incubation at 4 °C, the proteins were gel filtered using Superdex 200 and individual column fractions were immunoblotted for sec22b. Globular molecular size markers (above) are as in Fig. 4. In the condition shown in the bottom panel, all four of the proteins overlapped in fractions 15–19 (not shown).

**FIG. 3. Syntaxin 5 and rbet1 potentiate each other in binding to bead-immobilized GST-membrin.** Soluble syntaxin 5 (400 nM in A, 2 µM in B) and rbet1 (3.1 µM in A, 1.5 µM in B) were mixed with glutathione beads preloaded with either GST or GST-membrin (620 nM). After a 1-h incubation at 4 °C, beads were washed three times and analyzed by quantitative immunoblotting for syntaxin 5 (A) or rbet1 (B). Ponceau staining of blots indicated that equal amounts of GST or GST-membrin were loaded for each condition (not shown). At the doses used in part A, a maximum of 6.6% of the added syntaxin 5 bound to the beads with a molar ratio of 0.044 (syntaxin 5/GST-membrin). In part B, 14.3% of added rbet1 bound with a molar ratio of 0.36 (rbet1/GST-membrin). All values represent the mean of two duplicates ± S.E.

**FIG. 4. Formation of a highly cooperative ternary complex composed of rbet1, membrin, and syntaxin 5.** Purified soluble SNARE proteins were mixed in solution for 1 h in the combinations indicated (right), and gel-filtered on Superdex 200. Individual column fractions were immunoblotted for rbet1 (top four panels), membrin (fifth and sixth panels) and syntaxin 5 (bottom two panels). The lower band in the seventh and eighth panels is a degradation product of syntaxin 5 (see Fig. 1). Elution positions of native tryptoglobulin (669 kDa), catalase (232 kDa), BSA (67 kDa), and chymotrypsinogen A (25 kDa) are indicated with arrows above the fraction numbers.

**FIG. 5. Sec22b binds to bead-immobilized membrin in the presence of soluble syntaxin 5 and rbet1.** Soluble sec22b (1.2 µg, 255 nM), syntaxin 5 (1.2 µg, 190 nM), and rbet1 (1.2 µg, 545 nM) were mixed as indicated with glutathione beads preloaded with either GST or GST-membrin (620 nM). After a 1-h incubation at 4 °C, the beads were washed three times with buffer and analyzed by immunoblotting for bound sec22b (top panel), rbet1 (middle panel) and syntaxin 5 (bottom panel). Ponceau staining indicated equal quantities of GST or GST-membrin were loaded in each lane (not shown). In this experiment, soluble syntaxin 5 did not potentiate the binding of rbet1 to GST-membrin beads (as it did in Fig. 3B), because a much lower concentration of syntaxin 5 was used.

ER/Golgi SNARE complex formation and once again points out the remarkable conservation of SNARE complex structure and function between the first fusion in the secretory pathway and the last. It remains to be seen which precise step in the assembly mechanism is regulated by the syntaxin 5 amino-terminal domain.

**Relationship of the Isolated Ternary and Quaternary Complexes**—We wondered whether the stable ternary complex purified in Fig. 4 was the direct precursor to the quaternary complex produced in Fig. 6. It seemed plausible that quaternary complex formation might occur by sec22b binding to a stable complex of syntaxin 5, membrin, and rbet1. On the other hand, one could imagine that simultaneous assembly of all four proteins might be required to form a quaternary complex. To test these possibilities, we isolated the ternary complex by gel filtration as in Fig. 4 and incubated it with soluble sec22b. We then immunoprecipitated the ternary complex and tested whether sec22b coprecipitated. To our surprise, we saw absolutely no coprecipitation of sec22b with the ternary complex, which was efficiently precipitated (Fig. 8, A and B). The immunoprecipitation utilized an rbet1 monoclonal antibody (4E11, Ref. 12) that does not affect the formation of the ternary complex and does not affect the association of sec22b with the quaternary complex (Fig. 8B). Thus, it appeared that the ternary complex, formed in the absence of sec22b and then isolated, was not a substrate for sec22b binding. We confirmed these results by preparing two binding reactions containing identical concentrations of soluble syntaxin 5, membrin, rbet1 and sec22b. In the first reaction, syntaxin 5, membrin and rbet1 were added first, the mixture was incubated 2.5 h, and then sec22b was added (sequential reaction). In the second mixture, all four proteins were added essentially simultaneously (simultaneous reaction). After incubation for 1 h with

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A. L. Williams and J. C. Hay, unpublished observations.
thrombin. The samples were then adjusted to 1 mM phenylmethylsulfonyl fluoride to inactivate thrombin and either analyzed directly by SDS-PAGE and Coomassie Blue staining (C), or combined with rbet1, sec22b, and membrin overnight at 4°C followed by gel filtration and immunoblotting for sec22b (D). Syntaxin 5-(55–333) is cleaved by thrombin following residue 251 to produce fragments Syntaxin 5-(55–251) and Syntaxin 5-(252–333). Of these two fragments, only Syntaxin 5-(252–333) is incorporated into the quaternary SNARE complex (not shown). The band marked by an asterisk in panel C, lane 1, is an amino-terminal degradation product from the original syntaxin 5-(55–333) preparation (see the Fig. 1 legend), which is cleaved by thrombin to produce the band marked by an asterisk in lane 2 and syntaxin 5-(252–333).

