Cysteine-Disulfide Cross-linking to Monitor SNARE Complex Assembly during Endoplasmic Reticulum-Golgi Transport

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Assembly of cognate SNARE proteins into SNARE complexes is required for many intracellular membrane fusion reactions. However, the mechanisms that govern SNARE complex assembly and disassembly during fusion are not well understood. We have devised a new in vitro cross-linking assay to monitor SNARE complex assembly during fusion of endoplasmic reticulum (ER)-derived vesicles with Golgi-acceptor membranes. In Saccharomyces cerevisiae, anterograde ER-Golgi transport requires four SNARE proteins: Sec22p, Bos1p, Bet1p, and Sed5p. After tethering of ER-derived vesicles to Golgi-acceptor membranes, SNARE proteins are thought to assemble into a four-helix coiled-coil bundle analogous to the structurally characterized neuronal and endosomal SNARE complexes. Molecular modeling was used to generate a structure of the four-helix ER-Golgi SNARE complex. Based on this structure, cysteine residues were introduced into adjacent SNARE proteins such that disulfide bonds would form if assembled into a SNARE complex. Our initial studies focused on disulfide bond formation between the SNARE motifs of Bet1p and Sec22p. Expression of cysteine SNARE derivatives in the same strain produced a cross-linked heterodimer of Bet1p and Sec22p under oxidizing conditions. Moreover, this Bet1p-Sec22p heterodimer formed during in vitro transport reactions when ER-derived vesicles containing the Bet1p derivative fused with Golgi membranes containing the Sec22p derivative. Using this disulfide cross-linking assay, we show that inhibition of transport with anti-Sly1p antibodies blocked formation of the Bet1p-Sec22p heterodimer. In contrast, chelation of divalent cations did not inhibit formation of the Bet1p-Sec22p heterodimer during in vitro transport but potently inhibited Golgi-specific carbohydrate modification of glyco-pro-α factor. This data suggests that Ca2+ is not directly required for membrane fusion between ER-derived vesicles and Golgi-acceptor membranes.

In eukaryotic cells, transport between different organelles occurs through vesicular intermediates that originate from one membrane compartment and fuse selectively with another. These intracellular fusion events are mediated by a family of proteins termed soluble N-ethylmaleimide-sensitive factor attachment receptors (SNAREs) (1). SNAREs are membrane-associated proteins that contain a characteristic heptad repeat region called the SNARE motif. SNARE motifs are ~60 amino acids in length and participate in forming oligomeric complexes with other SNAREs (2–4). Structural information from neuronal and endosomal SNARE core complexes indicate that the SNARE motifs assemble into parallel, coiled-coil four-helix bundles (3, 4).

During intracellular fusion, SNAREs from one membrane compartment interact with cognate SNAREs on another membrane compartment. The assembly of trans-SNARE complexes from two opposing membranes is thought to provide the driving force for bilayer fusion (5, 6). Indeed, purified SNARE proteins reconstituted into liposomes are sufficient for fusion events between proteoliposomes when physiologically relevant SNARE combinations are used (7–9). Despite the importance of SNAREs for membrane fusion, the precise mechanism by which SNARE proteins catalyze bilayer fusion or how SNARE complex assembly is regulated remains unclear. Current models describe pathways where trans-SNARE protein pairs produce membrane stalk structures that then lead to hemifusion and fusion pore intermediates (10–12). Further speculation centers on the nature of the fusion pore and if it is a lipid intermediate or if the transmembrane segments of SNAREs or other proteins form a pore structure (13–15). Upstream factors, such as Rab GTPases, tethering complexes, and SM proteins, are also required for membrane targeting and SNARE-dependent fusion (16–18). How these upstream components regulate SNARE complex assembly and disassembly are not known.

To explore these questions, we have taken a cysteine-disulfide cross-linking approach to monitor SNARE protein contacts during a round of membrane fusion in a model fusion assay. In Saccharomyces cerevisiae, fusion of ER-derived transport vesicles with Golgi-acceptor membranes has been recapitated in vitro with washed membranes and purified cytotoxic factors (19, 20). Genetic and biochemical studies indicate that the SNAREs Sec22p, Bet1p, Sed5p, and Bos1p are required for anterograde trafficking between the ER and Golgi compartments (21–23). These four proteins, when mixed together, form a stable quaternary complex with their SNARE motifs predicted to be structurally arranged as observed in the neuronal and endosomal SNARE four-helix bundles (24, 25).

