Reconstitution of Protein Transport from the Endoplasmic Reticulum to the Golgi Complex in Yeast: The Acceptor Golgi Compartment is Defective in the *sec23* Mutant

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Abstract. Using either permeabilized cells or microsomes we have reconstituted the early events of the yeast secretory pathway in vitro. In the first stage of the reaction ∿50–70% of the prepro-α-factor, synthesized in a yeast translation lysate, is translocated into the endoplasmic reticulum (ER) of permeabilized yeast cells or directly into yeast microsomes. In the second stage of the reaction 48–66% of the ER form of α-factor (26,000 D) is then converted to the high molecular weight Golgi form in the presence of ATP, soluble factors and an acceptor membrane fraction; GTPγS inhibits this transport reaction. Donor, acceptor, and soluble fractions can be separated in this assay. This has enabled us to determine the defective fraction in *sec23*, a secretory mutant that blocks ER to Golgi transport in vivo. When fractions were prepared from mutant cells grown at the permissive or restrictive temperature and then assayed in vitro, the acceptor Golgi fraction was found to be defective.

THE transit of newly synthesized proteins along the secretory pathway involves a number of events which have been defined biochemically or morphologically. After synthesis in the cytoplasm and entry into the lumen of the endoplasmic reticulum (ER)[1], proteins are modified by cleavage of the signal sequence, glycosylated, and assume their native conformation (Blobel and Dobberstein, 1975; Kornfeld and Kornfeld, 1985). Morphological and cell fraction studies have suggested that the exit of proteins, from the lumen of the ER, is mediated by vesicles which bud from transitional elements and fuse with the Golgi complex (Palade, 1975; Farquhar and Palade, 1981; Saraste and Kuismanen, 1984; Saraste and Hedman, 1983; Saraste et al., 1986; Lodish et al., 1987). Although the earliest events of protein transport have been reconstructed biochemically (Walter et al., 1984), later events such as the formation, transport, and fusion of shuttle vesicles with the Golgi complex are not well understood.

In vitro assays have proven to be an important tool in analyzing complicated biological processes. A biochemical dissection of the secretory pathway will require the development of transport assays that reconstitute stages of this pathway in vitro. This approach has already led to the identification of several components that play a role in the transit of proteins through the cell. For example, eukaryotic in vitro translocation systems, that reproduce the events involved in the targeting and threading of proteins across the lipid bilayer of the ER membrane, have permitted the identification of several components involved in this process (Walter and Blobel, 1980; Meyer et al., 1982; Wiedmann et al., 1987; Waters et al., 1986). More recently, an assay that can mimic inter-Golgi transport in vitro (Balch et al., 1984), has led to the identification of an N-ethylmaleimide-sensitive protein involved in a later stage of the pathway (Glick and Rothman, 1987). Although specific factors which mediate intracellular protein transport between the ER and the Golgi complex remain to be discovered, in vitro transport assays that use permeabilized mammalian cell systems suggest that they exist. With this approach (Simons and Virta, 1987; Beckers et al., 1987) cells are radiolabeled with [35S]methionine, during infection with vesicular stomatitis virus, either before or after the cells are permeabilized. In the presence of an ATP regenerating system presynthesized G protein, a membrane glycoprotein residing in the ER, is transported to the Golgi complex as indicated by trimming of the ER-associated high mannose oligosaccharide form of G protein.

A molecular dissection of the events in intracellular protein transport would be facilitated by an analysis of the secretory pathway in an organism that is amenable to both a genetic and biochemical approach. The yeast *Saccharomyces cerevisiae* offers this advantage. The genetic flexibility of this organism has permitted the isolation of a large collection of mutants that block protein transport at various stages of the pathway. Of the many genes that have been implicated in these processes, mutations in 11 genes (called *SEC* or *BET*) have been shown to affect the transit of proteins from the lumen of the ER to the Golgi complex (Novick et al., 1980; Newman and Ferro-Novick, 1987). At 37°C (nonpermissive

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1. *Abbreviations used in this paper:* Endo H, endoglycosidase H; ER, endoplasmic reticulum. HSP, high speed pellet; HSS, high speed supernatant; Pic, protease inhibitor cocktail.

temperature), precursors to exported proteins that fail to be transported in these mutants, accumulate in the lumen of the ER. An assay that efficiently reconstitutes protein transport from the ER to the Golgi complex in vitro could elucidate the molecular defect in these mutants and lead to the identification of components that play an essential role in this process.

Here we introduce a new assay that uses translocation into microsomes, or the ER of permeabilized yeast cells, to reconstitute transport to the Golgi complex. A precursor form (prepro-α-factor) of the secreted pheromone α-factor serves as a marker protein in this assay. In vivo (Julius et al., 1984b), this protein receives three N-linked oligosaccharide chains (Glucose$_3$ Mannose$_9$ N-acetylglucosamine$_2$) in the ER and is further modified by an extensive addition of mannose units (outer chain carbohydrate) in the Golgi complex. In the transport assay we developed, these posttranslational modifications are performed in vitro. The passage of prepro-α-factor to the Golgi complex is assayed in two stages. In the first stage of the reaction preproprotein of high specific radioactivity is synthesized in vitro and is translocated into the lumen of the ER. Translocation can be achieved in two ways: by using either a permeabilized cell system or yeast microsomes. In the second stage of the reaction the ER form of α-factor is transported to the Golgi complex. Transit is detected by the addition of outer chain carbohydrate. In this report we show that reconstitution of transport from the ER to the Golgi complex in yeast is dependent upon the presence of ATP and a 3,000 g supernatant (S3) of a yeast lysate. This supernatant contains both soluble factor(s) and membrane-bound components that include the acceptor compartment. Furthermore we have demonstrated that an S3 fraction isolated from *sec23*, a mutant that blocks ER to Golgi transport in vivo, fails to support transport in vitro. Fractionation of this supernatant has shown that a defect in the *Sec23* protein destroys the activity of acceptor membranes without affecting the soluble factors required for transport.

Materials and Methods

Growth Conditions

Yeast used for the preparation of permeabilized cells and S3 fractions were grown at 25°C in YPD medium (1% yeast extract, 2% Bactopeptone, and 2% glucose) to an OD$_{599}$ = 1.5–4.0. Wickerham's minimal medium (1946) was used; for sulfate-free medium, all sulfate salts were replaced by chloride salts. To change growth medium, cells were harvested in a clinical centrifuge at room temperature and then resuspended in new medium. Cell densities were measured in a 1-cm quartz cuvette at 599 nm in a spectrophotometer (model 25; Beckman Instruments, Inc., Palo Alto, CA).

