Biochemical Requirements for the Targeting and Fusion of ER-derived Transport Vesicles with Purified Yeast Golgi Membranes

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Abstract. In order for secretion to progress, ER-derived transport vesicles must target to, and fuse with the *cis*-Golgi compartment. These processes have been reconstituted using highly enriched membrane fractions and partially purified soluble components. The functionally active yeast Golgi membranes that have been purified are highly enriched in the *cis*-Golgi marker enzymes α1,6 mannosyltransferase and GDPase. Fusion of transport vesicles with these membranes requires both GTP and ATP hydrolysis, and depends on cytosolic and peripheral membrane proteins. At least two protein fractions from yeast cytosol are required for the reconstitution of ER-derived vesicle fusion. Soluble fractions prepared from temperature-sensitive mutants revealed requirements for the Ypt1p, Sec19p, Sly1p, Sec7p, and Uso1 proteins. A model for the sequential involvement of these components in the targeting and fusion reaction is proposed.

TRANSPORT of proteins between membrane-bound compartments of the secretory pathway is a process common to all eucaryotic cells. Transport is believed to involve the production of coated vesicle intermediates from a donor compartment and the subsequent uncoating, targeting, and fusion of these vesicles with an acceptor compartment (for reviews see Pryer et al., 1992; Rothman and Orci, 1992; Barlowe et al., 1994). The general mechanism of vesicle budding, targeting and fusion and the principal protein players involved in these reactions appears to be conserved from yeast to human cells (for review see Rothman, 1994).

We have developed a cell-free assay that measures the transit of the soluble precursor of the yeast mating pheromone α factor from the ER to the *cis*-compartment of the Golgi apparatus in yeast (Rexach and Schekman, 1991; Wuestehube and Schekman, 1992). The nucleotide and protein requirements for the production of ER-derived vesicles, as well as the polypeptide composition of purified vesicle intermediates have recently been characterized in detail (Barlowe et al., 1994; Rexach et al., 1994). Sec proteins act upon ER membranes to form transport vesicles shaped by cytoplasmic coat molecules consisting of Sar1p, Sec13p, Sec23p, Sec24p, and Sec31p. Vesicles formed under these conditions contain a specific set of lumenal and membrane proteins that include core-glycosylated pro-α-factor, Bet1p, Sec22p, and Bos1p, but are depleted of ER-resident proteins Kar2p, Sec12p, and Sec61p. Transport vesicles derived from the ER rapidly fuse with the *cis*-Golgi via a vesicle targeting mechanism that is apparently both selective and efficient. The soluble *N*-ethylmaleimide-sensitive fusion (NSF)[1] attachment protein (SNAP) receptor (SNARE) hypothesis was postulated to explain this selective targeting (Söllner et al., 1993; Rothman, 1994). According to this hypothesis all eukaryotic cells have families of organelle-specific receptor molecules (*v*- and *t*-SNAREs) whose members form highly specific pairs that are essential for vesicle docking. ER-derived vesicles carry an ER *v*-SNARE, which presumably has a cognate *t*-SNARE on *cis*-Golgi membranes. Furthermore, after vesicle targeting mediated by the *v*/*t*-SNARE mechanism is achieved, additional cytosolic proteins such as NSF-like and SNAP-like proteins bind to the SNARE complex and facilitate the membrane fusion process.

The details of the proteins involved in targeting and fusion of transport vesicles with the Golgi apparatus , as well as when and how they act, are poorly understood. Yeast offers a powerful system for addressing these questions, especially since the purified ER-derived vesicles described above are competent for fusion with *cis*-Golgi membranes (Rexach et al., 1994). Through a combined genetic and

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1. *Abbreviations used in this paper*: CPY, carboxypeptidase; LSF, low salt fraction; HSF, high salt fraction; MSS, medium speed supernatant; NSF, *N*-ethylmaleimide-sensitive fusion protein; PGK, phosphoglycerate kinase; SNAP, soluble NSF attachment protein; SNARE, SNAP receptor.

biochemical approach a set of yeast gene products have been identified that appear to be involved in the late stages of ER-to-Golgi transport. Four of these are membrane proteins; three corresponding to ER *v*-SNARE-like molecules (Sec22p, Bet1p, Bos1p); and the fourth a Golgi *t*-SNARE-like molecule (Sed5p). Also involved are a number of soluble and/or peripheral membrane proteins: Sec7p, Sec17p (the yeast α-SNAP homolog), Sec18p (an NSF homolog), Sec19p, Ypt1p (small GTP-binding protein), Sly1p , Uso1p (yeast homolog of mammalian transport factor p115), and the yeast coatomer complex.

In this report we describe an adaptation of our in vitro system to study the targeting and fusion of ER-derived vesicles with the Golgi apparatus. Functional yeast Golgi membranes as well as ER-derived transport vesicles were purified. We find that ER-derived vesicles can fuse with the Golgi complex with high efficiency in the presence of ATP, GTP and soluble fractions enriched for Sec7p, Ypt1p, Sly1p, and Uso1p. ATP- and GTP-hydrolysis are required for the fusion process to occur. We have used two-stage incubations and the selective inhibitory effects of ATPγS, GTPγS, and cytosol prepared from yeast strains harboring specific temperature-sensitive mutations to study the biochemical order of involvement of different proteins in the targeting/fusion process. The sequential steps operationally defined by these reagents are likely to reflect transport intermediates in vesicle recognition, formation of a docking complex, and fusion.

Materials and Methods

Materials

The *Saccharomyces cerevisiae* strains used in these experiments are listed in Table I. Strains were grown in YPD medium containing 1% Bacto yeast extract, 2% Bacto peptone (Difco Laboratories Inc., Detroit, MI) and 2% glucose. Other reagents were obtained as described by Baker et al. (1990). Antibodies used were directed against Och1p (Nakayama et al., 1992), Sec7p (Franzusoff et al., 1992), Sec12p (Barlowe and Schekman, 1993), Sec17p (Griff et al., 1992), Sec18p (Eakle et al., 1988), Bet1p (Rexach et al., 1994), Mnn1p (Graham et al., 1994), Kex2p (Fuller et al., 1989), Sed5p (Hardwick and Pelham, 1992), Ypt1p (Segev et al., 1988), Sly1p (Dascher et al., 1991), GDPase (Abeijon et al., 1989), Arf1p (kindly provided by R. Kahn), Sec21p (Hosobuchi et al., 1992), Sec61p (Sterling et al., 1992), and Sec23p (Hicke and Schekman, 1989). Standard methods for yeast genetic manipulations (Sherman et al., 1974), SDS-PAGE (Laemmli, 1970), immunoblotting (Towbin et al., 1979), and immunodetection by enhanced chemiluminescence (Amersham Corp., Arlington Heights, IL) were used. Deuterium oxide, Nycodenz, GTP, GDP, GTPγS, and ATPγS were purchased from Sigma Chemical Co. (St. Louis, MO).

Table I. Yeast Strains Used in This Study

Strain	Genotype
RSY271	MATα *his*4-619 *ura*3-52 *sec*18-1
RSY298	MATa *ade*2 *his*3-11,15 *leu*2-3,112 *trp*1-1 *ura*3-1 *sec*7-1
RSY300	MATα *his*3-11,15 *leu*2-3,112 *tura*3-1 *sec*7-4
RSY448	MATa *his*3-11,15 *leu*2-3,112 *trp*1-1 PEP4::URA3 *sec*17-1
RSY607	MATα *leu*2-3,112 *ura*3-52 PEP4::URA3
RSY610	MATα *his*3-11,15 *ura*3-1 PEP4::URA3 *sec*19-1
RSY919	MATα *ura*3-1 *mnn*1 *mnn*2
RSY942	MATa *lys*2-801 *his*4-619 *sec*22-3
RSY944	MATa *lys*2-801 *ura*3-52 *bet*1-1
RSY946	MATa *lys*2-801 *bet* 2-1
RSY948	MATa *lys*2-801 *trp*1-1 *ura*3-52 *uso*1-1
RSY949	MATα *lys*2-801 *trp*1-1 *ura*3-52 *uso*1-1
RSY950	MATa *his*3-11,15 *leu*2-3,112 *ura*3-1 YPT1::*ypt1*^{ts}LEU2
RSY951	MATα *his*3-11,15 *leu*2-3,112 YPT1::*ypt1*^{ts}LEU2
RSY954	MATa *lys*2-801 *leu*2-3,112 *sec*32-1
RSY976	MATa *ura*3-52 *ypt*1-3
RSY1074	MATα *ade*2-1 *leu*2-3,112 *trp*1-1 *ura*3-1 *sly1*^{ts}