Previously, disulfide cross-linking has been used to map the spatial and dynamic arrangements of oligomeric membrane receptors such as the aspartate receptor (26) and the dopamine D2 receptor (27). In this study, we engineered unique cysteine residues into the SNARE motifs of Bet1p and Sec22p such that a disulfide-cross-linked heterodimer formed under appropriate conditions. This Bet1p-Sec22p heterodimer was present not only in membrane preparations expressing both SNARE cysteine derivatives, but more importantly, this cross-linked heterodimeric SNARE species was generated in vitro through the fusion of topologically distinct membrane compartments. By combining an established cell-free ER-Golgi transport assay with the disulfide cross-linking approach, we report on the kinetics and requirements for the assembly of nascent Bet1p-Sec22p heterodimers.
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**EXPERIMENTAL PROCEDURES**

*Plasmids and Plasmid Construction—* To generate plasmid pRS315-BET1, the BET1 coding sequence with ~300 bp of flanking upstream and downstream sequence was amplified from genomic DNA using primers containing XbaI and XhoI restriction sites, respectively. The PCR product was ligated into XbaI- and XhoI-digested pRS315 (28). The plasmid containing the SEC22 gene (pRS313-SEC22) has been described (29). Cysteine residues were introduced into the SNARE motifs of Sec22p and Bet1p by site-directed mutagenesis using the QuikChange kit (Stratagene). The sequences of oligonucleotide primers used in the construction of these cysteine-containing derivatives are available upon request. All of the constructs were sequence verified by automated fluorescent sequencing (Dartmouth Molecular Biology Core Facility).

**Yeast Strains and Media—** Yeast strains CBY740 (Mata his3 leu2 ura3lys2), CBY773 (Mata his3 leu2 ura3lys2 sec22Δ::KAN), and CBY1599 (Mata/a his3/3his3 leu2/leu2 ura3/ura3 lys2/lyS2 MET15/met15 BET1/bet1Δ::KAN) were purchased from Invitrogen. CBY1584 was generated from transforming CBY773 with pRS313-SEC22-(D153C). CBY1676 was generated from transformation of the heterozygous diploid strain CBY1599 with pRS315-BET1(183C), followed by sporulation, asci dissection, and scoring to obtain bet1Δ covered by the BET1(183C) plasmid. For double mutant strains used in Figs. 2 and 3, CYB773 was transformed with plasmids containing the SNARE cysteine derivatives of SEC22 and BET1. Yeast transformations were performed using the lithium acetate method (30). For the preparation of semi-intact cells or microsomal membranes, strains were grown in selective medium (0.67% nitrogen base without amino acids, 2% dextrose, and required supplements) and then back diluted and grown in rich medium (1% Bacto-yeast extract, 2% Bacto-peptone, and 2% dextrose) for several doublings until the A600 was between 0.6 and 0.8. Standard yeast protocols were used (31).

**Antibodies and Immunoblotting—** Antibodies directed against Sec22p (32), Bet1p (33), Och1p (34), and α1,6-mannose linkages (19) have been described previously. Polyclonal anti-Sly1p antibodies were raised against a maltose-binding protein-Sly1p (full-length) fusion protein (MBP-Sly1p) expressed from pMAL-c2x (New England Biolabs). Polyclonal anti-Sly1p antibodies were affinity purified by MBP-Sly1p to an Affi-Gel 15 column according to the manufacturer’s instructions (Bio-Rad). Preimmune IgGs were isolated by binding to protein A-Sepharose beads as previously detailed (35). Western blots were developed using the SuperSignal WestPico chemiluminescent substrate (Pierce Chemical) and developed on both film and with a UVP Bioimaging System.

**Homology Modeling of Yeast ER-Golgi SNARE Four-helix Bundle—** The structure of the yeast ER-Golgi SNARE complex was constructed by homology modeling using the program SWISS-PDB Viewer version 3.7 (36, 37). The template used for modeling was the mammalian endosomal SNARE complex (Protein Data Bank accession number 1GL2) (3). The template and target sequences were aligned based on a previous alignment strategy of the core SNARE motifs (38). Briefly, the SNARE motifs of Sec22p, Sed5p, Bos1p, and Bet1p were aligned with manually threaded onto the SNARE motifs of endobrevin, syntaxin 7, vti1b, and syntaxin 8, respectively. The initial model was further refined using the energy minimization tools provided with the SWISS-PDB program. The quality of the model was evaluated further by using the WHAT_CHECK verification routines (39) from the program WHAT IF (40). Following model refinement and verification, the program MODIP (41, 42) was used to identify potential sites for introduction of disulfide bonds between adjacent SNARE motifs of the yeast ER-Golgi SNARE complex.

**In Vitro Budding and Transport Assays—** Semi-intact cells from wild-type and SNARE mutant strains were prepared as previously described (20). Microsomes were isolated from CBY1676 (43) and used to generate ER-derived vesicles containing Bet1p(183C). Vesicle budding and transport assays following [35S]glyco-pro-α-factor (gpd) have been previously described (19, 33). Briefly, two-stage transport reactions were performed in which COPII-generated vesicles isolated from CBY1676 microsomes were incubated with CBY1584 semi-intact cells (i.e. acceptor membranes) in the presence (or absence) of fusion factors (Uso1p, LMA1) for the indicated times and temperatures. For experiments examining both the transport of gpd and the extent of formation of a Bet1p-Sec22p heterodimeric species, three parallel transport reactions (30 µl) were set up in which two of the reactions were processed and averaged for α,1,6-mannose modification (19), whereas the third reaction underwent oxidative cross-linking as described below to catalyze disulfide bond formation between adjacent Bet1p and Sec22p proteins. When plotting the percentage of [35S]gpd transport and amount of heterodimer formation within the same graph, the data were scaled to maximal transport and maximal heterodimer levels, respectively.