Preparation of Permeabilized Cells, S3, High Speed Supernatant (HSS) and High Speed Pellet (HSP) Fractions

Cells grown at 25°C were harvested and resuspended in YP medium containing 0.1% glucose (OD$_{599}$ = 1.5). After a 30-min incubation at 24°C, cells (150 OD$_{599}$ units) were pelleted, resuspended in 50 ml of spheroplasting medium (YP medium, 0.1% glucose, 50 mM potassium phosphate, 1.4 M sorbitol, 50 mM β-mercaptoethanol, pH 7.5, and 1 mg Zymolyase 100T [ICN Immunobiologicals, Plainview, NY]) and incubated at 37°C for 30 min (or 60 min at 24°C for the *sec23* permissive experiment). Spheroplasts formed during this time were incubated in 100 ml of regeneration medium (YP medium, 0.1% glucose and 1 M sorbitol) for 90 min at 37°C (or 90 min at 24°C for the *sec23* permissive experiment). The regenerated spheroplasts were split into two equal aliquots; an S3 fraction was prepared

from one aliquot and permeabilized cells were generated from the remaining portion. To prepare the permeabilized cells, the regenerated spheroplasts were harvested, resuspended in 5 ml of permeabilization buffer (0.1 M potassium acetate, 0.2 M sorbitol, 2 mM magnesium chloride, and 1/50 vol of 1 M Hepes [pH 7.2]), and centrifuged at 3,000 g for 5 min. The final pellet was resuspended in 50 μl of buffer I (250 mM sucrose, 2 mM dithiothreitol [DTT], 1 mM EGTA, and 1/50 vol of 1 M Hepes [pH 7.4]) supplemented with 1× Protease inhibitor cocktail (Pic; described by Waters and Blobel, 1986). To prepare the S3 fraction, the regenerated spheroplasts were resuspended in 206 μl of 20 mM Hepes (pH 7.2) and then centrifuged at 3,000 g for 5 min. The HSS and HSP fractions were generated from an S3 fraction (supplemented with 1× Pic) centrifuged at 100,000 g for 1 h; the pellet (HSP) was resuspended in an equal volume of 20 mM Hepes (pH 7.2). Before the transport assay–concentrated Hepes buffer, potassium acetate, magnesium chloride, and sorbitol were added to the S3, HSS, and HSP fractions. The final concentration of buffer in each fraction was: 115 mM potassium acetate, 2.5 mM magnesium chloride, 0.2 M sorbitol, 1× Pic, and 1/28 vol of 1 M Hepes (pH 7.2). The protein concentration of each fraction was measured using the Bradford assay (1976): permeabilized cells (19 mg/ml), S3 fraction (14 mg/ml), HSS (8 mg/ml), and HSP (6 mg/ml). Ovalbumin was used as a protein standard in this assay.

In Vitro Translation and Translocation

In vitro transcriptions, yeast translation lysates, and microsomes were prepared as described before (Hansen et al., 1986) with minor modifications, briefly summarized below. 20 μg of plasmid pDJ100 (obtained from D. Julius) was linearized with Xba I (Boehringer Mannheim Diagnostics, Inc., Houston, TX) and then transcribed with SP6 RNA polymerase. The RNA was precipitated and resuspended in 100 μl of water. Cells (~4,000 OD$_{599}$ units), used to prepare yeast translation lysates, were converted to spheroplasts during a 30-min incubation at 37°C with 30 mgs of zymolyase 100T. The spheroplasts formed during this incubation were regenerated for 60 min at 37°C and then lysed as described by Hansen et al. (1986) with half the volume of lysis buffer. The lysate was homogenized with a motor driven Potter homogenizer (40 strokes). Cells, used for the preparation of microsomes, were converted to spheroplasts as described above with the regeneration step eliminated. The protocol of Hansen et al. (1986) was used with minor modifications. The S10 supernatant was loaded onto a sucrose cushion (2.0 M sucrose, 2 mM DTT, 2× Pic, and 1/50 vol of 1 M Hepes [pH 7.4]), rather than a Percoll gradient, and centrifuged at 100,000 g for 1 h. The microsomes at the interface were collected, treated with Staphylococcal nuclease as described before (Hansen et al., 1986), and washed by passage through a Sephacryl S-500 (Sigma Chemical Co., St. Louis, MO) column as described earlier (Walter and Blobel, 1983).

The translation cocktail (25 μl/reaction) contained 10 μl of the translation lysate, 20 mM Hepes (pH 7.4), 150 mM potassium acetate, 3 mM magnesium acetate, 2.8 mM DTT, 1 mM ATP, 0.1 mM GTP, 20 mM creatine phosphate, 30 μM of each amino acid except methionine, 20 μCi of [^{35}S]methionine (Amersham Corp., Arlington Heights, IL; 1,000 Ci/mmol), 0.2 mg/ml yeast tRNA (Boehringer Mannheim Diagnostics, Inc.), 0.2 mg/ml creatine phosphate kinase, 10 U RNAsin (Promega Biotec, Madison, WI, 40,000 U/ml), 8% glycerol, 75 mM sucrose, 1× Pic, and 3 μl of RNA (1/10 dilution of preparation described above). Prepro-α-factor was translated in vitro during a 1-h incubation at 20°C. Permeabilized cells (60 μg of protein) or microsomes (16 μg of protein in 3 μl) were added to the lysate (supplemented with 20 mM creatine phosphate) and the reaction mix was incubated at 20°C for 20 min. The translocation reaction was terminated by chilling the samples on ice. The amount of prepro-α-factor translocated was determined by densitometric scanning (model GS 300; Hoefer Scientific Instruments, San Francisco, CA) of light exposures of autoradiograms from six different experiments. The values we obtained were in the linear range of the assay. The percentage of prepro-α-factor translocated was defined as the amount of 19-kD primary translation product converted to the 26-kD species. The sum of the untranslocated 19-kD species and the 26-kD species was approximately equal to the 19-kD primary translation product present before the translocation reaction was initiated.

In Vitro Transport Reaction

Permeabilized cells (60 μg of protein), containing radiolabeled proprotein in the lumen of the ER, were pelleted during a 15-s centrifugation in a microfuge. The cells were washed (25 μl/reaction) with transport buffer (115 mM potassium acetate, 2.5 mM magnesium chloride, 0.2 M sorbitol, 1× Pic, and 1/50 vol of 1 M Hepes [pH 7.2]) and then resuspended in the same buffer (25 μl). The transport reaction (81.7 μl final volume unless stated

otherwise) contained 25 µl of permeabilized cells (60 µg of protein), 50 µl of an S3 fraction (0.7–1.0 mg of protein), 0.92 mM GDP-mannose (Sigma Chemical Co.), and an ATP-regenerating system (1 mM ATP, 0.1 mM GTP, 20 mM creatine phosphate, 0.2 mg/ml creatine phosphate kinase). The mix was incubated at 20°C for 90 min and the reaction was terminated by chilling the samples on ice. To deplete samples of ATP, apyrase (5 U/ml, grade VIII, Sigma Chemical Co.) instead of the ATP-regenerating system, was added to the reaction mix. After the reaction, samples were immunoprecipitated with 2 µl of anti–α-factor antibody as described before (Ruohola and Ferro-Novick, 1987). When using the α1-6-Main antibody (obtained from C. Ballou), samples were first immunoprecipitated with anti–α-factor antibody, washed as described before (Ruohola and Ferro-Novick, 1987) and solubilized in 70 µl of sample buffer at 100°C for 5 min. Samples were then diluted as described earlier (Ruohola and Ferro-Novick, 1987), and reprecipitated with 10 µl of α1-6-Man antibody. Solubilized immunoprecipitates were subjected to electrophoresis in a 12.5% NaDodSO₄/polyacrylamide slab gel unless stated otherwise and fluorographed as described before (Chamberlain, 1979). The decrease in the ER form of α-factor, observed during the transport reaction, was determined either by densitometric scanning of light exposures of autoradiograms or by excising and solubilizing protein bands from gels and measuring the counts by liquid scintillation counting as described before (Bangs et al., 1986).

Protease Protection

Samples were incubated with trypsin (0.47 mg/ml) in the presence or absence of 0.1% Triton X-100 during a 30-min incubation on ice. Trypsin inhibitor (final concentration 0.94 mg/ml) was added, and after 5 min, samples were boiled in 1% SDS and then immunoprecipitated as described before (Ruohola and Ferro-Novick, 1987).