Purification of Golgi-enriched Membranes

RSY919 cells were grown at 25°C in 1l YPD medium to early exponential phase (OD_{600} about 1). Cells (1,200 OD_{600} units) were harvested by centrifugation and washed twice with buffer A (20 mM Hepes, pH 6.5, 150 mM KOAc, 250 mM sorbitol). All subsequent steps were performed at 4°C. Cell pellets were resuspended in a Corex 30-ml glass tube in 3 ml buffer A containing 1 mM DTT, 1 mM ATP, 0.1 mM $MnCl_2$ and 10 μl of a proteinase inhibitor cocktail (pepstatin, aprotinin, leupeptin, antipain (1 mg/ml each), 100 mM phenylmethylsulphonyl fluoride, and 100 mM benzamidine). Glass beads (4 *g*) were added and cells were lysed by 4 1-min periods of agitation in a VWR vortex at full speed. The homogenate was cleared by centrifugation at 500 *g* for 5 min. The supernatant fraction was subjected to 10,000 *g* for 10 min to yield a postmitochondrial supernatant fraction, S10, which was then centrifuged at 50,000 *g* for 15 min in a TLA100.3 rotor (Beckman Instruments, Inc.) to yield a pellet, P50, and a supernatant, S50. The S50 fraction was loaded on top of a sucrose step gradient made in buffer B (20 mM Hepes, pH 6.5, 1 mM ATP in D_2O), consisting of 1 ml 60% sucrose (wt/wt), 3 ml 20% sucrose (wt/wt), and 3 ml 7% sucrose. The gradient was centrifuged for 2 h at 150,000 *g* in a SW41 rotor (Beckman Instruments, Inc., Palo Alto, CA). Membranes were collected from the sample/7% sucrose interface (fraction B1), the 7%/20% sucrose interface (fraction B2), and the 20%/60% sucrose interface (fraction B3). Fraction B2, containing the main portion of *cis*-Golgi membranes, was mixed with 1 ml of 60% sucrose in D_2O, placed in a centrifuge tube, and overlaid with 1 ml each of 30, 25, and 10% sucrose in D_2O. This gradient was centrifuged for 3 h at 150,000 *g* in a SW55 rotor (Beckman Instruments, Inc.). Golgi enriched membranes (fraction FB2) were collected from the interface between the 10% and 25% sucrose layers and frozen in liquid nitrogen in 100-μl aliquots for storage at −80°C.

Preparation of Membrane-free Cytosol and ER-derived Transport Vesicles

Membrane-free cytosol was prepared as described previously (Rexach and Schekman, 1991) with some modifications. A postmitochondrial supernatant was prepared by centrifugation of a cleared total homogenate at 100,000 *g* for 30 min. An equal volume of 60% Nycodenz (Sigma Chemical Co.) in buffer B was added, and the mixture (3 ml) was loaded in a 5 ml centrifuge tube (SW55 rotor) and overlaid with 1 ml of 30% Nycodenz in buffer B followed by 1 ml buffer B. The gradient was centrifuged at 100,000 *g* for 3 h to allow light membranes to float. Membrane-free cytosol (2.8 ml) was collected from the bottom of the tube and desalted by filtration on a Sephadex G25 column (15 ml) equilibrated in B88 (Baker et al., 1988) containing 1 mM ATP. The eluted protein peak was pooled and 100-μl aliquots were frozen in liquid nitrogen for storage at −80°C. The preparation of ER-enriched microsomal membranes and the loading of these membranes with [^{35}S]methionine-labeled prepro-α-factor by in vitro translocation has been described by Wuestehube and Schekman (1992). ER-derived vesicles were purified either from a Ypt1p-dependent transport reaction (Rexach et al., 1994) or from a vesicle formation reaction utilizing purified protein components (Barlowe et al., 1994). In the latter case the reaction mixture contained 100 μl washed microsomes (120 μg protein), an ATP regenerating system (Baker et al., 1988), 0.1 mM GTP, Sar1p (3 μg), Sec23/Sec24p complex (20 μg), and Sec13/Sec31p complex (5 μg) in a total volume of 600 μl. Reactions were incubated for 30 min at 30°C. A medium speed supernatant (MSS) fraction, prepared by centrifugation for 5 min at 13,000 *g* in a TOMY refrigerated microcentrifuge (Tomy Seiko, Tokyo, Japan) at 4°C, was mixed with 500 μl 50% Nycodenz in D_2O (wt/wt) and placed on the bottom of a 2.3 ml centrifuge tube (TLS55 rotor). A discontinuous gradient was prepared by overlaying the sample with 0.5 ml each of 27 and 10% sucrose in D_2O/buffer88. The gradient was centrifuged for 4 h at 150,000 *g*. Floated vesicles (300 μl) were collected at the 27/10% sucrose interface and frozen in liquid nitrogen in 100-μl aliquots for storage at −80°C. Vesicle release was quantified by determining the amount of protease-protected, Con A–precipitable [^{35}S]gp-

α-factor contained in the 12,000 *g* supernatant (Rexach and Schekman, 1991).

In Vitro Targeting and Fusion of ER-derived Vesicles with Golgi Membranes

Vesicle chase reactions were performed using floated ER-derived vesicles, containing a cargo of core-glycosylated [^{35}S]methionine-labeled pro-α-factor, as donor membranes. Vesicles (5 μl) were mixed on ice with 0.8 μg of Golgi membranes, 60 μg cytosolic protein, an ATP regeneration system (Baker et al., 1988) and 0.5 mM GTP in a total volume of 30 μl and incubated for 60 min at 20°C. After incubation the mixture was processed for anti-α1,6-mannose immunoprecipitation or for Con A–Sepharose precipitation (Rexach and Schekman, 1991). The percentage of transport was calculated as the anti-α1,6-mannose immunoprecipitable c.p.m. divided by the total Con A–Sepharose precipitable c.p.m. multiplied by 100. Other additions to reactions included: 1 μg per reaction anti-Ypt1p Fab fragments, 1 μg per reaction yeast Ypt1p isolated from *Escherichia coli* (Baker et al., 1990), 100 μM GTPγS ± 1 mM GTP, 200 μM GMPPNP ± 1 mM GTP, 1 mM ATPγS, or 5 mM EGTA/600 μM $MnCl_2$ ± 500 μM $CaCl_2$.

In "two stage" transport experiments vesicles and Golgi membranes were first incubated (20 min, 20°C) in the presence of 60 μg of cytosol, prepared from the cells of different mutant strains (stage 1). Then samples were chilled for 5 min before addition of 20 μg of wild type cytosol (with or without various inhibitors). Samples were then incubated for 40 min at 20°C and processed as above.

Enzyme Assays

The standard assay (20 μl) for α-1,6mannosyltransferase contained 0.1 μCi GDP-[^{3}H]mannose (New England Nuclear, Boston, MA), 1 mM GDP-mannose, 1 mM ATP, 1 mM $MnCl_2$, B88, and was initiated by addition of membranes. After a 10-min incubation at 20°C, the reaction was stopped by the addition of 20 μl 2% SDS. The sample was then incubated for 5 min at 100°C and processed for immunoprecipitation using anti-α1,6-mannose antibodies. The incorporation of [^{3}H]mannose into α1,6 mannoproteins was linear with respect to the incubation time (0–15 min) and membrane concentration (0.1–20 μg of protein). Enzyme activity is expressed in cpm of [^{3}H]mannose incorporated per min and μg protein. All other enzymes were measured using standard assays for guanosine diphosphatase (Abeijon et al., 1989), α-mannosidase (Opheim, 1978), and cytochrome c oxidase (Mason et al., 1973), vanadate-sensitive Mg^{2+}-ATPase (Willsky, 1979). Total protein was measured with the D_c protein assay (Bio-Rad Laboratories, Richmond, CA) according to the manufacturer's instructions.

Electron Microscopy and Immunolocalization

For routine electron microscopy, the sample fractions were pre-fixed with 1% glutaraldehyde, and then centrifuged to obtain pellets. The samples were fixed in 2% glutaraldehyde in cacodylate buffer, washed, post-fixed in 1% osmium tetroxide, followed by 0.5% tannic acid mirdant. En bloc staining with 2% uranyl acetate was done before ethanol dehydration and embedding in epoxy resin. Thin sections contrasted with uranyl acetate and lead citrate were viewed in the Philips 301 electron microscope.

Samples for immunoelectronmicroscopy were fixed in 3% formaldehyde – 0.2% glutaraldehyde, washed, dehydrated, and embedded in London Resin White. Thin sections were incubated first with affinity-purified antibody to Ypt1p, washed, and then incubated with goat anti–rabbit Fab fragments conjugated to 5 nm colloidal gold. As a control, similar sections were incubated without the primary antibody.

Results

Isolation of Golgi-enriched Membranes

The first step in reconstruction of the targeting/fusion reaction involved obtaining highly enriched donor (ER vesicles) and acceptor (Golgi) membrane fractions. We developed a method for the rapid isolation of yeast Golgi membranes that are enriched for specific marker proteins and highly active as an acceptor compartment in our in vitro transport reaction. As a marker for Golgi membranes we used α1,6-mannosyltransferase activity (α1,6-MT), measured by incorporation of [^{3}H]mannose from GDP-[^{3}H]mannose into the outer chains of endogenous yeast glycoproteins. We found that lysates produced by glass bead agitation of intact yeast cells, rather than by homogenization of yeast spheroplasts, produced a higher activity Golgi membrane fraction. We also found that functional Golgi membranes were labile; acceptor activity (see below) was lost with a half-time of 6 h at 0°C. Therefore the purification was designed to be completed in one day so that Golgi membranes could be frozen in aliquots. Acceptor activity remained active in samples stored at −80°C. As diagrammed in Fig. 1 Golgi membranes were isolated from strain RSY919 which carries the *mnn1* and *mnn2* mutations. This mutant has wild-type secretory properties, but does not contain α1,2- or α1,3-mannosyltransferases whose activity would interfere with the α1,6MT assay. Differential centrifugation of a total yeast lysate revealed that more than 80% of α1,6MT activity was present in the S50 fraction (Fig. 2 *a*). After density centrifugation on a sucrose/D_2O gradient the majority (65%) of α1,6MT activity was found in the B2 fraction (Fig. 2 *b*). Most of this activity (95%) floated in a sucrose gradient as a single peak (Fig. 2 *c*).