**Oxidative Cross-linking of Cysteine-containing SNAREs—** Disulfide cross-linking between adjacent cysteine residues was induced by the addition of Cu(1,10-phenanthroline)SO4(Cu²⁺/Phen) (26, 44). For initial cross-linking experiments (Figs. 2 and 3), an equivalent amount of membranes from the indicated strains were washed three times with buffer 88 (20 mM HEPES, pH 7.0, 150 mM potassium acetate, 250 mM sorbitol, and 5 mM magnesium acetate) to remove cytosol. Each wash was followed by a brief centrifugation at 20,000 × g to pellet washed semi-intact cells. After the last wash, the semi-intact cells were resuspended in buffer 88, and a freshly prepared Cu²⁺/Phen solution was added to a final concentration of 0.2 mM. The stock Cu²⁺/Phen solution was made by adding equal volumes of freshly prepared 50 mM CuSO4 (dissolved in buffer 88) and 200 mM 1,10-phenanthroline (dissolved in ethanol) into buffer 88. The reactions were incubated on ice for 15 min. Following oxidative cross-linking, the reactions were mixed with a one-half volume of 5× SDS-PAGE non-reducing sample buffer that contained 150 mM N-ethylmaleimide to quench free sulfhydryls. The reactions were then heated at 70 °C for 5 min, followed by a brief 20,000 × g centrifugation to pellet insoluble material. After centrifugation, a portion of the sample was removed and resolved by non-reducing SDS-PAGE, transferred onto nitrocellulose, and probed with the indicated antibodies.

For cross-linking of two-stage transport reactions, at the indicated times, the reactions were placed on ice for 2 min, followed by the addition of Cu²⁺/Phen to a final concentration of 0.2 mM. The reactions were incubated on ice for 15 min and then mixed with an equal volume of quenching solution (buffer 88 containing 100 mM N-ethylmaleimide) for an additional 15 min. The reactions were then centrifuged at ~148,000 × g in a TLA 100.3 rotor (Beckman Coulter) for 10 min at 4 °C to concentrate membranes. After centrifugation, the resulting membrane pellets were solubilized in 15 µl of 2× non-reducing SDS-PAGE sample buffer. The samples were heated at 70 °C for 5 min and then centrifuged briefly at 20,000 × g. The samples were resolved by non-reducing SDS-PAGE, transferred onto nitrocellulose, and probed with the indicated antibodies. For densitometric analysis, bands on immunoblots were quantified using the Labworks software package (UVP).
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RESULTS

Rationale—Transport between the ER and Golgi involves a number of distinct steps that include tethering and fusion of vesicles to Golgi-acceptor membranes. During vesicle fusion, SNARE complex assembly between donor and acceptor membranes is an essential feature of this intracellular transport event. ER-Golgi transport assays in yeast have primarily used [35S]gp fusions to monitor the fusion stage and to investigate mechanisms involved in this process. However, [35S]gp fusions report indirectly on membrane fusion events because outer chain glycosylation relies on lumenal content mixing between donor and acceptor membranes. During vesicle fusion, SNARE complex assembly occurs at the plasma membrane, whereas no pairs were predicted involving residues from opposing SNARE helices (i.e. no Sec22p-Bos1p or Sed5p-Bet1p).

To capture disulfide-linked SNARE species from bona fide fusion events, one cysteine-containing SNARE from ER-derived vesicles must interact with a cysteine-containing SNARE located on Golgi-acceptor membranes. Ideally, the two cysteine-containing SNARE derivatives would originate on the membrane compartment in which they are functionally required during transport. In previous studies using thermosensitive alleles of SNAREs, we focused our attention on three predicted disulfide pairs involving residues from opposing SNARE helices (i.e. no Sec22p-Bos1p or Sed5p-Bet1p).