Treatment with Endoglycosidase H (Endo H)

Solubilized immunoprecipitates were resuspended in 85 µl of buffer A (100 mM ammonium acetate [pH 5.5], 0.45% SDS, 3% β-mercaptoethanol, 0.03% sodium azide and 2× Pic) and boiled for 5 min. Each sample was divided into two tubes containing 120 µl of buffer B (100 mM ammonium acetate [pH 5.5], 3% Triton X-100, 0.03% sodium azide, and 2× Pic). Endo H (7.5 mU; ICN Immunobiologicals) was added to one of the two tubes and the samples were incubated at 37°C for 13 h. The reaction was terminated by the addition of 6 vol of acetone (20 µg of cytochrome c was added as carrier protein). After a 2-h incubation at −20°C, samples were centrifuged for 15 min in a microfuge and the pellet was resuspended in sample buffer.

Electron Microscopy

Spheroplasts and permeabilized yeast cells (50 OD₅₉₉ units) were fixed for 3.5 h in 1% paraformaldehyde, 0.8% glutaraldehyde, 0.15 M cacodylate buffer (pH 7.2), 0.1 M potassium acetate, 0.2 M sorbitol (1 M for spheroplasts), and 2 mM magnesium chloride. Samples were washed three times with 0.15 M cacodylate buffer (pH 7.2) and then postfixed for 1 h on ice with 1% osmium tetroxide, 0.1 M cacodylate buffer (pH 7.2). Samples were then washed three times with 0.15 M sodium chloride and stained for 2 h on ice with uranyl acetate in 0.5% sodium chloride. The samples were washed again with 0.15 M sodium chloride, resuspended in 2% Bacto-agar, and formed into blocks. The blocks were dehydrated with ethanol and embedded in Spurr medium (Polysciences, Inc., Warrington, PA). Thin sections were stained with uranyl acetate and lead citrate, then viewed in an electron microscope (model 301; Philips Electronic Instruments, Mahwah, NJ) at 80 kV.

In Vivo Radiolabeling of sec Mutants

The mutants were grown overnight, at 25°C (OD₅₉₉ = 1–1.5), in sulfate-free minimal medium supplemented with 100 µM ammonium sulfate and 2% glucose. For radiolabeling, a modification of the protocol described by Reid and Schatz (1982) was used. Cells were resuspended in sulfate-free medium containing 2% glucose (1 OD₅₉₉ U/0.1 ml) and labeled with [³⁵S]sulfate (200 µCi/OD₅₉₉) for 2 min during vigorous aeration. The incubation was terminated by the addition of 2.5 vol of cold ammonium sulfate (35 mM final concentration) and the cells were harvested and flash frozen in liquid nitrogen. Thawed cells were resuspended in 100 µl of 1% SDS and lysed by vortexing for 2 min, on a vortex mixer in the presence of glass beads (0.2 g of glass beads, 425–600 µm in size). The lysate was diluted as described before (Ruohola and Ferro-Novick, 1987) and immunoprecipitated with α-factor antibody.

Immunoblot Analysis

Spheroplasts were resuspended in permeabilization buffer and centrifuged at 3,000 g for 5 min. The amount of carboxypeptidase Y and hexokinase B released into the supernatant and retained within the permeabilized cells was determined by Immunoblot analysis. Samples were subjected to electrophoresis in a 10% NaDodSO₄/polyacrylamide slab gel and then transferred overnight at 4°C onto nitrocellulsoe (BA 83, 0.2 µm Schleicher & Schuell, Keene, NH). Carboxypeptidase Y and hexokinase B were detected with antisera and radioiodinated staphylococcal protein A (30 mCi/mg, Amersham Corp.) as previously described (Goud et al., 1988). Anti-carboxypeptidase antiserum was used at a dilution of 1:400 and anti-hexokinase B antiserum was used at a dilution of 1:200. The nitrocellulose filters were dried and exposed to Kodak XAR-5 film at −70°C for 4–6 h. The protein bands were excised from the nitrocellulose filter and counted in a Gamma counter (model 4000; Beckman Instruments, Fullerton, CA). The data points reported in Table II were in the linear range of the assay.

Results

Prepro-α-factor Enters Permeabilized Yeast Cells and is Translocated Across the ER Membrane

To develop an efficient assay that can reconstitute the early events of protein transport in yeast, we used permeabilized yeast cells. Permeabilized cells (SFNY26-6A, see Table I) were prepared by removing the yeast cell wall enzymatically and osmotically shocking spheroplasts. Electron microscopic analysis of thin sections revealed that cytoplasmic contents were depleted from the permeabilized cells (Fig. 1, compare C with A and B). Although holes in the plasma membrane were not visible, it appeared that this lipid bilayer no longer served as a barrier between contents normally retained within the cell and its environment. Nuclear contents were preserved in these cell preparations, however, the nuclear envelope was dilated (Fig. 1 A). The dark granular material of the vacuole was also retained (Fig. 1 B). Vesicles studded with ribosomes, seen at higher magnifications (not shown), were most likely derived from the ER membrane. To biochemically assay the extent of permeabilization, Immunoblot analysis was used to quantitate the release of two different enzymes: hexokinase B, a cytoplasmic enzyme, and carboxypeptidase Y, a vacuolar constituent. The results in Table II demonstrate that most of the hexokinase B was depleted from the permeabilized cells, while the majority of the carboxypeptidase Y was retained in the cell preparations.

Previous studies with yeast have shown that prepro-α-factor is efficiently translocated across the ER membrane (Hansen et al., 1986; Rothblatt and Meyer, 1986; Waters and Blobel, 1986). Accordingly, we added permeabilized cells to a yeast lysate containing radiolabeled prepro-α-factor translated in vitro. As is evident from the results presented in Fig. 2, A and B (lane 2), the 19-kD primary translation product entered these cells and was efficiently translocated across the ER membrane. Approximately 50–70% of the prepro-α-factor was converted to the 26-kD ER form (three N-linked oligosaccharide units). This form was protected from proteases in the absence of detergent (Fig. 2 A, lane 3), but was fully degraded in the presence of detergent (Fig. 2 A, lane 4). Minor species containing 1 and 2 N-linked core oligosaccharide units were also observed (Fig. 2 B, lane 2) as previously reported (Hansen et al., 1986; Rothblatt and Meyer, 1986; Waters and Blobel, 1986). This finding indicated that the ER membrane in these cell preparations was biologically active, intact and accessible to exogenously added substances. To determine if the ER form of α-factor was contained within
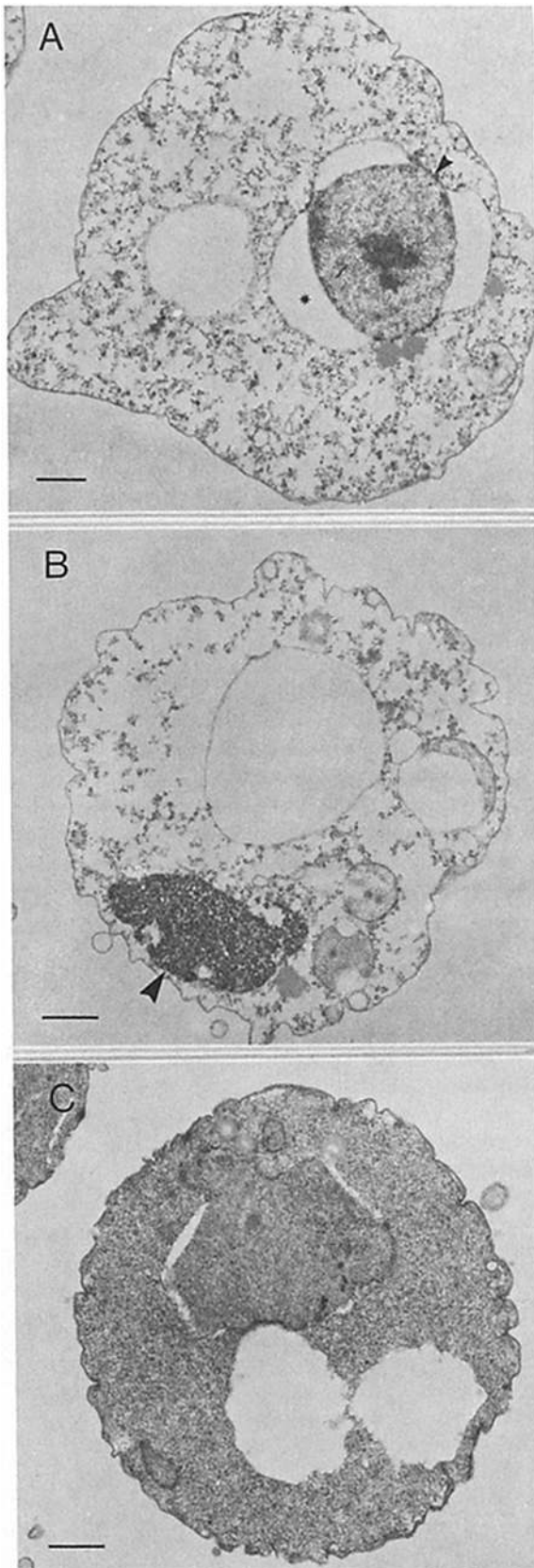
Figure 1. Morphology of yeast spheroplasts and permeabilized cells. Spheroplasts from wild-type cells (SFNY 26-6A, see Table I) are shown in *C* and permeabilized cells are shown in *A* and *B* for comparison. The small arrowhead points to a nucleus, the large arrowhead denotes a vacuole, and the asterisk (*) marks the lumen of the nuclear envelope. Bar, 0.5 μm.