Table II is a purification table for a typical Golgi membrane isolation. The specific activity of the Golgi marker enzyme α1,6MT increased after sedimentation in the sucroseD_2O gradient 17-fold over the activity in the total cell lysate. Overall enrichment of α1,6MT after flotation was 518-fold with a 19% yield.

Biochemical and Morphological Characterization of Golgi-enriched Membrane Fraction

We used biochemical and immuno electron-microscopical criteria to document the enrichment of Golgi membranes

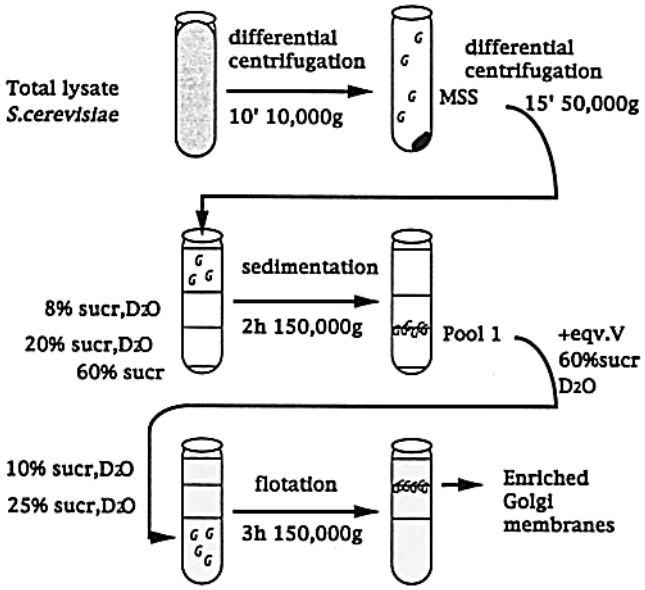

Figure 1. Flow diagram for purification of yeast *cis*-Golgi membranes. The general scheme used to isolate Golgi membranes is presented. Details are described in Materials and Methods.

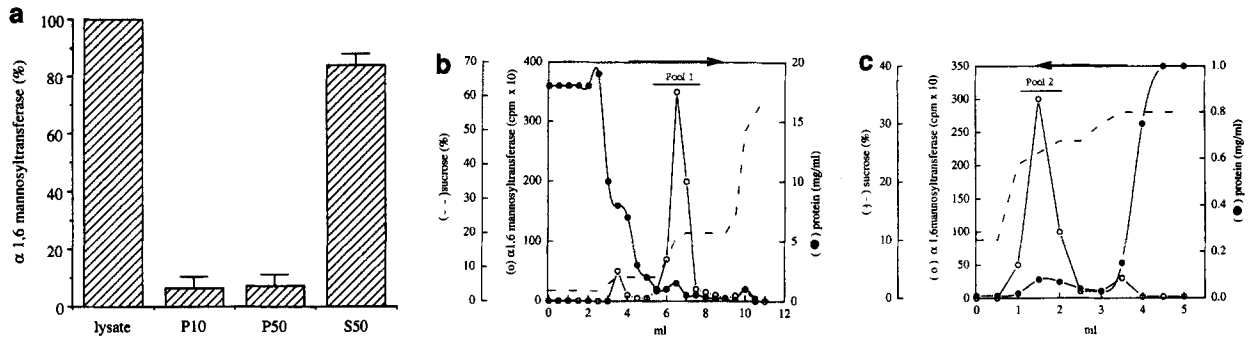

Figure 2. Purification of α1,6MT-containing membranes. (*A*) Crude yeast lysate was fractionated as in Materials and Methods and the amount of the 1,6MT activity in the original lysate and early fractions was determined. (*B*) S50 was loaded on top of a sucrose/D_2O step gradient. Membranes were sedimented for 2 h at 150,000 *g* and 23× 0.4 ml fractions were collected from the top. Protein concentration (*filled circles*), enzyme activity (*open circles*), and sucrose concentration (*dashed line*) were measured in every fraction. The peak of 1,6MT activity (fractions 13–16) was pooled. (*C*) The sample density was adjusted to 35% sucrose, loaded on the bottom of sucrose/D_2O step gradient, and membranes were floated for 3 h at 150,000 *g*. Fractions (11 × 0.5 ml) were collected from the top. The Golgi peak was pooled as before and analyzed. The arrows in the top x axis point to the direction of membrane movement during sedimentation and flotation.

in our preparations. Characteristic proteins and enzyme activities are associated with the different cellular organelles and may be used to assess the purity of fractions. The result of marker protein assays from a typical fractionation are shown in Table III and Fig. 3. The markers used are as follows: cytochrome *c* oxidase as a marker for the inner membrane of mitochondria (Mason et al., 1973); Sec61p and Sec12p as ER markers; the membrane-bound enzyme α-mannosidase as a vacuole marker (Opheim, 1978); vanadate-sensitive Mg^{2+}-ATPase as an integral membrane protein of the plasma membrane (Willsky, 1979); and phosphoglycerate kinase (PGK) as a soluble cytosolic protein. As markers for the *cis*, *medial*- and *trans*-Golgi compartments we used the α1,6MT structural protein (Och1p), GDPase, α1,3-mannosyltransferase structural protein (Mnn1p) and endoproteinase Kex2p (Nakayama et al., 1992; Graham et al., 1994; Redding et al., 1991). The specific activities of cytochrome c oxidase, α-mannosidase, Mg^{2+}-ATPase, GDPase and α1,6MT were determined from enzyme and protein assays. Other proteins were detected by immunoblotting as indicated in Table III.

Less than 1% of the total cell lysate content of the markers for plasma membrane, ER, mitochondria, and cytosol were found in the Golgi pool. Markers for the different Golgi compartments (Franzusoff et al., 1989) were enriched to different extents: Kex2p, a marker for *trans*-Golgi, was enriched 60-fold; Mnn1p, a marker for the medial Golgi, was enriched 94-fold; and Och1p, a marker for the *cis*-Golgi, was enriched more then 800-fold. Another Golgi marker, GDPase, was enriched 205-fold which reflects the broad distribution of this enzyme among different Golgi sub-compartments (Witters et al., 1994). Among the tested peripheral membrane proteins we found greater than 30-fold enrichment for the small GTP-binding protein, Ypt1p, and Sec17p in the Golgi pool (data not shown). Detectable amounts of Sec18p, Sed5p, Bet1p, Sly1p, Sec7p, Sec22p, Bos1p, and Sec21p (γ-subunit of yeast coatomer complex; Hosobuchi et al., 1992) were also found to be tightly associated with the purified membranes (Fig. 3).

To assess the purity of the Golgi membranes morphologically, the enriched Golgi pool was examined by thin section and immunoelectron microscopy. Membranes in the Golgi-enriched fraction (Fig. 4, *a* and *b*) appeared mainly as an array of spherical to discoid vesicles and tubules, 50–300 nm in diameter or length. Most of the larger tubular structures (*arrowheads*, Fig. 4 *b*) resemble cross-sectional views of Golgi cisternae as observed in the intact

Table II. α1,6-Mannosyltransferase Distribution during Golgi Purification

Fraction	Total protein	α1,6-Mannosyltransferase			
		Total activity*	Specific activity	Fold purification	Yield
	mg				%
Lysate	200.1	46	231	1.0	100
S50	170.2	42	251	1.1	92
Pool 2	15.1	20	1,900	8.2	43
Fl. Golgi	0.09	11	130,000	562	25

*α1,6-Mannosyltransferase activity is expressed as cpm [^{3}H]mannose × 10^3 incorporated into mannoproteins per minute.

Table III. Distribution of Marker Enzymes and Proteins in the Total Yeast Lysate and Enriched Golgi Fraction

Marker protein	Organelle	Lysate (specific activity)	Golgi fraction (fold enrichment)	
Och1p*	*cis*-Golgi	0.14	112.5	804
GDPase (units)	Golgi	0.47	96.3	205.1
Mnn1p*	*med*-Golgi	7.0	655.6	93.6
Sec12p*	ER	29.3	64.5	2.2
α-Mannosidase (milliunits)	vacuole	1.4	8.4	5.9
PM ATPase (units)	PM	0.27	0.8	2.9
Cyt *c* oxidase (units)	mitochondria	0.03	0.07	2.2
PGK*	cytosol	53.2	0.5	0.01

*Aliquots of total lysate and enriched Golgi fractions were subjected to SDS-PAGE, and immunoblotted with different antibodies. Immuno-complexes were detected by 125I-protein A and quantified using a PhosphoImager.