Disulfide Cross-linking of Bet1p and Sec22p Expressed within the Same Yeast Strain—We performed initial experiments to validate our experimental design and determine which predicted SNARE pairs, if any, could generate a disulfide-cross-linked species. Yeast strains were constructed in which cysteine residues were introduced near the –3, –1, and +3 hydrophobic layers in the SNARE motifs of Sec22p and Bet1p (Fig. 1). These cysteine-containing SNARE derivatives were expressed under their endogenous promoters from CEN-based plasmids, and semi-intact cell membranes were prepared from strains co-expressing both cysteine-containing SNAREs. After incubation of membranes with Cu²⁺/Phen to promote an oxidizing environment, cross-linking reactions were quenched with N-ethylmaleimide, and proteins were resolved by non-reducing SDS-PAGE. Disulfide cross-linked proteins were detected by immunoblotting to identify species recognized by both anti-Bet1p and anti-Sec22p antibodies and having an electrophoretic mobility near the theoretical size of a Bet1p-Sec22p heterodimer (~41 kDa). For the initial evaluation of Bet1p-Sec22p pairs (Fig. 2), cysteine-containing SNARE derivatives were expressed from CEN-based plasmids in a sec22Δ strain (CBY773). Therefore, in these membranes, a single copy of SEC22 was provided by the plasmid, whereas two copies of BET1 (endogenous and cysteine-containing) were expressed. Because the primary objective at this stage was to test which pairs of SNARE proteins were capable of forming disulfide-cross-linked products, the presence of endogenous wild-type Bet1p should not prevent the identification of such pairs. Three of the predicted Bet1p-Sec22p
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**FIGURE 2.** Formation of various Bet1p-Sec22p cysteine-disulfide heterodimers expressed within the same strain. Washed semi-intact cells expressing the indicated cysteine derivatives of Bet1p and Sec22p were incubated in the absence (−) or presence (+) of 0.2 mM Cu²⁺/Phen as described under “Experimental Procedures.” After 15 min on ice, the reactions were terminated with sample buffer (containing N-ethylmaleimide) and incubated at 70 °C for 5 min. Proteins were resolved by nonreducing SDS-PAGE (10%) and immunoblotted with anti-Sec22p or anti-Bet1p. The position of the Bet1p-Sec22p heterodimer is indicated by the solid arrowhead, whereas the asterisk indicates a breakdown product of a Sec22p homodimer. In addition to the cysteine derivative of Bet1p, the semi-intact cells also contain wild-type Bet1p.

pairs were tested and two of the pairs formed disulfide-cross-linked species (Fig. 2, solid arrowhead) that were both Cu²⁺/Phen-dependent and recognized by anti-Bet1p and anti-Sec22p antibodies. Although the Bet1p(S76C)-Sec22p(I146C) pair is predicted to form a disulfide bond by MODIP, this particular pair failed to generate a detectable heterodimeric cross-linked species. We also tested other SNARE pair combinations (Sec22p-Sed5p and Sed5p-Box1p) for disulfide bond formation, but were unable to unambiguously detect corresponding heterodimers from these predicted pairs (data not shown). At this point we cannot explain why these predicted SNARE pairs failed to produce cross-links but speculate that either our SNARE model contains local inaccuracies or that the arrangement of SNARE proteins in the context of cellular membranes is different from the crystal structure.

Of the two positive pairs identified from Fig. 2, we chose the SNARE pair Bet1p(I83C)-Sec22p(D153C) for our in vitro transport experiments described below. However, before proceeding with any transport experiments, we further characterized these derivatives as well as generated a strain expressing only the Bet1p-cysteine derivative. To create the Bet1p(I83C) strain, a heterozygous diploid (Bet1I/bet1Δ) strain was obtained and transformed with a CEN-based plasmid containing the Bet1 gene. After sporulation and tetrad analysis, we recovered washed semi-intact cells expressing the indicated cysteine-containing derivatives (Bet1p(I83C) and Sec22p(D153C)).

Membranes containing wild-type or only one of the two cysteine-containing SNARE derivatives did not generate a Cu²⁺/Phen-dependent disulfide-cross-linked heterodimer recognized by both anti-Bet1p and anti-Sec22p on immunoblots (Fig. 3) when compared with oxidized membranes expressing both cysteine-containing derivatives. These results provided further evidence that the formation of a Bet1p-Sec22p disulfide-cross-linked heterodimer required expression of both cysteine derivatives within the same strain. Approximately 3–5% of the total amount of Sec22p was cross-linked to Bet1p when both cysteine-containing derivatives were expressed in the same strain. This cross-linked percentage is in accord with the amount of Sec22p associated with Bet1p when measured by native co-immunoprecipitation (29).

While conducting these disulfide-cross-linking experiments, we detected a Cu²⁺/Phen-dependent species appearing in membranes expressing only Sec22p-cysteine derivatives. In experiments that go beyond the scope of this paper, we determined that this species is an ~50-kDa Sec22p homodimer (not shown on gels) and are currently examining its functional significance in ER-Golgi transport. In Fig. 2 and other anti-Sec22p immunoblots, the protein band indicated by an asterisk is a breakdown product of the ~50-kDa Sec22p homodimeric species.

Bet1p-Sec22p Cross-linked Dimers Are Formed during in Vitro Two-stage Transport Assays—Having identified pairs of SNARE residues capable of disulfide bond formation when expressed in the same strain, we next investigated whether a Bet1p-Sec22p cross-linked species could be generated in cell-free transport assays. In two-stage transport reactions, COPII vesicles generated from Bet1p(I83C) microsomes were incubated with Sec22p(D153C)-acceptor membranes in the presence of purified Uso1p and LMA1. At the end of transport, parallel reactions were processed to assess Golgi-specific [35S]gp modification or were oxidized with Cu²⁺/Phen to promote disulfide bond formation between any Bet1p(I83C)-Sec22p(D153C) SNARE complexes. Inclusion of [35S]gp in the transport experiments not only provided an internal standard for vesicle fusion, but also allowed us to compare the functional requirements for [35S]gp transport and Bet1p-Sec22p disulfide bond formation.