Table I. Yeast Strains

Strain	Genotype	Source
SFNY 26-6A*	MAT α, his 4-619	This study
NY 429	MAT α, ura 3-52, sec14-3	P. Novick‡
NY 432	MAT α, ura 3-52, sec 18-1	P. Novick
LHY 3-8C	MAT α, ura 3-52, leu 2-3, 112 his 3, sec23-1	R. Deshaies§
LB 1-3B	MAT a, mnn 2-1	C. Ballou§
LB 347-1C	MAT α, mnn9	C. Ballou

* SFNY26-6A is essentially isogenic to DBY 877 (obtained from D. Botstein, Department of Biology, Massachusetts Institute of Technology, Cambridge, MA).
‡ Department of Cell Biology, Yale University School of Medicine.
§ Department of Biochemistry, University of California at Berkeley, Berkeley, CA.

the permeabilized cells after the translocation reaction, we sedimented the cells by performing a 15-s centrifugation in a microfuge. Since the majority of the 26-kD form of α-factor was found to pellet (Fig. 2 *B*, lanes *2–4*), it must have resided inside the permeabilized cells.

## The ER Form of α-factor is Converted to a High Molecular Weight Species in the Presence of an S3 Fraction and ATP

Transport and processing of prepro-α-factor through the yeast secretory pathway has been previously examined in vivo (Julius et al., 1984*b*). When the transit of this protein is arrested in the Golgi apparatus, the major form which accumulates is a high molecular weight species that migrates as a heterogeneous smear on SDS–polyacrylamide gels (Julius et al., 1984*a*). Analysis of this material has shown that this form of α-factor results from the addition of outer chain carbohydrate to the proprotein. Proteolytic processing is thought to begin in the Golgi complex and completed in secretory vesicles. Mature α-factor is secreted into the medium as a 13 amino acid peptide (Julius et al., 1984*b*).

To determine if transport to the Golgi complex could occur in vitro, we pelleted the permeabilized yeast cells after the translocation reaction, and resuspended them in a buffer that contained an ATP regenerating system, GDP-mannose (a substrate for mannosyltransferases; Nakajima and Ballou, 1975) and a 3,000 *g* supernatant of a yeast lysate (S3). After a 90-min incubation at 20°C, the 26-kD ER form of α-factor diminished in intensity when the high molecular weight species appeared (Fig. 3, lane *4*). This conversion was not observed when the incubation was carried out at 0°C (Fig. 3,

*Table II. Amount of Hexokinase B and Carboxypeptidase Y Remaining in the Permeabilized Cell Preparations*

Marker enzyme	Spheroplasts	Permeabilized cells	Supernatant
	%	%	%
Hexokinase B	100	29 ± 19	71 ± 18
Carboxypeptidase Y	100	81 ± 5	19 ± 5

Spheroplasts were resuspended in permeabilization buffer and centrifuged at 3,000 *g* for 5 min. The levels of hexokinase B and carboxypeptidase Y (cytoplasmic and vacuolar enzyme markers, respectively) were determined in intact spheroplasts, the permeabilized cell pellet and supernatant by quantitative Immunoblot analysis. The data in this table were the result of two separate experiments.
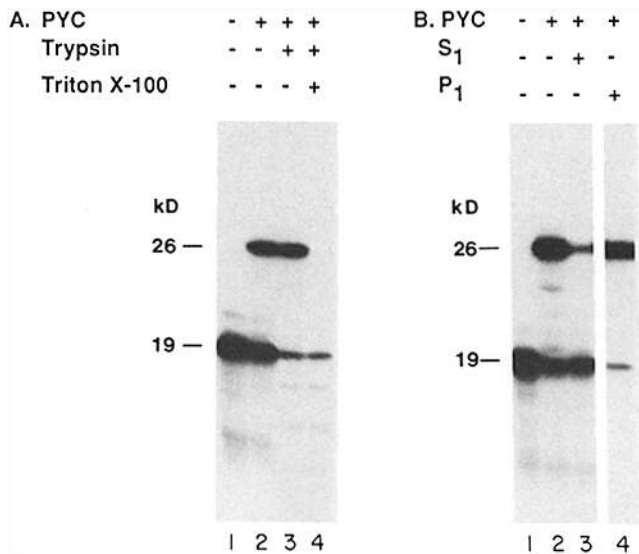
Figure 2. Prepro-α-factor enters permeabilized yeast cells (PYC) and is translocated. In vitro translated prepro-α-factor (A and B, lane 1) was incubated with permeabilized yeast cells (60 μg of protein) for 20 min at 20°C in the presence of an ATP-regenerating system (A and B, lane 2). (A) Samples were treated with trypsin (0.47 mg/ml) in the absence (lane 3) and presence (lane 4) of 0.1% Triton X-100. (B) At the end of the translocation reaction samples were centrifuged in a microfuge for 15 s to generate supernatant (S_1) and pellet (P_1) fractions. Prepro-α-factor was largely found in the supernatant (A and B, lane 3), while the majority of the ER form of α-factor was in the pellet fraction with the permeabilized cells (A and B, lane 4). All samples were immunoprecipitated with anti-α-factor antibody raised against the secreted form of this protein (Newman, A., and S. Ferro-Novick, unpublished results). Solubilized immunoprecipitates were subjected to electrophoresis in a 12.5% NaDodSO4/polyacrylamide slab gel and fluorographed.