Sec22b, both reactions were gel filtered to determine the efficiency of sec22b incorporation into quaternary complexes. As shown in Fig. 5C, much more sec22b was incorporated in the simultaneous mixture than in the sequential mixture, indicating that efficient sec22b binding required simultaneous addition of the proteins.

How can we explain these results, considering that Figs. 5 and 6 clearly implied that sec22b binds to the combination of syntaxin 5, membrin, and rbet1? One possibility is that once syntaxin 5, membrin, and rbet1 have begun to assemble to create a sec22b binding site, there is a limited time during which sec22b must bind, or its binding site becomes inaccessible for steric reasons. Alternatively, a second copy of syntaxin 5, membrin or rbet1 might take the place of sec22b to create a ternary complex containing four helices. A “place-holder” extra syntaxin 5:sec22b:rbet1:membrin) when quantitated by either scintillation counting. Thus, two independent means were used for quantitation. As documented in Fig. 9A, the stoichiometry of the quaternary complex was very suggestive of 1:1:1:1 (syntaxin 5:sec22b:rbet1:membrin) when quantitated by either method, consistent with a four-helix bundle structure as has been determined for the synaptic complex. The substoichiometric amounts of sec22b can be explained by “contamination” of the quaternary preparation with ternary complexes, which are not separated from quaternary complexes by gel filtration. Fig. 9B demonstrates that the isolated ternary complex had a stoichiometry of 1:1:1 (syntaxin 5:rbet1:membrin). Hence, the inability of sec22b to bind to the ternary complex was not a result of an extra Q-helix taking its place and instead must have been because of a unique kinetic aspect of the assembly process. It thus appears unlikely that ER/Golgi SNARE complexes contain duplicate copies of any of the SNAREs, as has been suggested (14).

**ER/Golgi SNARE Complexes Containing Syntaxin 5 and Sec22b Accumulate in Living Cells**—Because yeast sec22 temperature-sensitive mutants are able to engage in anterograde ER/Golgi transport, it seemed important to verify that the syntaxin 5- and sec22b-containing complex was a natural complex that exists in living cells and not simply an *in vitro* curiosity. Although the sec22b-containing complex appears to be abundant in detergent extracts of rat liver (11, 12), it could...
not be eliminated that the complexes formed after cell lysis. To prove unambiguously that the sec22b-containing complex exists in cells, we expressed the epitope-tagged full-length SNAREs, Myc-sec22b and HA-tagged syntaxin 5 (HA-syntaxin 5) in normal rat kidney (NRK) cells, lysed the cells, and performed anti-epitope tag immunoprecipitations. In one set of cells, Myc-sec22b and HA-syntaxin 5 were transfected into cells separately, and the two populations were mixed prior to lysis and anti-epitope tag immunoprecipitation. In another population of cells, Myc-sec22b and HA-syntaxin 5 were cotransfected into the same cells, and the cells were lysed and subjected to anti-epitope tag immunoprecipitation. As shown in Fig. 10A, anti-Myc precipitation of Myc-sec22b resulted in coprecipitation of endogenous syntaxin 5 in either case; however, significant coprecipitation of HA-syntaxin 5 occurred only in the cotransfected cell extract. This demonstrates that the epitope-tagged sec22b and syntaxin 5 are in a complex together prior to cell lysis and do not form complexes during incubations in detergent solution.

A similar experiment demonstrates that endogenous sec22b and syntaxin 5 also must exist in a complex prior to cell disruption. We lysed untransfected NRK cells in detergent either in the presence or absence of a ~200-fold excess of purified recombinant sec22b cytoplasmic domain prepared as in Fig. 1. If SNARE complex formation occurs after cell lysis, then the soluble sec22b should effectively compete with the endogenous sec22b for binding to the other SNAREs, because there is a huge excess of the soluble competitor, and because we know this competitor is active for complex formation (see Fig. 6). In this case we would expect soluble competitor, rather than endogenous sec22b, to coprecipitate in anti-syntaxin 5 immunoprecipitations. But as shown in Fig. 10B, the presence of competitor did not significantly affect the efficiency of endogenous sec22b coprecipitation with syntaxin 5, indicating that the complexes of the endogenous proteins existed prior to cell lysis. A small amount of competitor sec22b coprecipitated as well, indicating that some SNARE complexes may have formed post-lysis when one of the reactants was present at an extremely high concentration. We also demonstrated that the endogenous SNARE complexes did not increase with time post-lysis, as might be expected if the binding took place in detergent solu-