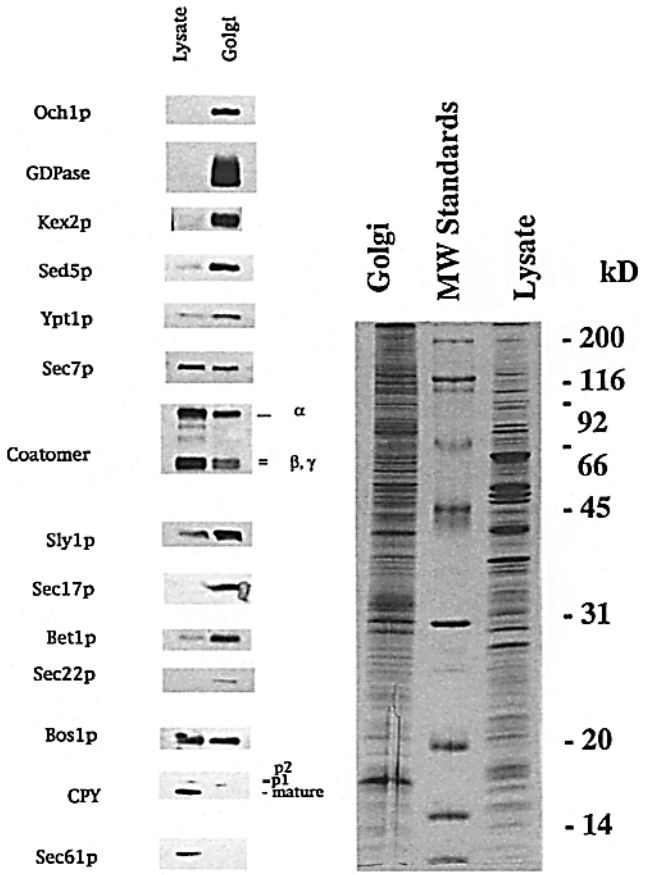

Figure 3. Polypeptide and antigenic composition of *cis*-Golgi enriched membranes. Samples of total yeast lysate and floated Golgi membranes were solubilized in sample buffer and separated on an SDS-polyacrylamide (10%) gel. The right panel shows the silver stain (equal protein amount loaded), and the left panel shows the blot immunostaining for the different marker proteins (protein concentration in the total lysate sample was 10 times higher than in the Golgi membrane sample).

yeast cell (Preuss et al., 1992). Small fragments of Golgi membranes are not easily identified because they may be indistinguishable from other membrane vesicles especially if the lumen of these fragments dilates during the purification process. Since specific labeling of yeast Golgi membranes by antibodies to Ypt1p has been previously described (Preuss et al., 1992), immunolocalization of this marker provided a better assessment of the efficiency of our purification procedure. A significant fraction (43%) of the membrane profiles were labeled by affinity-purified antibodies to Ypt1p (Fig. 4 *d*). Occasional linear arrays of gold label (*small arrowheads*, Fig. 4 *d*) suggest staining over a cisternae-like structure.

Enriched Membranes Reconstitute Sec Protein-dependent Targeting/Fusion

Because our goal was to use isolated Golgi membranes for the identification and characterization of components that mediate ER–Golgi vesicular transport, we tested the specific activity of enriched Golgi membranes in an in vitro transport assay. Specific activity was expressed as the ability of Golgi membranes to allow purified ER-derived vesicles loaded with [^{35}S]glyco-pro-α-factor to target and fuse. After fusion [^{35}S]gp-α-factor acquires specific α1,6-mannose residues (Rexach and Schekman, 1991). We showed before that the isolated ER-derived transport vesicle fraction is devoid of ER marker proteins (Barlowe et al., 1994; Rexach et al., 1994). Furthermore, the transport vesicles fuse with *cis*-Golgi membranes, but not with ER membranes. Thus, no new ER vesicles form, and the isolated Golgi membranes are the only potential target in the course of the fusion reaction. Conversion of the [^{35}S]gp-α-factor contained in the vesicle fraction to the α1,6-mannose modified form required the addition of both cytosol and an acceptor membrane fraction (Fig. 5). Minimal (<10%) vesicle chase was observed when cytosol or GTP was omitted from the reaction. Purified Golgi-enriched membranes contained about 100 times more acceptor activity compared to total yeast membranes (maximum activity for the transport in 30 μl reaction required 0.8 μg purified Golgi membranes and about 80–100 μg of total membranes). The transport activity was linear with respect to the membrane concentration and about 30 μg/ml Golgi membranes was sufficient to convert 47% of the [^{35}S]gp-α-factor to the outer-chain modified form (data not shown). We showed previously that a late stage in the total ER to Golgi transport reaction requires ATP hydrolysis and Ca^{2+} (Rexach and Schekman, 1989). Transport with purified membrane components required GTP and ATP hydrolysis and was inhibited by 74% in the presence of 100 μM GTPγS or 200 μM GMPPNP and by 51% in the presence of 2 mM ATPγS (Fig. 6). As in an overall ER–Golgi transport reaction in the crude cell lysate the targeting/fusion reaction with enriched membranes was Ca^{2+}-dependent, and inhibited 80% by the chelators 5 mM EGTA or 1 mM BAPTA.

In vitro targeting and fusion of purified vesicles with Golgi membranes required the addition of yeast cytosol. We used our reconstituted system and temperature sensitive yeast mutants to determine the role of the different soluble proteins facilitating targeting and fusion. We found that cytosols prepared from *sec7-1*, *sec7-4*, *sec18-1*, *uso1-1*, *sly1^{ts}*, *sec19-1*, *ypt1-3*, and *ypt1^{ts}* mutant cells grown at permissive (24°C) temperature supported transport. However, a short pre-incubation of mutant cytosols at 30°C inactivated their ability to support transport (Fig. 7 and data not shown). Cytosols prepared from wild type cells or strains defective in ER-vesicle budding (*sec*12-1 and *sec*23-1) did not show any defect before or after preincubation at elevated temperatures. Purified Golgi membranes from *sec7-4*, *sec18-1*, *uso1-1*, *sly1^{ts}*, *sec19-1*, and *ypt1^{ts}* mutant cells grown at the permissive (24°C) temperature had wild-type acceptor activity in the presence of cytosol and donor vesicles prepared from wild type yeast (data not shown). Unfortunately, pre-treatment of wild type or mutant Golgi membranes at elevated temperature resulted in a dramatic loss of acceptor activity. Inactivation of Sec18p by NEM treatment was used before in the ER–Golgi in vitro transport reaction (Rexach and Schekman, 1991). However, with purified Golgi membranes such treatment resulted in a dramatic inactivation of glycosylation machinery (or GDP-mannose transport system), as measured by incorporation of [^{3}H]mannose from