lane 2). The areas of the gel containing the 19-kD, 26-kD, and high mol wt forms of α-factor were excised, solubilized, and measured by scintillation counting. The increase in counts in the high molecular weight species (5,060 cpm) was largely accounted for by the decrease in counts in the 26-kD ER form of α-factor (3,750 cpm). Thus, the high molecular weight species (5,060 cpm) was primarily a direct product of the 26-kD species. A small decrease in counts (650 cpm) in the 19-kD species was also observed, suggesting that some translocation had occurred. Since the total decrease in radioactivity in the 19-kD and 26-kD species was approximately the same as the increase in the high molecular weight form, a likely interpretation of the data is that the 19-kD primary translation product was converted to the 26-kD ER form which was the precursor to the high molecular weight form of α-factor. Conversion to the high molecular weight form of α-factor was efficient as shown by the fact that the ER form of α-factor diminished in intensity by ~54% (Fig. 3, lanes 1 and 4); a decrease of 48–66% was generally observed. (The decrease in the ER form of α-factor was determined by densitometric scanning of lightly exposed autoradiograms). No requirement for GDP-mannose was seen (Fig. 3, lane 3), however, GDP-mannose was required when S3 fractions and permeabilized cells were prepared from glucose starved cells (not shown). Enzymatic degradation of ATP with apyrase (Fig. 3, lane 5) or hexokinase and glucose (not shown)
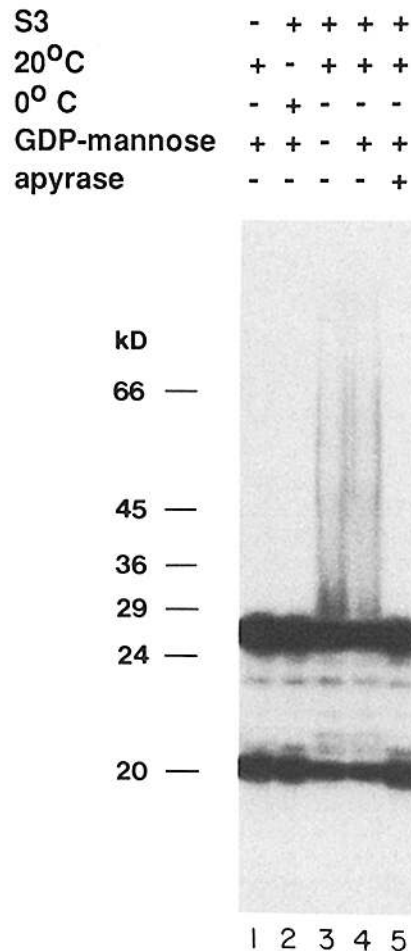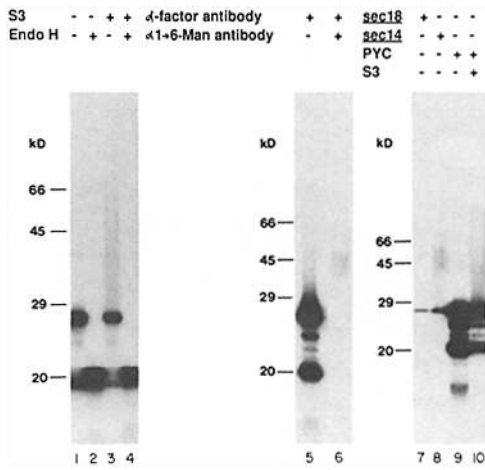

Figure 3. The ER form of α-factor is converted to the high molecular weight form in the presence of an S3 fraction and ATP. Permeabilized yeast cells (60 μg of protein), containing the ER form of α-factor, were incubated with an S3 fraction (0.7 mg of protein) and an ATP-regenerating system in the presence of GDP-mannose (lane 4) or in its absence (lane 3). No high molecular weight α-factor was detected when the sample was incubated on ice for 90 min (lane 2), or when the incubation was performed in the absence of an S3 fraction (lane 1). Depletion of ATP with apyrase (5 U/ml) inhibited the reaction (lane 5).

prevented the formation of the high molecular weight form of α-factor, indicating that ATP was required for this event.

High Molecular Weight α-factor is in the Golgi Complex

In vivo studies have shown that the high molecular weight species is the result of the addition of mannose units to the N-linked oligosaccharide chains (Julius et al., 1984a). These oligosaccharides are sensitive to digestion by Endo H, an enzyme that cleaves between the two N-acetylglucosamine residues (Chu et al., 1978). To determine whether the shift in molecular weight observed in vitro was due to an extension of N-linked oligosaccharide units, we digested immunoprecipitated species of α-factor with Endo H. The results in Fig. 4 (lanes 3 and 4) demonstrate that high molecular weight α-factor was sensitive to digestion, indicating that as observed in vivo, this increase in molecular weight was the result of the extension of N-linked carbohydrate and not to other posttranslational modifications.

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1469

kD
66 —
45 —
29 —
20 —

1 2 3 4   5 6   7 8 9 10

Figure 4. High molecular weight α-factor is transported to the Golgi complex. Permeabilized yeast cells, containing the ER form of α-factor, were incubated in the presence (lanes *3* and *4*) or absence (lanes *1* and *2*) of an S3 fraction. Samples were immunoprecipitated with α-factor antibody and then treated with Endo H (lanes *2* and *4*) as described in Materials and Methods. In lanes *5* and *6*, the in vitro transport assay was performed with permeabilized yeast cells and an S3 fraction prepared from a strain harboring the *mnn2* mutation. At the end of the reaction, samples were immunoprecipitated with α-factor antibody and then subjected to a second immunoprecipitation with α1-6-Man antibody (lane *6*). Solubilized immunoprecipitates were subjected to electrophoresis in a 12.5% NaDodSo4/polyacrylamide slab gel (lanes *1–6*) and fluorographed. In lanes *7–10*, the ER (lane *9*) and Golgi forms (lane *10*) of α-factor, synthesized in vitro, were compared with the forms of α-factor that accumulated in two different secretory mutants. The *sec18* mutant (lane *7*), blocks transport from the ER to the Golgi complex, and *sec14* (lane *8*) blocks transit through the Golgi apparatus. These mutants were radiolabeled in vivo with [³⁵S]sulfate at 37°C. Lysates were prepared as described in the Materials and Methods and immunoprecipitated with α-factor antibody. Solubilized immunoprecipitates were subjected to electrophoresis in a 15% NaDoSO4/polyacrylamide slab gel (lanes *7–10*) and fluorographed.

A detailed analysis of bulk glycoproteins in *Saccharomyces cerevisiae* has revealed the structure of these mannoproteins (Kukuruzinska et al., 1987; Ballou, 1982; Trimble and Atkinson, 1986). The inner core of N-linked oligosaccharide has the same structure as in mammalian cells and is added to glycoproteins in the lumen of the ER, while outer chain carbohydrate is added in the Golgi apparatus. The latter is a highly branched structure consisting of 50–150 mannose residues. It has an α1-6-backbone with numerous sidechains attached in the α1-2-linkage and terminal residues in the α1-3-linkage. Antibody directed against the α1-6-Man backbone has been used to determine if an exported protein has obtained this Golgi-specific modification (Esmon et al., 1981; Ferro-Novick et al., 1984). The α1-6-Man antibody does not efficiently recognize the α1-6-backbone unless this structure is devoid of sidechains. In the yeast *mnn2* mutant, mannoproteins lack these sidechains and the backbone is exposed (Ballou, 1982). To determine if the α1-6-backbone had been added to the high molecular weight species, we prepared permeabilized yeast cells and an S3 fraction from a strain harboring the *mnn2* mutation (see Table I). The high molecular weight species formed during the reaction was first immunoprecipitated with α-factor antibody (Fig. 4, lanes *5* and *6*)

and then with antibody directed against the α1-6-Man backbone (Fig. *4*, lane *6*). As seen in Fig. *4*, high molecular weight α-factor was immunoprecipitated by the α1-6 Man antibody, but not all of the heterogeneous material was recognized. This finding was not surprising since the antibody we used only recognizes long α1-6-polymannose chains (Ballou et al., 1980). In addition, some of the high molecular weight α-factor recognized by the antibody was not readily observed (Fig. 4, lane *6*), because IgG (50 kD) migrated in this region of the gel and quenched fluorographic detection. The specificity of the α1-6-Man antibody for outer chain carbohydrate was apparent since negligible amounts of the ER form of α-factor were detected. Although the transport reaction did not work well when fractions were prepared from the *mnn2* mutant, high molecular weight α-factor was still seen (Fig. 4, lane *5*). Since this species of α-factor was precipitated by antibody directed against the α1-6-Man backbone (Fig. 4, lane *6*), the high molecular weight form must have gained access to the Golgi complex to obtain this modification.