As shown in Fig. 4A, the addition of Uso1p and LMA1 to the transport reactions stimulated [35S]gp modification ~3.0-fold (black bars). The addition of Uso1p and LMA1 to the transport reactions also produced a ~41-kDa species (solid arrow) recognized by both anti-Sec22p and anti-Bet1p (Fig. 4B). From the immunoblots, the relative amounts of the Bet1p-Sec22p heterodimer were measured by densitometry and graphically represented in Fig. 4A (gray bars). The graph clearly shows that addition of Uso1p and LMA1 to transport reactions stimulated formation of a Bet1p-Sec22p disulfide-cross-linked product to a similar extent as [35S]gp transport (~2.7-fold from anti-Sec22p panel and ~3.0-fold from anti-Bet1p panel).

Additional controls confirmed that the Bet1p-Sec22p cross-linked species was a direct result of transport. First, in the absence of Uso1p and LMA1, some [35S]gp transport (14.8%) occurred probably because of residual transport factors remaining associated with acceptor membranes even after washing (Fig. 4A). A similarly low level of cross-linked Bet1p-Sec22p was detected under this condition (Fig. 4B). However, if transport reactions were incubated on ice, [35S]gp transport was negligible, and subsequently, no cross-linked Bet1p-Sec22p was detected (Fig. 4). Second, in the absence of Sec22p(D153C)-acceptor membranes, a small amount of [35S]gp transport occurred because of trace amounts of Bet1p(I83C)-Golgi membranes present in the vesicle fraction (Fig. 4A). However, because the Golgi were from Bet1p(I83C) membranes, no Bet1p-Sec22p cross-linked species was formed (Fig. 4B). As a negative control, we observed that fusion reactions using the non-predicted SNARE pair of Bet1p(S76C) vesicles with Sec22p(D153C)-acceptor membranes produced no detectable disulfide-
linked heterodimer (supplemental materials Fig. 1). Taken together, these results demonstrate that a specific Bet1p-Sec22p disulfide-cross-linked species forms when Bet1p(I83C) vesicles fuse with Sec22p(D153C)-acceptor membranes. The formation of this Bet1p-Sec22p heterodimeric species requires the same conditions as transport of [35S]gpf.

Time Course of α-Factor Transport and Bet1p-Sec22p Heterodimer Formation Is Similar—During the course of ER-Golgi transport, vesicle SNAREs engage with acceptor membrane SNAREs to catalyze membrane fusion. Because the previous experiment only compared the extent of α-factor modification and Bet1p-Sec22p heterodimer formation at the end of a 60-min transport reaction, we investigated the kinetics of α-factor transport and Bet1p-Sec22p heterodimer formation using the two-stage fusion assay. Again, parallel reactions were set up in which Bet1p(I83C) vesicles were incubated with Sec22p(D153C)-acceptor membranes in the presence of Uso1p and LMA1. At specific time intervals, the reactions were either processed for Golgi-specific 1,6-mannose modification or were incubated with Cu2+/Phen to promote disulfide bond formation. As shown in Fig. 5, within the first few minutes of the reaction, a small amount of transport (~10% of maximum) occurred as measured by both α-factor modification and Bet1p-Sec22p heterodimer formation. In addition, throughout the 60-min reaction, the kinetics of α-factor transport and Bet1p-Sec22p disulfide bond formation were very similar, suggesting that the mixture of luminal compartments and SNARE complex assembly occur on a similar time scale and report on the same fusion event.

Addition of Uso1p Alone Does Not Produce Maximum Levels of Bet1p-Sec22p Heterodimer—Vesicle transport between ER and Golgi proceeds through defined stages of vesicle budding, vesicle tethering, and membrane fusion. We have demonstrated that addition of Uso1p to cell-free transport assays produces a dilution-resistant intermediate consisting of ER-derived vesicles tethered to Golgi-acceptor membranes (19). Because vesicle- and Golgi-SNARE associations can now be monitored using this disulfide-cross-linking technique, two-stage transport reactions were performed to assess the extent of Bet1p-Sec22p heterodimer formation when Uso1p-tethered intermediates were formed. In experiments similar to those described in Fig. 4, addition of Uso1p alone caused a 2.2-fold increase in [35S]gpf transport when compared with reactions conducted in the presence or absence (Fig. 6A, black bars) of Uso1p and LMA1. Moreover, Uso1p addition decreased the levels of freely diffusible vesicles in our assay by ~30% (data not shown), a reduction that reflects vesicle tethering and fusion with Golgi membranes. Interestingly, when Bet1p-Sec22p heterodimer formation was assessed in these reactions, the stimulation of disulfide-cross-linked products (Fig. 6B, gray bars) was not observed. These results indicate that in the presence of Uso1p, vesicles tether to Golgi membranes, yet the amount of Bet1p-Sec22p heterodimer formation mirrored the amount of vesicle fusion.