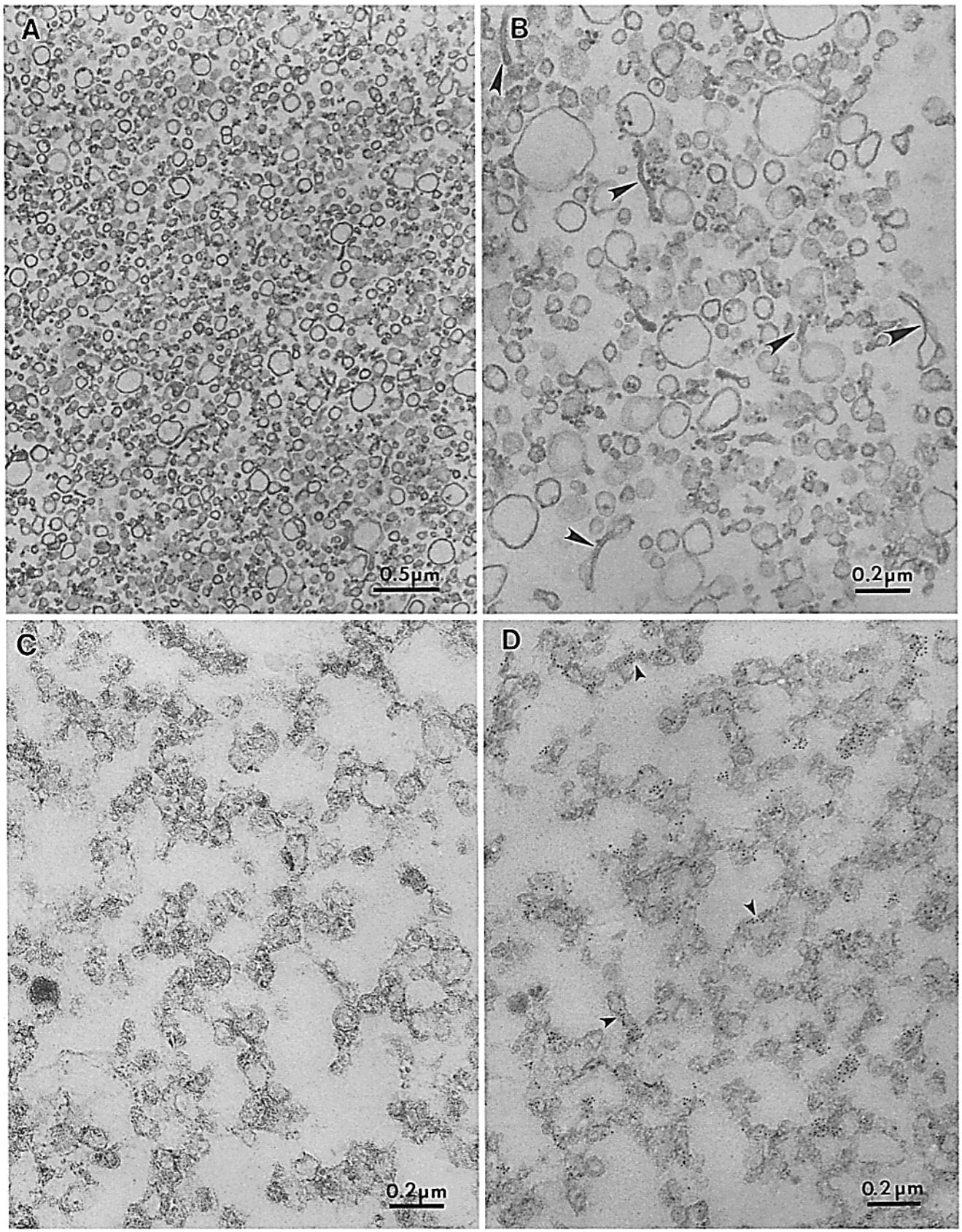

Figure 4. Electron micrographs of enriched *cis*-Golgi membranes. (*A*) A representative view of the pooled Golgi fraction from the sucrose flotation gradient. (*B*) Higher magnification of the enriched Golgi membranes showing several cisternae resembling that seen in the intact yeast (*arrowheads*). (*C*) Control for immunolocalization of Ypt1p where primary antibody was omitted and no gold label is seen. (*D*) Immunolocalization of Golgi marker, Ypt1p, shows gold label on many membranes of the Golgi-enriched fraction. Small arrowheads note linear display of gold particles over cisternae-like structures. Bars: (*A*) 0.6 μm; (*B–D*) 0.2 mm.

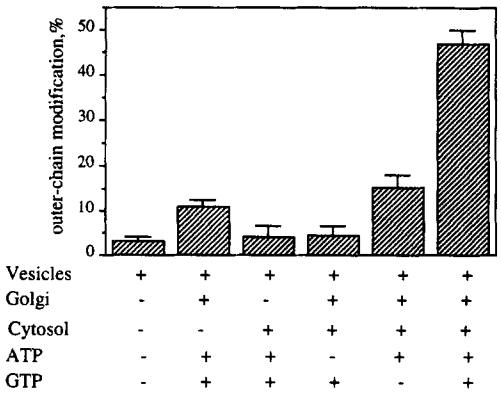

Figure 5. Transport reaction with purified donor (ER-derived vesicles) and acceptor (*cis*-Golgi membranes) components. Aliquots of floated transport vesicles (5 μl) were mixed with fresh membrane-free cytosol (60 μg), purified Golgi membranes (0.8 μg), an ATP regeneration mix, and 0.2 mM GTP as indicated. Samples were incubated for 1 h at 20°C, and reactions terminated by addition of 2% SDS and heated to 95°C. Samples were processed for precipitation with Con A and anti-outer chain antibodies as described in Materials and Methods. The average of four experiments and standard error are indicated.

GDP-[^{3}H]mannose into the outer chains of endogenous yeast glycoprotein (data not shown). Because the assay used in our experiments relied on efficient conversion of the [^{35}S]gp-α-factor contained in the vesicle fraction to the α1,6-mannose modified form, we were unable to use NEM-treated membranes in our experiments.

The transport defect of mutant cytosols in half of the cases (*uso1-1*, *sly1^{ts}*, *sec7-4*) was rescued by supplementing the reaction with a portion of wild-type cytosol (Fig. 8).

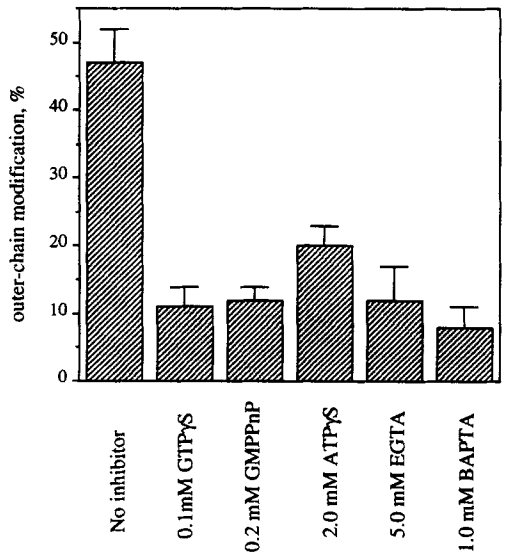

Figure 6. Inhibition of targeting/fusion reaction with non-hydrolyzable analogs of GTP and ATP and by chelation of Ca^{2+} ions. All reaction components were mixed as in Fig. 5 with the addition of inhibitors as shown. Samples were processed as described for Fig. 5.

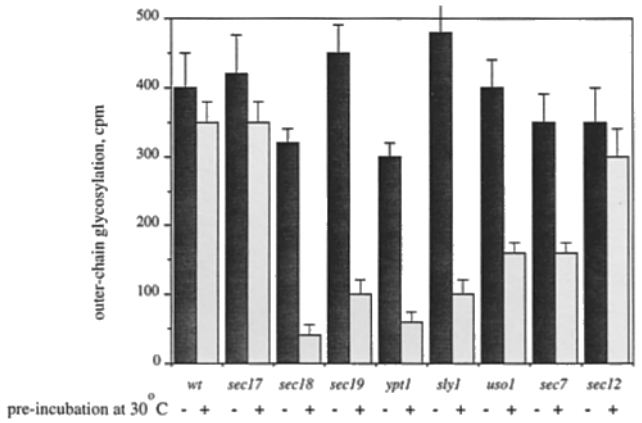

Figure 7. Transport-stimulating activity in the cytosol, prepared from *ts*-mutant cells is sensitive to a 15 min pre-incubation at 30°C. Membrane-free cytosol was prepared from the mutant cells grown at 24°C as described in Materials and Methods. Portions of cytosol were pre-incubated for 15 min either on ice (*dark bars*) or at 30°C (*light bars*) and the aliquots (60 μg of protein) were added to the transport reaction. Samples were processed as in Fig. 5.

This rescue was inefficient in the case of *sec18-1*, *sec19-1* and *ypt1^{ts}* cytosols, probably because of a "dominant–negative" behavior of the mutant protein in the in vitro reaction.

Fractionation of Soluble Components on Mono Q Column

To resolve the cytosolic requirement for ER-derived vesicle targeting and fusion, we applied desalted yeast cytosol on an FPLC Mono Q anion exchanger. Protein was eluted with a step gradient of 0.9 and 2.0 M of KOAc, and pooled fractions were tested for transport-stimulating activity (Fig. 9). No significant activity was detected in the flow

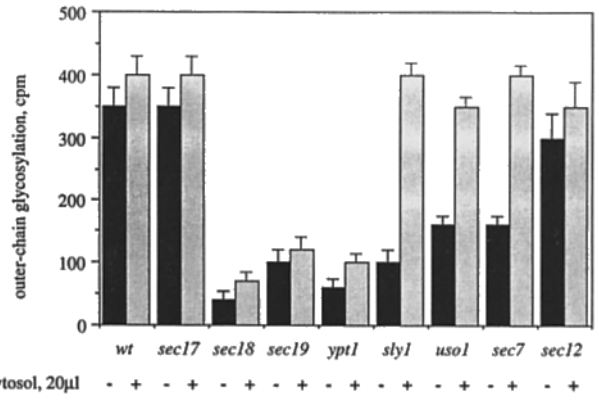

Figure 8. Rescue of the heat-inactivated, transport-stimulating cytosol activity by wt cytosol. Cytosol was prepared from mutant cells, inactivated at 30°C, and the aliquots were added in the transport reaction (*dark bars*). For some samples (*light bars*) wild-type cytosol was added to the transport reactions before the incubation. Samples were processed as above. Addition of the wild-type cytosol (20 μg) alone did not support the transport reaction (110 cpm after immunoprecipitation with anti-outer chain antibodies).

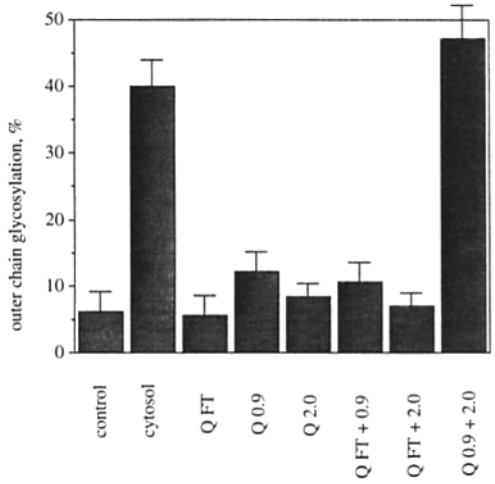

Figure 9. Transport-stimulating activity requires two MonoQ fractions. Membrane-free cytosol (20 mg) prepared from *Saccharomyces cerevisiae* RSY607 cells was dialyzed against B88 and loaded on a FPLC MonoQ 5/5 column equilibrated with the same buffer. Proteins were eluted by 0.9 M KOAc and then by 2.0 M KOAc in B88. The flow through pool (*FT*), 0.9 M eluate (*Q 0.9*), and 2.0 M eluate (*Q 2.0*) were collected, dialyzed against B88, and concentrated to 1 ml using Centricon-10 (Amicon Corp.) concentrators. Samples (5 μl) of each fraction (8–60 μg of protein) were added to the transport assay in the absence of cytosol. *Control*, no cytosol; *cytosol*, whole cytosol (60 μg protein) added.