To indirectly assess how far this proprotein had traversed the pathway in vitro, we compared the high molecular weight species detected in vitro with the form synthesized in vivo at the nonpermissive temperature (37°C) in *sec4*, a mutant that blocks protein transit in the Golgi complex (Novick et al., 1981). For this purpose cells were grown overnight at 24°C, shifted to 37°C for 1 h, and then pulse labeled for 2 min with [³⁵S]sulfate. Radiolabeled cells were then harvested, lysed, and incubated with α-factor antibody. As seen in Fig. 4, high molecular weight α-factor was not detected in vitro in the absence of an S3 fraction (lane *9*) or in *sec18* (lane *7*), a secretory mutant that blocks transit from the ER to the Golgi complex in vivo. However, the molecular weight of the proprotein detected in vitro, in the presence of an S3 fraction, was similar to the species synthesized at 37°C in *sec4* (Fig. 4, compare lanes *8* and *10*). Although the gel system we normally used did not allow for the detection of the mature form of α-factor, a band which migrated with the completely processed peptide could occasionally be seen when gels were appropriately fixed. In 13 separate in vitro reactions using wild-type cells we observed this peptide three times. The appearance of this species was dependent

S3   + + + +
Trypsin   - + + -
Triton X-100   - - + -
Rxn + Triton X-100   - - - +

kD
66 —
45 —
29 —
20 —

1 2 3 4
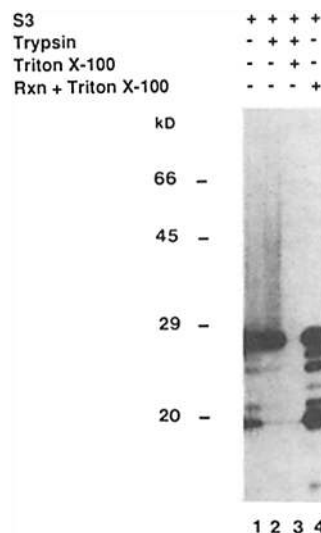
Figure 5. Transport from the ER to the Golgi complex requires sealed compartments. The transport assay was performed in the presence (lanes *1–4*) of an S3 fraction. At the end of the reaction samples were incubated with trypsin (0.47 mg/ml) without (lane *2*) or with (lane *3*) 0.1% Triton X-100 (see Materials and Methods). Triton X-100 (0.1%) was added to the sample in lane *4* before the transport assay.

upon the presence of an S3 fraction (not shown). Thus, when this reaction was working at maximal efficiency, a minor portion of the prepro-α-factor appeared to be completely processed. Alternatively, the appearance of this peptide could be the result of nonspecific proteolysis.

Transport of α-factor from the ER to the Golgi Complex Occurs Between Sealed Membrane Compartments

When the transport product was incubated with trypsin, no degradation of the high molecular weight Golgi form or the 26-kD ER form of α-factor was observed (Fig. 5, compare lanes 1 and 2), unless detergent was simultaneously added (Fig. 5, lane 3). In contrast, most of the 19-kD primary translation product, which pelleted with the cells, was degraded regardless of the presence of detergent (Fig. 5, compare lanes 2 and 3). These results indicated that both the ER and Golgi complex were intact after the transport reaction. Since the 19-kD primary translation product was accessible to protease in the absence of detergent, the permeabilized cells were not resealing during the assay. No addition of outer chain carbohydrate was detected if sealed membrane compartments were disrupted during the reaction by the addition of detergent (Fig. 5, lane 4). These findings indicate that the addition of outer chain carbohydrate to the ER form of α-factor requires the presence of sealed membrane compartments.

Transport from the ER to the Golgi Complex Requires Cytoplasm and Membranes from the S3 Fraction

The S3 fraction, required to support transport to the Golgi complex, contains both cytoplasm and membranes. This fraction was subfractionated during a 100,000 g centrifugation for 1 h to generate HSS and HSP fractions. Fig. 6 shows that neither fraction alone was sufficient to support transport (Fig. 6, lanes 2 and 3); however, when the two fractions were combined, activity was restored (Fig. 6, compare lanes 1 and 4). In the presence of a fixed concentration of HSP, transport increased as the amount of HSS added to the reaction mix was increased (Fig. 6, lanes 4, 5, and 6). Conversely, when the concentration of HSS was maintained, the efficiency of the reaction was diminished as the amount of HSP added to the assay was decreased (not shown).

To determine if proteins in both the HSS and HSP were required for transport, we treated each fraction with trypsin, inactivated the trypsin with trypsin inhibitor, and then added the remaining components to the assay mix. Since the factors present in each fraction were inactivated during this treatment (Fig. 7, compare lane 2 with lanes 3 and 4), we concluded that proteins in both the HSS and HSP were required for transport. Efficient transport was observed when trypsin was inactivated with trypsin inhibitor before incubation with either the HSS or HSP fractions (not shown). These studies indicate that both soluble and membrane-bound proteins are required for the in vitro transport of the 26-kD form of α-factor to the Golgi complex in yeast.

The Acceptor Compartment is Provided by the S3 Fraction

To determine the identity of the HSP requirement, we have used the mnn9 mutant (see Table I) which is defective for the addition of outer chain carbohydrate to glycoprotein (Tsai et
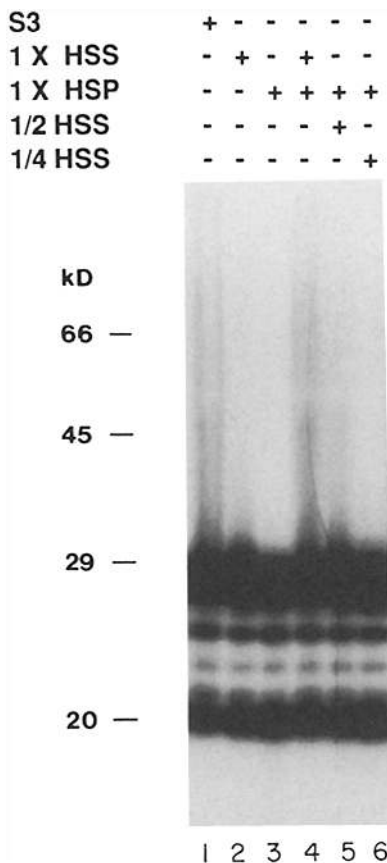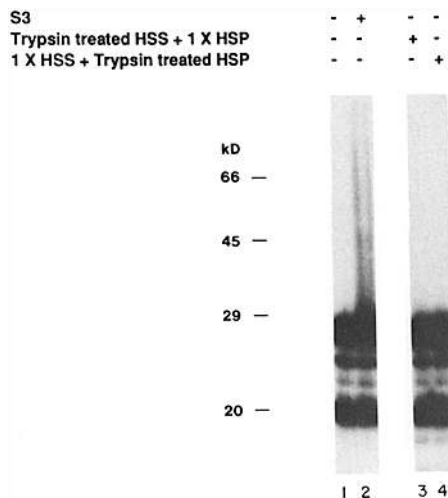

Figure 6. Factors in the cytoplasm and membranes are required for transport. Transport was observed when wild-type permeabilized cells (60 μg of protein) were assayed (136.2 μl) in the presence of an S3 fraction (0.7 mg of protein) (lane 1). This supernatant was subfractionated at 100,000 g for 1 h to yield a high speed supernatant (HSS) and high speed pellet (HSP). When either the HSS (0.4 mg of protein) or HSP (0.3 mg of protein) was assayed, in the presence of wild-type permeabilized cells (60 μg of protein), little or no transport was observed (lanes 2 and 3); if these two fractions were combined (lane 4), activity was restored (compare lanes 1 and 4). Transport to the Golgi complex was diminished if the concentration of HSS added to the reaction was decreased to 1/2 (lane 5) or 1/4 (lane 6).

al., 1984). When a mnn9 S3 fraction was assayed in the presence of wild-type permeabilized cells, the ER form of α-factor was not converted to the high molecular weight species (Fig. 8, lane 2). Fractionation experiments indicated that soluble factors isolated from the mnn9 supernatant could support this conversion, when assayed in the presence of a wild-type HSP; however, the mutant S3 membrane fraction, when assayed in the presence of wild-type soluble factors, did not (not shown). This finding implies that the S3 fraction contains the acceptor membrane compartment that is responsible for converting the 26-kD species of α-factor to the Golgi form.