Anti-Sly1p Antibodies Inhibit [35S]gpf Transport and Bet1p-Sec22p Heterodimer Formation—Although SNAREs are central components of membrane fusion, additional proteins are required to ensure proper regulation and fidelity of vesicle transport and SNARE complex assembly. One such regulatory protein, Sly1p, is a member of the Sec1 family of proteins (10). Sly1p is peripherally associated with membranes through its interactions with Sed5p and is required for ER-Golgi transport in vivo and in vitro (46–49). Previously, we used thermosensitive alleles to show that Sed5p and Sly1p are not required for Uso1p-dependent vesicle tethering, but rather their activity is needed for vesicle fusion (50). Given the requirement of Sly1p for fusion, we performed two-stage transport reactions in the presence of inhibitory antibodies specific for Sly1p. Affinity purified anti-Sly1p antibodies were titrated into transport reactions to determine the optimal amount of antibody to use during the studies (data not shown). As seen in Fig. 7, two-stage transport reactions were sensitive to anti-Sly1p antibodies, whereas preimmune IgGs at comparable concentrations did not inhibit transport (black bars). Furthermore, when the transport reactions were processed for Bet1p-Sec22p heterodimer formation, it was clear that Sly1p antibodies decreased the amount of disulfide-cross-linked product (Fig. 7, immunoblot). In addition, the stimulation of heterodimer by Uso1p and
FIGURE 6. Uso1p-dependent vesicle tethering alone does not promote maximal Bet1p-Sec22p heterodimer formation. Two-stage transport reactions were set up as described in the legend to Fig. 4, except Uso1p and LMA1 were individually tested in the transport reactions. After 60 min, in A, the amount of [35S]gp transport was quantified (black bars) and in B, parallel transport reactions were processed for Bet1p-Sec22p heterodimer formation. The solid arrow indicates Bet1p-Sec22p heterodimer. The amount of Bet1p-Sec22p heterodimer was quantified by the software package Labworks (UVP) and graphed in A (gray bars).

FIGURE 7. Antibodies directed against Sly1p inhibit both in vitro transport of [35S]gp and the formation of Bet1p-Sec22p heterodimer. Bet1p(B3C) vesicles containing [35S]gp were mixed with Sec22p(D133C)-acceptor membranes in the presence of affinity purified anti-Sly1p antibodies or preimmune (PI) IgGs. The amount of Golgi-modified [35S]gp was measured to determine transport efficiency in the presence of the antibodies. Also, the extent of Bet1p-Sec22p heterodimer formation was assayed by immunoblotting the transport reactions with anti-Sec22p. The amount of Bet1p-Sec22p heterodimer for each reaction condition is indicated under the lanes in the immunoblot as integrated optical density (IOD). The solid arrow indicates the position of the Bet1p-Sec22p heterodimer and the asterisk is a proteolytic fragment of a Sec22p-homodimer species.

FIGURE 8. Bet1p-Sec22p heterodimer formation is not inhibited by metal chelators. Two-stage transport reactions using Bet1p(B3C) vesicles and Sec22p(D133C)-acceptor membranes were assembled in the absence or presence of either 3 mM EGTA or 0.5 mM BAPTA. After 60 min, in A, the amount of [35S]gp transport in the absence (black bars) or presence (gray bars) of Uso1p and LMA1 under the different chelator conditions was quantified. B, parallel transport reactions were processed for Bet1p-Sec22p heterodimer formation. The solid arrow indicates the Bet1p-Sec22p heterodimer. The Sec22p-homodimer breakdown product is indicated by the asterisk. In C, standard two-stage transport reactions were incubated with or without 3 mM EGTA for 40 min at 23 °C. The reactions were placed on ice and CaCl2, MnCl2, or both were added to the reactions at the indicated concentrations. The reactions were then incubated for an additional 30 min at 23 °C, and the amount of outer chain-modified [35S]gp was measured.

LMA1 paralleled that of [35S]gp transport as in the experiments shown above. In summary, inhibition by affinity purified anti-Sly1p antibodies indicates that Sly1p is required for membrane fusion and Bet1p-Sec22p heterodimer formation.

Formation of Bet1p-Sec22p Heterodimer Is Not Inhibited by Metal Chelators—Previous studies examining ER-Golgi transport in mammalian and yeast systems have implicated Ca2+ in a late-stage requirement for vesicle fusion (51, 52). Additionally, these studies suggested that Ca2+ chelation causes the accumulation of ER-derived vesicles firmly docked to Golgi membranes (53). As a means to block transport and further characterize the Bet1p-Sec22p heterodimer in the fusion pathway, metal chelators were used to inhibit transport. Two-stage transport reactions were performed in the absence or presence of the metal chelators, EGTA and BAPTA. When either EGTA (3 mM) or BAPTA (0.5 mM) was included in the reactions, the overall amount of [35S]gp transport was significantly reduced (~83–89%; Fig. 8A). From the [35S]gp transport data alone, it appeared that EGTA or BAPTA inhibited ER-Golgi transport, consistent with previous reports (51, 52). Strikingly, when Bet1p-Sec22p heterodimer formation was measured in fusion reactions that contained EGTA or BAPTA, no reduction was detected (Fig. 8B). Moreover, the amount of Bet1p-Sec22p heterodimer and the extent of Uso1p and LMA1 stimulation were similar under all three reaction conditions (Fig. 8B).