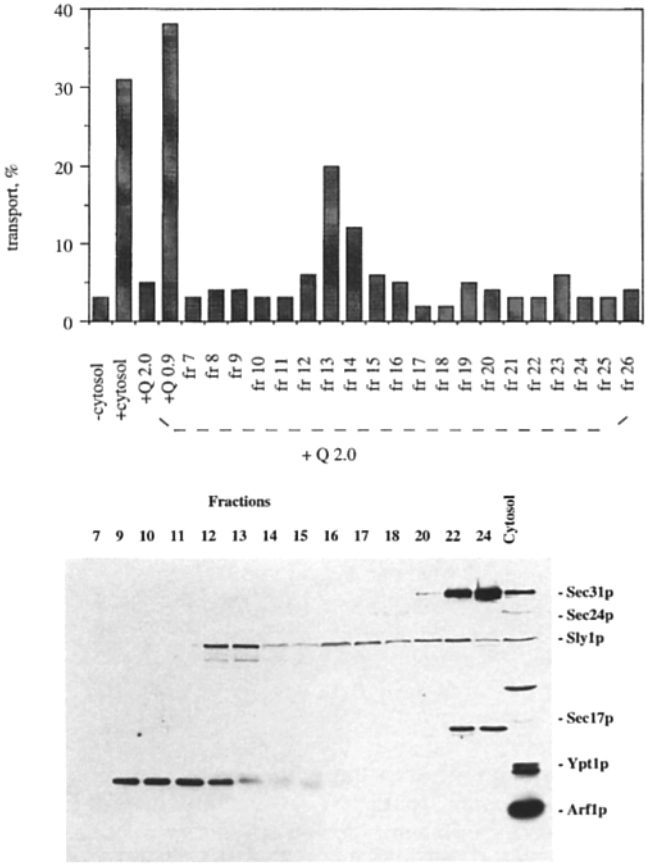

Figure 10. Mono Q chromatography of LSF activity. Dialyzed 0.9 M eluate (as in Fig. 9) was loaded on a FPLC Mono Q column and fractionated with a 0.15–0.9 M KOAc gradient. Fractions (1 ml) were collected, dialyzed against B88, and assayed in the transport reaction in the presence of 5 μl of 2.0 M eluate (*A*); − and + cytosols as in Fig. 9. Aliquots of each fraction were loaded on 12.5% SDS-PAGE and various proteins were detected by immunoblotting (*B*).

through (unbound) nor in individual eluted fractions when assayed alone, indicating that multiple components had been separated. When the 0.9 M pool was mixed with the 2.0 M pool, the activity of whole cytosol was restored, indicating that the unbound fraction contained no transport-stimulating activity. Further fractionation of the 0.9 M pool was achieved using an FPLC MonoQ column, eluted with a KOAc gradient (Fig. 10 *a*). Fractions were combined with the 2.0 M pool and tested for activity in the in vitro assay. The highest activity was detected in fractions 13 (approximately four times background) and fraction 14 (approximately two times background). These fractions were pooled and are referred to as the low salt fraction (LSF). Fractions (7–25) from the same gradient were analyzed for Ypt1, Sly1, Sec7, Sec17, Sec18, Sec21, Sec24, and Arf1 protein distribution by immunoblotting (Fig. 10 *b*). Only two of these proteins, Ypt1p and Sly1p co-eluted with the transport-stimulating activity. Attempts to separate LSF further, by ion-exchange and gel-filtration chromatography yielded no significant activity in any resulting fraction when assayed alone or mixed with the 2.0 M pool, indicating that either a component was inactivated or multiple components were separated (data not shown). We next examined the fractionation behavior of the 2.0 M pool. This fraction was reapplied to an FPLC MonoQ column, and protein was eluted with a 0.9–2.0 M KOAc gradient, and fractions were tested in our assay (Fig. 11 *a*). When LSF was included in the assay mix one peak of activity (~2.5 times background) was detected, eluting at 1.4 M KOAc. We termed this pool high salt fraction (HSF). All fractions after the MonoQ column were analyzed by immunoblotting for the same set of proteins as described above (Fig. 11 *b*). Sec7p, Sec18p, and Sec21p were detected in the active 2.0 M pool. However only Sec7p completely co-eluted with the active fractions. After an additional separation of HSF by gel-filtration chromatography on an FPLC Superose 6 column only 20% of the original activity was recovered. Again all the transport-stimulating activity co-eluted with Sec7p and the peak of activity was separated from Sec18p (not shown). The activity peak and Sec7p eluted as a high molecular weight (700–800 kD) protein complex. Separation of the activity from Sec21p and thus yeast coatomer (COP I) was achieved by velocity sedimentation in a glycerol gradient. HSF was loaded on the gradient and all activity (~30% from original) co-sedimented with Sec7p with a sedimentation coefficient of about 10S (data not shown).

The combination of LSF and HSF supported transport in our assay to the same level as whole cytosol indicating that all necessary transport-stimulating proteins have been recovered.

The ability of the HSF and the LSF to rescue the activity of mutant cytosols was also examined (Fig. 12 *a*). Addition of the HSF to the assay rescued only the defect in *sec7-4*

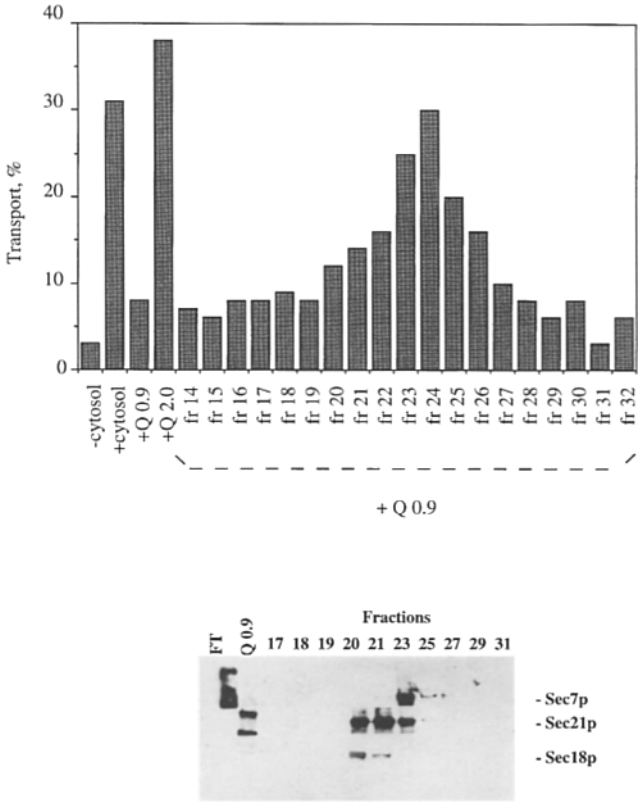

Figure 11. Mono Q chromatography of HSF activity. Dialyzed 2.0 M eluate (as in Fig. 9) was loaded on FPLC Mono Q column and fractionated with 0.9–2.0 M KOAc gradient. Fractions (1 ml) were collected, dialyzed against B88 and assayed in the transport reaction in the presence of 0.9 M eluate (4 μl) (*A*); − and + cytosols as in Fig. 9. Aliquots of each fraction were separated on 12.5% SDS-PAGE and various proteins were detected by immunoblotting (*B*).

cytosol, supporting the idea that Sec7p is one of the active components of HSF. The HSF, prepared from the *sec7-4* mutant strain was unable to support transport in the presence of wt LSF (not shown). Addition of the LSF to the in vitro reaction completely rescued the defect in *sly1*^{ts} cytosol and partially rescued the defect in the cytosol prepared from *uso1-1* mutant cells (Fig. 12 *b*). Soluble Sec17p (yeast α-SNAP), Sec18p (yeast NSF), Sec21p (a component of yeast coatomer [COPI] complex) or Sec24p (a component of COPII protein complex) were not required to drive the targeting and fusion reactions reconstituted in our in vitro assay. Because the membranes used in our assay were not salt-treated, a large portion of Sec17p, Sec18p, and Sec21p was tightly associated with the Golgi membranes; this pool may be sufficient to stimulate the targeting/fusion reaction.

Sequential Intermediates in the Targeting/Fusion Reaction

We used two-stage incubation experiments to study the order of involvement of different factors during targeting and fusion of ER-derived vesicles with Golgi membranes. In the first stage of the reaction membranes were pre-incubated for 20 min in the presence of 60 μg of heat-inactivated mutant cytosol and an ATP-regeneration system. In the second stage, 20 μg of wt cytosol was added to the reaction in combination with different inhibitors (Fig. 13). This addition of wt cytosol (20 μg of protein) was sufficient to support only 25% of targeting and fusion activity when mutant cytosol was omitted from the first stage. When GTPγS, ATPγS, EGTA, or Ypt1 F_{ab} were added in the first stage, significant inhibition of transport was observed (Fig. 13, *stage 1*). Similar results were obtained when *sly1*^{ts} cytosol was present during the first stage of the reaction and the different inhibitors were added together with *wt* cytosol during the second stage. The second stage of the reaction was not inhibited by GTPγS and Ypt1 F_{ab} when *uso1-1* or *sec7-4* cytosol was present during the first stage, but transport reaction remained sensitive to ATPγS and EGTA (Fig. 13, *stage 2*). None of the inhibitors were effective at the second stage of the reaction if wt cytosol was used at the first stage.