The ER and Golgi Forms of α-factor are Enriched in Separate Compartments
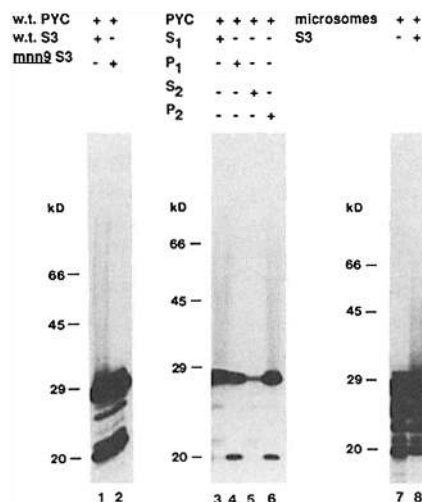
After the first stage of the reaction the ER form of α-factor was predominantly retained within the permeabilized yeast

Figure 7. The cytoplasmic and membrane-bound factors are trypsin sensitive. Permeabilized yeast cells, containing the ER form of α-factor, were assayed (136.2 μl total volume) in the absence (lane *1*) or presence (lane *2*) of an S3 fraction. HSS and HSP fractions generated from this S3 fraction were incubated with trypsin (1.2 μg of trypsin/50 μg HSS; 1 μg of trypsin/50 μg HSP) for 10 min on ice, trypsin inhibitor was then added (8 μg), and the samples were incubated an additional 5 min. Transport was blocked when the trypsin-treated HSS was assayed in the presence of an untreated HSP (lane *3*); or, the trypsin-treated HSP was assayed in the presence of an untreated HSS (lane *4*). Control experiments demonstrated that efficient transport was observed when trypsin was inactivated with trypsin inhibitor, before incubation with either the HSS or HSP fractions (not shown).

Figure 8. The Golgi form of α-factor is in a sealed compartment outside the permeabilized cells. Microsomes can serve as the donor compartment. The high molecular weight form of α-factor (lane *1*) was detected when wild-type permeabilized cells were assayed in the presence of a wild-type S3 fraction, but not if an S3 fraction was isolated from a strain harboring the *mmn9* mutation (lane *2*). After the transport assay, a portion of the reaction product was centrifuged for 15 s in a microfuge and the forms of α-factor present in the supernatant and pellet fractions were immunoprecipitated with α-factor antibody. The Golgi form was found in the supernatant (S_1; lane *3*) and not in the pellet (P_1) with the permeabilized cells (lane *4*). When the reaction product was centrifuged at 100,000 g for 1 h in an ultracentrifuge, the Golgi form of α-factor was found in the pellet (P_2; lane *6*) and not in the supernatant (S_2; lane *5*). In lanes *7* and *8*, the transport assay was performed with microsomes instead of permeabilized cells. Prepro-α-factor was translocated into microsomes in a yeast translation lysate. After translocation, the lysate was supplemented with an ATP-regenerating system (1 mM ATP, 0.1 mM GTP, 20 mM creatine phosphate, and 0.2 mM creatine phosphate kinase) and GDP-mannose (0.92 mM). Samples were then incubated for 90 min at 20°C in the absence (lane *7*) or presence (lane *8*) of an S3 fraction.

cells (Fig. 2 *B*). Our findings with the *mnn9* mutant indicate that the functional acceptor must be added exogenously to the permeabilized cells. Based on this result one expectation is that, after the second stage of the reaction, the high molecular weight reaction product would be associated with the exogenous acceptor rather than with the cells. When we pelleted the permeabilized cells for 15 s in a microfuge, after the transport assay, the high molecular weight form of α-factor was recovered in the supernatant fraction and not in the pellet (Fig. 8, lanes *3* and *4*). When the supernatant, containing the Golgi form of α-factor, was recentrifuged for 5 min in a microfuge the high molecular weight species was still recovered in the supernatant. Approximately 50% of the NADPH cytochrome *c* reductase activity, a marker for the ER membrane (Kubota et al., 1977), pelleted during the 15-s centrifugation and an additional 25% pelleted after 5 min of centrifugation. Thus, differential centrifugation separated most of the NADPH cytochrome c reductase (~75%) from the Golgi form of α-factor. The distribution of the 26-kD species correlated with the distribution of NADPH cytochrome *c* reductase activity. As expected, most of the ER and Golgi forms of α-factor did pellet when the reaction product was centrifuged at 100,000 g for 1 h (Fig. 8, lanes *5* and *6*). This finding implied that these forms of α-factor were retained within membrane-bound organelles and confirmed earlier results that had been obtained through protease protection experiments (Fig. 5).

To further establish that the 26-kD species and Golgi form of α-factor reside in separate compartments, we centrifuged

the reaction product in a 30–50% sucrose gradient to resolve these species. The ER form of α-factor and NADPH cytochrome *c* reductase activity peaked in the pellet of this gradient. In contrast, the Golgi form of α-factor peaked at a concentration of 40–41% sucrose (not shown). Thus, we have used differential centrifugation and a sucrose density gradient to demonstrate that the ER and Golgi forms of α-factor are enriched in separate compartments.

The Donor Compartment Can be Provided by Microsomes Rather than Permeabilized Yeast Cells

The addition of outer chain carbohydrate to the ER form of α-factor occurs in a compartment which does not fractionate with the permeabilized cells. Since this compartment is provided by the S3 fraction, then prepro-α-factor translocated into yeast microsomes, may be converted to the Golgi form in the presence of an S3 fraction. When this experiment was performed, the addition of outer chain carbohydrate to the 26-kD species of α-factor was observed (Fig. 8, lane *8*). This reaction required ATP, since apyrase blocked this event (not shown). Thus, the donor can be provided in the form of microsomes rather than permeabilized yeast cells.

GTPγS Inhibits ER to Golgi Transport In Vitro but not Translocation

It has recently been suggested that guanine nucleotide–binding proteins participate in intracellular protein transport (Salminen and Novick, 1987; Melancon et al., 1987; Goud et al., 1988; Segev et al., 1988). The nonhydrolyzable GTP analog GTPγS has been shown to inhibit transport through the Golgi in vitro causing the accumulation of vesicles (Melancon et al., 1987). As seen in Fig. 9, 1×10^{-4} M GTPγS can inhibit transport from the ER to the Golgi complex in vitro. Low concentrations of GTPγS (1×10^{-5} M) also affect transport. These affects were partially reversible if 1 mM GTP was added with GTPγS at the beginning of the reaction. When 1×10^{-4} M GTPγS was added during the first stage of the reaction no affect on translocation was observed (Fig. 9, compare lanes 4 and 5). Thus, GTPγS inhibits the second stage but not the first stage of this two stage reaction.