The formation of cross-linked Bet1p-Sec22p in the presence of EGTA or BAPTA could reflect SNARE complexes that have arrested "in trans" prior to bilayer fusion (14). Alternatively, bilayer fusion could proceed normally under these conditions and the apparent inhibition of [35S]gp transport could be because of inhibition of outer chain carbohydrate addition. The mannosyltransferase enzymes responsible for Golgi-specific carbohydrate modifications are known to require Mn2+ (54, 55). To address divalent cation requirements for in vitro outer chain modification of [35S]gp, two-stage transport reactions were initially incubated in the presence of inhibiting amounts of EGTA and then...
supplemented with Ca\(^{2+}\), Mn\(^{2+}\), or both, in an attempt to reverse the inhibition. As shown in Fig. 8C, addition of Ca\(^{2+}\) alone failed to restore EGTA inhibition even when an excess of Ca\(^{2+}\) (5 mM) over chelator was provided. In contrast, transport reactions supplemented with a high concentration of Mn\(^{2+}\) (5 mM) completely reversed the inhibition, and similar levels of outer-chain-modified \([^{35}S]gpa\)f were observed as in a standard transport reaction without EGTA. EGTA inhibition was not relieved when a lower concentration (0.1 mM) of Mn\(^{2+}\) was provided. This was probably because of a 30-fold excess of EGTA to Mn\(^{2+}\) in the reaction. Interestingly, when transport reactions were supplemented with low concentrations of both Mn\(^{2+}\) (0.1 mM) and Ca\(^{2+}\) (0.5 mM), a modest restoration of outer chain modification was observed as previously reported (51). Although this low concentration of Mn\(^{2+}\) could not rescue the inhibition alone, the additional presence of Ca\(^{2+}\) may compete for binding to EGTA and allow free Mn\(^{2+}\) to participate in outer chain modification reactions. Taken together, the maximal formation of Bet1p-Sec22p heterodimers in the presence of metal chelators and the reversal of Golgi-specific outer chain modification by free Mn\(^{2+}\) suggest that ER–Golgi transport may not directly require Ca\(^{2+}\).

**DISCUSSION**

The centrality of SNARE proteins in catalyzing fusion of intracellular membranes is well appreciated (10). However, the mechanisms that govern SNARE complex assembly and disassembly in successive rounds of membrane fusion are not well understood. In this report, we establish a new disulfide cross-linking approach to monitor the status of two ER–Golgi SNAREs during fusion of ER-derived vesicles with Golgi-acceptor membranes. This cross-linking approach relies on the placement of single cysteine residues within the SNARE motifs of Bet1p and Sec22p such that when assembled into a SNARE complex, oxidizing conditions produce an intermolecular disulfide bond.

As predicted from a molecular model of the four-helix ER–Golgi SNARE bundle, we found that cysteine pairs near the -1 or +3 interaction layers in Bet1p and Sec22p produced disulfide cross-linked heterodimers when co-expressed in yeast cells. Expression of the -1 pair in separate yeast strains allowed us to prepare ER-derived vesicles containing the Bet1p(I83C) derivative and Golgi-acceptor membranes containing the Sec22p(D153C) derivative. When these membranes were incubated in cell-free transport assays, a disulfide-cross-linked Bet1p-Sec22p adduct was detected under conditions of membrane fusion. Formation of the cross-linked heterodimer was temperature and time dependent and required the same fusion components (e.g. Uso1p, LMA1, and Sly1p) known to regulate fusion of ER-derived vesicles with Golgi-acceptor membranes (19, 50). Moreover, the rate of Bet1p-Sec22p heterodimer formation mirrored the rate of Golgi-specific outer chain modification of gpaf used in cell-free fusion assays to assess luminal content mixing (20). Based on these results, we conclude that the formation of the cross-linked Bet1p-Sec22p species is an authentic reporter for fusion of ER-derived vesicles with Golgi membranes.

Once the validity of the SNARE cross-linking approach was established, we used the assay to examine the role of Ca\(^{2+}\) and other divalent cations in formation of the Bet1p-Sec22p heterodimer during fusion of ER-derived vesicles with Golgi-acceptor membranes. Ca\(^{2+}\) performs a well established role in regulated fusion of synaptic vesicles with presynaptic membranes. Membrane depolarization in neuronal cells causes a rapid Ca\(^{2+}\) influx and is thought to trigger synaptic vesicle exocytosis through binding to synaptotagmin. Upon influx, calcium-bound synaptotagmin undergoes conformational changes that alter its affinity for lipids and SNARE proteins at the neural synapse and stimulate fusion. In addition to synaptotagmin, there are likely other targets of Ca\(^{2+}\) involved in synaptic vesicle exocytosis that remain to be characterized (10). Ca\(^{2+}\) has also been proposed to function in several other intracellular membrane fusion events in the early secretory pathway (51, 52, 56) and between endocytic organelles (57, 58). In contrast to neuronal exocytosis, the role for specific Ca\(^{2+}\)-binding proteins during these intracellular fusion reactions remains unclear. For in vitro ER–Golgi transport assays in yeast, the divalent cation chelators EGTA (51, 53) and BAPTA (46) are reported to inhibit a late fusion stage of transport and are reversed by the addition of Ca\(^{2+}\) and Mn\(^{2+}\).