**Fig. 9. Relative molar stoichiometries of the quaternary and ternary ER/Golgi SNARE complex.** Purified soluble syntaxin 5 (residues 252–333), membrin, rbet1, and sec22b were purified from bacteria grown on [14C]glucose. These uniformly radioactive proteins were mixed and incubated to form the quaternary and ternary complexes. After purification by gel filtration, the complexes were concentrated by acetone precipitation (A) or immunoprecipitation with an anti-rbet1 monoclonal antibody that does not affect complex formation (B). A, the purified concentrated quaternary complex was analyzed by SDS-PAGE followed by Coomassie Blue staining (left) and autoradiography (right). The molar stoichiometry of each protein, relative to syntaxin 5, was calculated using Coomassie Blue staining intensities as well as by radioactivity (after excision of gel slices and scintillation counting). B, the purified concentrated ternary complex was analyzed by SDS-PAGE, autoradiography, and scintillation counting of excised protein bands. Molar stoichiometries calculated from the radioactivity are expressed relative to syntaxin 5. The band in A marked by an arrowhead was not radioactive and was derived from the BSA carrier in the gel filtration buffer. The band marked by an asterisk, though radioactive, was not part of the complex as it was not immunoprecipitated with anti-rbet1 antibody (not shown).

**Fig. 10. SNARE complexes containing both syntaxin 5 and sec22b exist in cells and do not form post-lysis in detergent extracts.** A, NRK cells were either not transfected (NRK), transfected with either Myc-sec22b or HA-syntaxin 5 and then mixed and co-cultured in the same dish (Transfect, mix), or cotransfected with both Myc-sec22b and HA-syntaxin 5 (Cotransfect). Cells were lysed in Triton X-100, and cellular detergent extracts were either directly immunoblotted for syntaxin 5 and sec22b (Extracts), or they were subjected to immunoprecipitation with anti-Myc (Myc I.P.) antibodies and the immunoprecipitates were immunoblotted for syntaxin 5 and sec22b. The two natural isoforms of syntaxin 5 created by alternative protein initiation sites are indicated by syn5-42 and syn5-34. B, NRK cells were lysed in Triton X-100 with or without ~300 µg/ml purified recombinant sec22b cytoplasmic domain (as in the legend to Fig. 1). The recombinant sec22b-lacking extract (NRK) and the recombinant sec22b-containing extract (NRK + competitor) and a 100-fold dilution of the latter were immunoblotted for sec22b or immediately immunoprecipitated with preimmune or anti-syntaxin 5 antibodies, and the immunoprecipitates were immunoblotted for sec22b. The molecular mass difference between endogenous sec22b and the recombinant sec22b cytoplasmic domain was easily resolvable on these gels (arrows). C, PC12 cells were lysed in Triton X-100 and cellular detergent extracts were either directly immunoblotted for the indicated R-SNAREs (Extract) or immunoprecipitated with preimmune or anti-syntaxin 5 antibodies, and the immunoprecipitates were immunoblotted as indicated. Each extract lane contained 5 µl of extract, and each immunoprecipitate lane represented 60 µl of extract (all from the same autoradiographic exposure).
To demonstrate the specificity of the ER/Golgi SNARE complex in vivo (regardless of the mechanism(s) ensuring specificity), we performed anti-syntaxin 5 immunoprecipitations on PC12 cell extracts that contain both ER/Golgi and synaptic SNARE complexes. As shown in Fig. 10C, sec22b coprecipitated very efficiently with syntaxin 5, whereas a synaptic R-SNARE, VAMP, does not coprecipitate within our detection limits. This specificity demonstrates that random unrelated R-SNAREs do not associate with syntaxin 5 in cells and further argues for the authenticity of the characterized ER/Golgi quaternary complex.

DISCUSSION

Our results demonstrate many potential parallels between an ER/Golgi quaternary SNARE complex and the well characterized synaptic counterpart. Both complexes appear to involve four interacting amphipathic helices, although different numbers of polypeptide chains are involved (four in ER/Golgi and three in exocytosis). In both cases, three helices combine to create a high affinity binding surface for a fourth helix. The extremely potent binding of sec22b implies that in vivo, sec22b binding is the membrane-bridging step in complex assembly, just as potentiated VAMP binding is the membrane bridging step in exocytosis. Thus, sec22b would oppose syntaxin 5, membrin, and rbet1 on opposite membranes just as VAMP opposes syntaxin and SNAP-25. Sec22b and VAMP contain an arginine in the zero-layer position (3, 5), whereas all of the SNAREs comprising their binding sites are Q-SNAREs. In exocytosis (28, 29), as well as in ER/Golgi complexes (Fig. 7), the syntaxin amino-terminal domains act as potent inhibitors of SNARE complex assembly. What relieves this inhibition in vivo is not known, though Sec1-related proteins are a good candidate based upon their structure (31) and binding characteristics (32, 33). In summary, based upon the two SNARE complexes for which subunit structures are now known, a pattern emerges where SNARE complexes are composed of four-helix bundles with three Q-helices opposed by a highly potentiated R-helix and are negatively regulated by the amino-terminal syntaxin domain (Fig. 11).