Fig. 14 summarizes the results of the inhibitor studies. Sly1p, a GTPγS-sensitive step and Ypt1p (the function likely to be inhibited by GTPγS) may act at an early stage in the reaction; Uso1p and Sec7p appear to act in a subsequent or a parallel step. The last step is inhibited by ATPγS, probably due to the involvement of the Sec17p and Sec18p. We showed previously that a Sec18p-dependent step is followed by a Ca^{2+}-dependent step in the vesicle–Golgi fusion reaction (Rexach and Schekman, 1991). The biochemical complementation assay developed here could not be exploited to refine this assignment because cytosol from *ypt1*^{ts}, and *sec18-1* mutant cells were inhibitory in a mixed incubation.

Genetic Interactions of Soluble and Membrane Components of the Targeting/Fusion Apparatus

To corroborate the biochemical data on the involvement of specific soluble proteins in targeting/fusion of ER-derived vesicles with Golgi membranes we also tested genetic interactions between the genes encoding Ypt1p, Bet2p, Sly1p, Uso1p, and Sec7p. We crossed pairs of temperature-sensitive mutants, dissected tetrads from the resulting diploid strains, tested spore growth at permissive (24°C) and sub-restrictive (30°C) temperatures and confirmed our results by complementation analysis in a search for synthetic lethal interactions between these mutations. When *sec7-4* was crossed to *ypt1*^{ts}, the majority of tetrads had only three viable spores. This is the expected result if the double mutant is inviable. The same results were obtained with the *ypt1-3* mutant. When *sec7-4* was crossed to *bet2-1* the double mutant grew at 24°C but, unlike the parental strains, failed to grow at 30°C. In contrast, in all other crosses to *sec7-4*, the majority of tetrads had four viable spores at 30°C. These data, together with our biochemical data on an involvement of Ypt1p and Sec7p in the same process, support the idea that the *SEC7* and *YPT1* gene products interact functionally, and possibly physically, and suggest that the *BET2* gene product may also be involved in this interaction.

YPT1 showed synthetical lethal interactions not only with *SEC7* but also with *SLY1* and *USO1* (Sapperstein et al., 1996). In all cases double mutants of the genotype *ypt1*^{ts}*sly1*^{ts} and *ypt1*^{ts}*uso1-1* were inviable. We did not de-

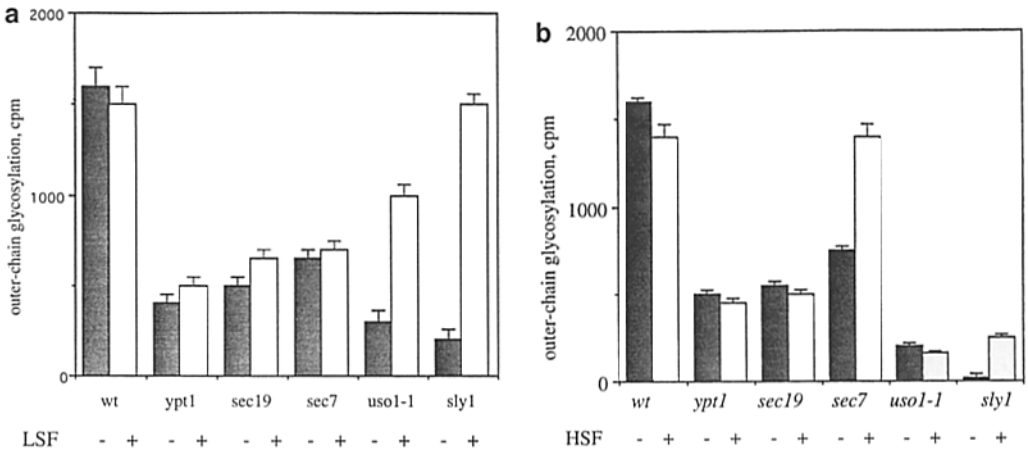

Figure 12. Mutant cytosol rescue by LSF and HSF fractions. Cytosol prepared from mutant cells was heat inactivated (15 min at 30°C) and added to the transport reaction (*dark bars*). (*a*) 5 μl of LSF pool (Fig. 10, fractions *12–14*, 5 mg protein/ml) was added to the reaction (*light bars*). (*b*) 5 μl of HSF pool (Fig. 11, fractions *22–26*, 0.9 mg protein/ml) was added to the reaction (*light bars*). After incubation (1 h, 20°C) samples were processed as above.

tect any synthetically lethal interactions between *SEC7* and *SLY1* or any of the genes encoding membrane components of targeting/fusion complex (*SEC22*, *BET1*, and *BOS1*).

Discussion

We have purified functionally active yeast Golgi membranes and used them to better define the biochemical requirements for the targeting and fusion of transport vesicles budded from the ER. Current biochemical and genetic data suggest that the yeast Golgi complex is organized into three distinct functional compartments: *cis*, containing α1,6 mannosyltransferases; *medial*, containing α1,2 and α1,3 mannosyltransferases; and *trans*, populated by the late Golgi enzymes Kex1p, Kex2p, and dipeptidylaminopeptidase A (reviewed by Franzusoff, 1992). The Golgi purification procedure was designed to be rapid because we found that transport acceptor activity in crude or purified membranes decayed rapidly. The Golgi membrane fraction purified in this study is operationally defined as an early Golgi compartment, and had the highest enrichment (600-fold) for the *cis*-Golgi membrane marker, α1,6 MT. The purified Golgi membrane fraction was also enriched, to a lesser degree, for the late Golgi marker, Kex2p (Red-

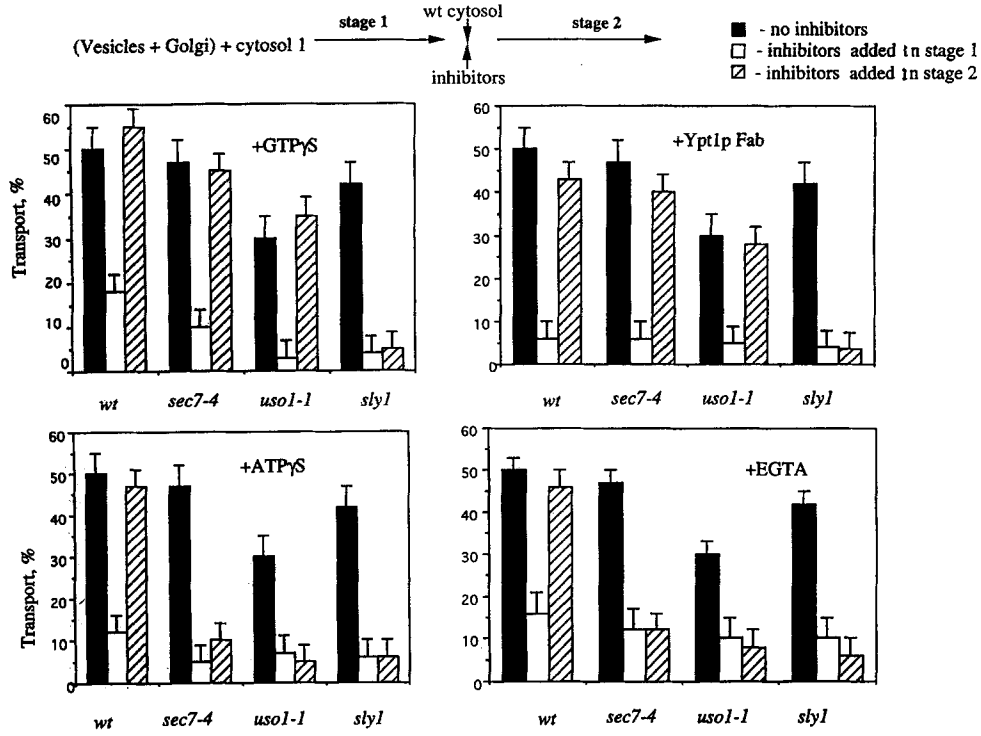

Figure 13. Two-stage transport reaction. Wt or heat inactivated (15 min at 30°C) cytosol samples (60 μg of protein, cytosol 1) were added to the transport reactions which were incubated for 20 min at 20°C. Reactions were transfered to ice and 2 μl (20 μg of protein) of wt cytosol was added. Reactions were incubated for an additional 40 min at 20°C (stage 2) and samples were processed as above. Inhibitors: 0.2 mM GTPγS (*A*), 0.2 μg of anti Ypt1p Fab (*B*), 2.0 mM ATPγS (*C*), 5 mM EGTA/0.6 mM $MnCl_2$ (*D*) in 2 μl of B88 were added to the reactions before the first or second incubations as indicated.