During two-stage transport reactions, we observed that the level of Bet1p-Sec22p heterodimer formed was equivalent in the presence or absence of EGTA and BAPTA even though Golgi-specific outer chain modification of \([^{35}S]gpa\)f was completely inhibited by both chelators. Importantly, Mn\(^{2+}\), but not Ca\(^{2+}\), was most effective in reversing the EGTA block with regard to \([^{35}S]gpa\)f modification. Both EGTA and BAPTA have a high affinity \((K_d = 10^{-8}–10^{-9} \text{ M})\) for Mn\(^{2+}\) (59). Many glycosylation reactions require divalent cations for their activity and, more specifically, the Golgi-resident mannosyltransferases are known to require Mn\(^{2+}\) for the addition of outer chain carbohydrate (54, 55, 60), a reaction that is critical for monitoring in vitro transport of \([^{35}S]gpa\)f to Golgi compartments. Previous work has shown that both Ca\(^{2+}\) (0.5 mM) and Mn\(^{2+}\) (0.1 mM) were needed to reverse EGTA inhibition of the \([^{35}S]gpa\)f-based ER–Golgi transport assay (51). Under similar conditions, we observed a partial restoration of EGTA inhibition of outer chain-modified \([^{35}S]gpa\)f (Fig. 8C). However, a higher concentration of Mn\(^{2+}\) alone (5 mM) was sufficient to completely reverse EGTA inhibition, whereas an equivalent concentration of Ca\(^{2+}\) alone did not rescue the block. Based on these observations, we hypothesize that EGTA chelation does not interfere with SNARE complex assembly and the subsequent fusion of ER-derived vesicles with Golgi membranes. The observed inhibition of outer chain-modified \([^{35}S]gpa\)f by EGTA is probably because of inhibition of the Mn\(^{2+}\)-dependent mannosyltransferase reaction. We speculate in previous studies (51, 53), that the apparent late-stage fusion was stimulated by Ca\(^{2+}\) because under suboptimal Mn\(^{2+}\) concentrations added Ca\(^{2+}\) displaced sufficient Mn\(^{2+}\) from EGTA to support an increased level of outer chain modification.

What exactly is the molecular arrangement of the Bet1p-Sec22p heterodimer we detect by cross-linking? In cells, SNARE proteins undergo dynamic cycles during membrane fusion reactions and exist in a variety of disassembled and assembled states. Monomeric and oligomeric forms of SNARE proteins in topologically distinct compartments are thought to assemble into four-helix trans-SNARE intermediates that convert to cis-SNARE complexes when opposed membrane bilayers fuse (10). We speculate that most of the Bet1p-Sec22p heterodimer detected with the cysteine-disulfide cross-linking approach reflects Bet1p and Sec22p assembled into a four-helix cis-SNARE complex produced from membrane fusion. In support of this idea, it should be noted that our molecular model to predict suitably positioned cysteine pairs was based on the structure of a stable four-helix SNARE bundle. Furthermore, we observed a strict correlation between the kinetics of Bet1p-Sec22p cross-linking with luminal content mixing, an indication that the Bet1p-Sec22p heterodimer measured was a product of membrane fusion. In addition, Bet1p-Sec22p cross-linked adducts were detected in native Bos1p immunoprecipitates from detergent-solubilized membranes after immunoprecipitates were exposed to oxidant (data not shown). This result indicates that at least some of Bet1p-Sec22p heterodimer exists in an ER–Golgi SNARE complex with Bos1p. However, whereas we favor the interpretation that our assay measures cis-SNARE complexes, we cannot exclude the possibility that a fraction of the Bet1p-Sec22p cross-linked species detected originates from...
trans-SNARE complexes or binary and ternary SNARE complexes. Further experimentation will be required to determine the precise molecular arrangement of Bet1p-Sec22p heterodimers detected in this assay. The ability to cross-link cognate SNAREs from two distinct membrane compartments during a fusion reaction provides a new approach to decipher the mechanisms of SNARE complex assembly and dynamics. The MODIP program used to predict sites for introduction of disulfide cross-links identified 21 potential pairs in our ER-Golgi SNARE complex model. At this time only a few of the pairs have been characterized in cell-free fusion reactions and further analyses should provide insight into other molecular contacts during SNARE-mediated fusion. It may be possible to identify conformation specific pairs that form during distinct stages of the SNARE cycle and distinguish from cis-SNARE complexes. This cross-linking method could also be used to follow the fate of cis-SNARE complexes as they are disassembled or captured into vesicles by coat protein complexes. Finally, this approach may be generally applicable to dissect other SNARE-dependent membrane fusion reactions and to establish assays for organellar fusion events for which in vitro techniques are not currently available.

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