It should be noted that our results do not directly prove that a parallel four-helix bundle per se is at the core of the ER/Golgi quaternary complex, only that the four proteins are involved. However, a four-helical bundle is the most likely arrangement because we showed that only the predicted coiled coil domain is sufficient for assembly in the case of syntaxin 5, rbet1 has only one predicted helical region, and others have shown that the membrane-proximal coiled coils of yeast Bos1p and Sec22p are sufficient for SNARE interactions in vitro (19). Still, the precise structural relationship of the ER/Golgi and exocytic complexes will need to be determined in biophysical studies.

Our results do not fit easily with recent findings from reconstituted yeast ER to Golgi transport, where Bet1p and Bos1p were required only on ER-derived vesicles, and Sed5p was required only on Golgi membranes (14). In contrast, our results would be most consistent with syntaxin 5, membrin and rbet1 functioning on the same membrane. Perhaps mammalian ER/Golgi transport requires additional fusion steps not required in yeast because syntaxin 5, membrin and rbet1 function on the same membrane. Perhaps mammalian ER/Golgi transport requires additional fusion steps not required in yeast because syntaxin 5 on COPII-coated vesicles was definitely required for reconstituted mammalian ER to Golgi transport (13). Alternatively, other proteins may have substituted for Bet1p, Bos1p, or Sed5p in an asymmetric fashion in the yeast temperature-sensitive mutants. For example, perhaps the Q-SNARE Gos1p could substitute at a low level for Bet1p on Golgi membranes but not on vesicles, which lack Gos1p.

It has been demonstrated by two studies that the sec22-3 temperature-sensitive allele in yeast does not block anterograde ER to Golgi transport in in vitro reconstitutions (14, 17). Although these results were interpreted to mean that Sec22p is not functionally involved in anterograde transport, a different explanation would be that another ER/Golgi R-SNARE, for example Ykt6p (9), can substitute its function. Another possibility is that the sec22-3 mutation does not eliminate the ability of Sec22p to function with Sed5p, Bet1p, and Bos1p in an anterograde complex, even though this mutation appeared to inhibit binary binding between Sec22p and Sed5p (19). The sec22-3 mutation affects only the zero-layer (residue 157) in the coiled coil domain, substituting the arginine with a glycine. Although zero-layer residues are conserved and therefore must have an important role in aspects of SNARE function, zero-layer mutations in the SNAP-25 carboxyl-terminal coil have no effect on the synaptic fusion complex stability or ability to reconstitute exocytosis (34). Furthermore, an essential role for sec22b in anterograde ER/Golgi transport is suggested by transport reconstitutions in semi-intact NRK cells, where anti-sec22b antibodies block cargo transport at an early stage (35).
ER/Golgi SNARE Complex

Results from both yeast and mammals agree that sec22 is found in SNARE complexes with the anterograde Q-SNAREs (10, 11, 36). The physiological relevance of these sec22-containing SNARE complexes is strengthened by their dependence on upstream docking factors like Ypt1p and Uso1p (10, 36). If sec22 were not involved in anterograde transport, then these well documented protein interactions would represent a puzzling enigma.

Why does sec22b not bind to the preassembled, isolated, ternary complex of syntaxin 5, membrin, and rbet1? One possibility is that there is a limited kinetic window after ternary complex formation that VAMP binds to the already assembled, stable Q-SNARE pre-complex (30, 37). It is not yet clear whether these differences have an important physiological consequence. The inability is that there is a limited kinetic window after ternary complex formation is strengthened by their dependence on SNAP-232, NSF, rabs, sec1, or LMA1 proteins, that may play an important role in sequentially assembling the ER/Golgi complex may stem from the lack, of simultaneous assembly is possible. In the synapse, VAMP also binds with highest affinity to the combination of the three Q-SNARE helices. However, it appears to be possible, at least in vitro, to stage the assembly such that VAMP binds to the already assembled, stable Q-SNARE pre-complex (30, 37). It is not yet clear whether these differences have an important physiological consequence. The inability to sequentially assemble the ER/Golgi complex may stem from the lack, in vitro, of important SNARE chaperones such as NSF, rabs, sec1, or LMA1 proteins, that may play an important role in regulating the binding activities of the Q-SNARE ternary complex and the R-SNARE sec22b.

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