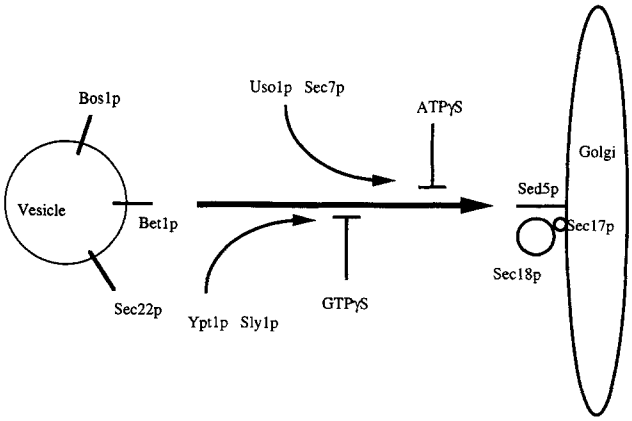

Figure 14. Model for the sequence of involvement of soluble components in the targeting/fusion reaction.

ding et al., 1991), but was depleted for marker proteins of the ER, plasma membrane, the vacuole, and mitochondria. Membranes were free of the ER peripheral proteins Sec13p and Sec24p. Two peripheral proteins, the small GTP-binding protein, Ypt1p, and the yeast α-SNAP homolog, Sec17p, were significantly enriched in the Golgi fraction. We also found that Sed5p, Sec7p, Sec18p, and yeast coatomer co-purified with *cis*-Golgi membranes, but to a lesser extent than Ypt1p and Sec17p. After treatment of isolated membranes with high salt (1 M KOAc) some peripheral proteins (Sec17p, Sec18p, coatomer) were removed whereas the majority of Ypt1p and Sec7p remained tightly associated with the Golgi membranes (Fig. 4 *d* and data not shown). Analysis of untreated and salt-treated purified membranes by thin-section electron microscopy revealed that the purified Golgi consists mainly of a mixed population of vesicular and tubular membranes ranging from 50 to 300 nm in diameter and length. About 40% of these structures were densely labeled with antibodies to Ypt1p on thin sections prepared for immunoelectron microscopy. These results are similar to immunoelectron microscopic observations by Preuss et al. (1992), who found morphologically similar structures labeled with anti-Ypt1p IgG and anti α-1,6-mannose antibodies in wild type yeast cells.

We found that vesicle targeting and fusion with the purified Golgi membranes required ATP, GTP, Ca^{2+}, and cytosol. Nonhydrolyzable nucleotide analogs ATPγS and GTPγS specifically blocked vesicle consumption in the reconstituted in vitro reaction. The primary target for the GTPγS inhibition is most probably the Ypt1 protein. Novick and Brennwald (1993) proposed that Ypt1p can load on the surface of the transport vesicles in the GTP-bound form and be released to the cytoplasm in the GDP-bound form by the action of GDI (guanine nucleotide dissociation inhibitor). Thus GTP hydrolysis appears to be required for the proper function and recycling of Ypt1p. It is notable that GTP hydrolysis is required at least twice during one round of ER to Golgi transport, first for transport vesicle uncoating mediated by Sar1p (Barlowe et al., 1994) and later during the targeting/fusion step, probably for the formation of the targeting complex (Söllner et al., 1993; Søgaard et al., 1994) involving Ypt1p. ATP hydrolysis, which is inhibited by ATPγS, is probably required for Sec18p function during the maturation of a targeting complex (Wilson et al., 1992; Rothman, 1994). In the presence of ATPγS a specific 20S complex can form in vitro in a mammalian transport assay (Söllner et al., 1993). The 20S particle contains SNAREs, SNAP-25, NSF, and α-SNAP and is disassembled when ATP is hydrolyzed (Wilson et al., 1992).

Specific protein requirements for ER-derived transport vesicle targeting and fusion with *cis*-Golgi membranes were revealed by inhibition of Sec protein function in mutant cells. Cytosol prepared from temperature-sensitive mutant cells was inactivated by brief incubation at an elevated temperature; in some cases transport activity was rescued by the addition of a small amount (20 μg) of wild type cytosol. These studies showed that Sec7p, a peripheral membrane protein (Achstetter et al., 1988), Ypt1p, a peripheral and cytosolic GTP-binding protein (Segev et al., 1988), Sec19p, a cytosolic protein (Garrett et al., 1994), Sly1p, a peripheral membrane protein (Dascher et al., 1991), Uso1p, a peripheral membrane protein (Nakajima et al., 1994; Seog et al., 1994), and Sec18p, yeast NSF homolog (Eakle et al., 1988) are required for vesicle targeting and fusion.

Most of the targeting Sec proteins are highly enriched in the transport stimulating fractions purified from yeast cytosol on the FPLC MonoQ column. We found that the cytosolic requirement for the transport reaction was satisfied by a mixture of two protein fractions eluted from FPLC MonoQ columns; one at low (0.2–0.3 M KOAc) salt, termed LSF, and one eluted in high (1.4 M KOAc) salt, termed HSF. LSF is enriched for Ypt1p and Sly1p. Soluble Sec17p was not required to drive the transport reaction in vitro, but like Sec18p, the bulk of Sec17p was found already associated with Golgi membranes. The most likely active components of LSF are Ypt1p and Sly1p. It is possible that this fraction also contains Sec19p. We found that the LSF, but not HSF, prepared from *sec19-1* cytosol was defective in our transport assay (data not shown). Addition of wild type LSF to the transport reaction rescued *sly1*^{ts} and *uso1-1* mutant cytosols. We could not determine the fractionation properties of Sec19p or Uso1p due to the lack of specific antibodies. The precise biochemical function of Uso1p or its mammalian homolog p115 (Sapperstein et al., 1996) is currently unknown. p115 is required for the docking or fusion step in intra-Golgi transport (Waters et al., 1992) and for binding of transcytotic vesicles to plasma membrane (Barroso et al., 1995). Uso1 is required for the assembly of the ER to Golgi SNARE complex (Sapperstein et al., 1996).

HSF was enriched for Sec7p, and also contained some coatomer complex and Sec18p. Additional purification of HSF by gel-filtration and glycerol velocity sedimentation revealed that the activity co-purified only with Sec7p and that coatomer and Sec18p were not functional components of HSF activity.

The main candidate for the active component of HSF is Sec7 protein (and possibly Sec7p-associated proteins). Sec7 immunoreactive material co-purified with HSF activity on every ion-exchange and gel-filtration column used, as well as on a glycerol velocity gradient. It appears that Sec7p forms a complex with several other proteins (Fran-

zusoff, A., personal communication) and this complex could be time and condition sensitive. Cytosol prepared from *sec7-4* mutant cells was defective in the transport assay, and its activity was restored by addition of HSF prepared from *wt* cytosol. ER to Golgi transport of [^{35}S]gp-α-factor in the *sec7-4* or *sec7-1* semi-intact cells was temperature-sensitive. At the restrictive temperature accumulation of core-glycosylated pro-α-factor in a functional vesicle intermediate was observed (data not shown).

Franzusoff et al. (1992) observed accumulation of a transport vesicle intermediate as a result of an ER to Golgi transport block in vitro induced by addition of anti-Sec7p IgG, which affects cytosolic, but not Golgi-associated Sec7p. These authors proposed a role for Sec7p as a coat protein. However, the Sec7p is not required to form ER-derived transport vesicles (Barlowe et al., 1994), and instead it is mainly found associated with the Golgi membrane surface (Preuss et al., 1992; and data not shown). It is possible that Sec7p (and probably other proteins, like Uso1p and Ypt1p) binds to the surface of the transport vesicles in exchange for the COPII-type protein coat just before targeting to the acceptor membrane. Such re-coating could be required to facilitate correct targeting of the vesicles to the acceptor membrane. If this model is correct then it may be possible to reconstitute such a reaction using purified ER-derived transport vesicles.

Alternatively, Sec7p, and probably Ypt1p and Uso1p bind to the *cis*-Golgi membrane surface prior the vesicle targeting and are required for the docking of uncoated transport vesicles. If this model is correct, then previously described immuno-purified membranes enriched for Sec7p (Franzusoff et al., 1992) and/or Ypt1p (Lian and Ferro-Novick, 1993) were not the ER-derived transport vesicles but rather Golgi fragments.

Surprisingly, soluble Sec18p and Sec17p were not required for our in vitro targeting/fusion reaction, although a similar activity (NSF and α-SNAP) is required in the reconstituted mammalian intra-Golgi transport reaction (Wilson et al., 1989; Clary et al., 1990). In the mammalian system this requirement was revealed only when the membrane-bound Sec18p-like protein, NSF, was inactivated by NEM treatment or after salt extraction of the membranes. The significant amounts of Sec17p and Sec18p tightly associated with our purified Golgi membranes were probably sufficient to provide this essential activity during the targeting/fusion reaction. Salt extraction, which removed approximately 80% of the membrane bound Sec17p and Sec18p from our enriched Golgi membranes (data not shown), may be sufficient to reveal a requirement for these and other peripheral proteins in our targeting/fusion reaction.

We used two-step transport reactions to study the biochemical sequence of involvement of different soluble activities in the targeting/fusion reaction. Sly1p, Ypt1p, and GTP hydrolysis are required during the early stages of the reaction. Sec7p and Uso1p are involved in a parallel or in a later step possibly after Ypt1p has already been loaded with GTP. We suggest that all these proteins are recruited to the surface of uncoated ER-derived vesicles as a prelude to recognition of the acceptor membrane and formation of a pre-fusion complex. The biochemical complementation assay described here allows a functional reconstruction of this targeting process with enriched cytosolic proteins. Further effort is required to achieve a fully resolved system.

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