The Acceptor Compartment is Defective in the sec23 Mutant

The authenticity of the transport reaction can be tested by using secretory mutants that have been shown to block transport from the ER to the Golgi complex in vivo. To initiate an analysis of a *sec* gene product in this reaction, we tested in vitro fractions (an S3 fraction and permeabilized cells) derived from *sec23* (see Table I), a mutant that blocks ER to Golgi transport in vivo at 37°C. Fractions were prepared from mutant cells that were incubated at the restrictive temperature in vivo by following the protocol we developed to
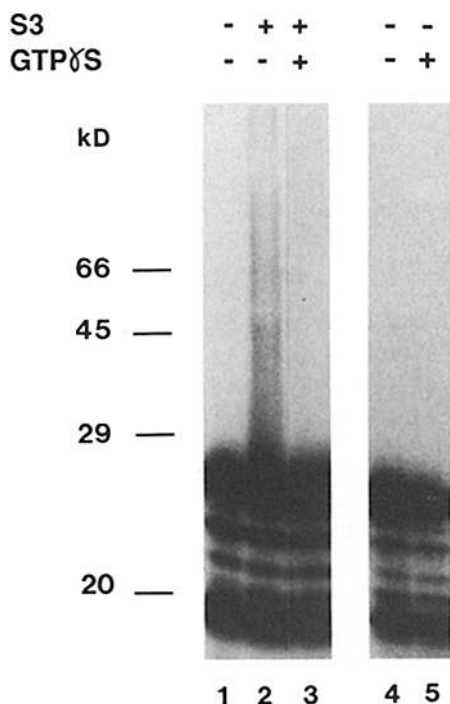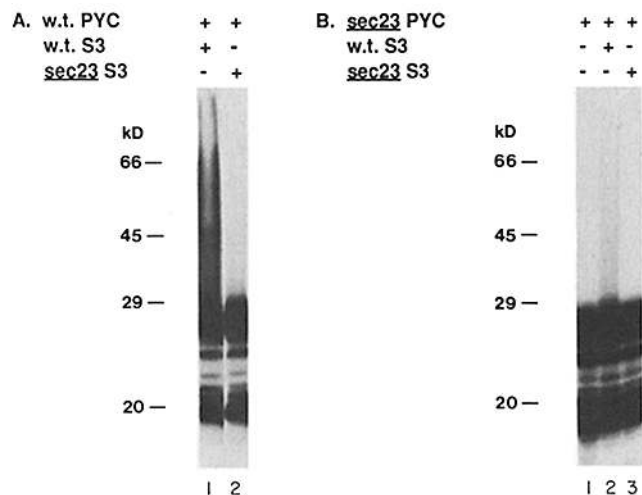
reconstitute transport with wild-type cells. When this was done transport was completely blocked if *sec23* permeabilized cells were assayed in the presence of a *sec23* S3 fraction (Fig. 10 *B*, lane *3*).

Since the function of donor, acceptor, and soluble fractions can be assayed separately, we proceeded to determine which fraction is defective in the *sec23* mutant. When an S3 fraction, isolated from this mutant, was assayed in the presence of wild-type permeabilized cells (Fig. 10 *A*, lane 2) transport failed to occur. However, permeabilized *sec23* cells were capable of supporting transport when assayed in the presence of a wild-type S3 fraction, but were less effective than wild-type cells (Fig. 10 *B*, lane 2). Not all functions of the ER membrane were effected in *sec23*, since mutant permeabilized cells could efficiently translocate prepro-α-factor into the lumen of the ER (not shown). The diminished capacity of the donor compartment may be a secondary consequence of shifting the mutant in vivo. This possibility is discussed below.

To determine whether a soluble or membrane-bound factor in the S3 fraction of *sec23* is defective, this supernatant was centrifuged at 100,000 *g* for 1 h to generate HSS and HSP fractions. Mutant HSS in combination with a wild-type HSP effectively supported transport in wild-type permeabilized cells (Fig. 11, compare lanes *1* and 2). However, a mutant HSP assayed in the presence of a wild-type HSS failed to support transport (Figure 11, lane *3*). These results indicate that the *sec23* mutant is defective in a component that is normally provided by the HSP. Findings described above suggest that the acceptor membrane compartment is provided by this fraction.

Since the *sec23* mutant was incubated at the restrictive temperature before fractions were prepared, the phenotype we observed in vitro may be a secondary consequence of this incubation. Therefore, we repeated these experiments with

Figure 9. GTPγs inhibits transport. Wild-type permeabilized cells, containing the 26-kD form of α-factor, were incubated without (lane *1*) or with an S3 fraction in the absence (lane 2) or presence (lane *3*) of 1×10^{-4} M GTPγS. In vitro translated prepro-α-factor was incubated with permeabilized yeast cells in the absence (lane *4*) or presence (lane *5*) of 1×10^{-4} M GTPγS.

Figure 10. An S3 fraction from *sec23* does not support transport. (*A*) Wild-type permeabilized cells, containing radiolabeled proprotein in the lumen of the ER, were incubated in the presence of a wild-type (lane *1*) or *sec23* S3 fraction (lane 2). (*B*) Permeabilized *sec23* cells, containing radiolabeled proprotein in the ER, were incubated in the absence (lane *1*) or the presence of a wild-type S3 fraction (lane 2). Permeabilized *sec23* cells did not support transport when incubated with a *sec23* S3 fraction (lane *3*).

w.t. HSS	+	-	+	-
sec23 HSS	-	+	-	+
w.t. HSP	+	+	-	-
sec23 HSP	-	-	+	+

kD

66 —

45 —

29 —

20 —

1 2 3 4
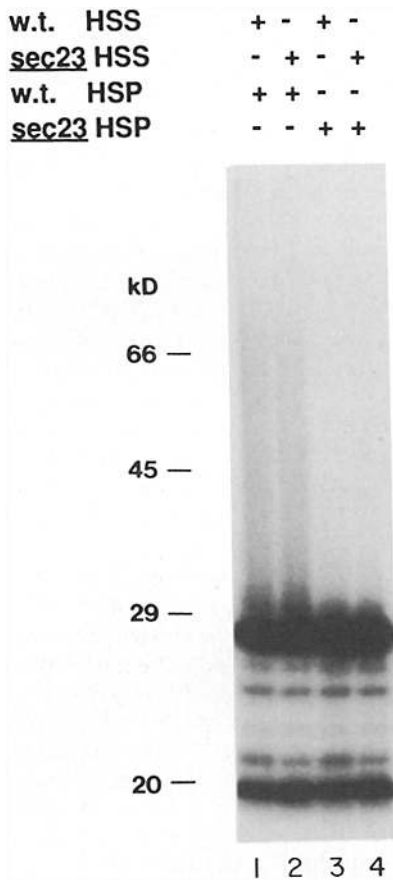
Figure 11. The acceptor membrane is defective for transport in *sec23.* A *sec23* S3 fraction was centrifuged at 100,000 *g* for 1 h to generate HSS and HSP fractions that were assayed (136.2 μl total reaction volume) for activity in the presence of wild-type permeabilized cells (60 μg of protein). A *sec23* HSS (0.4 mg of protein) effectively supported transport to the Golgi complex, when assayed in the presence of a wild-type HSP (compare lanes *1* and *2*). However, a *sec23* HSP (0.3 mg of protein) failed to transport the ER form of α-factor, when assayed in the presence of wild-type HSS (compare lanes *1* and *3*). No transport was observed if the HSS and HSP fractions were both isolated from the *sec23* mutant and assayed in the presence of wild-type permeabilized cells (lane *4*).

fractions generated from cells that were only incubated at the permissive temperature in vivo. Using these fractions we observed that the acceptor compartment was defective for transport while the donor and soluble fractions were fully functional (not shown). Further experiments will be needed before the effects observed on the donor compartment, after an in vivo shift, can be evaluated.

Discussion

In vitro assays that faithfully reproduce the stages of intracellular protein transport will lead to the identification of components that are essential for these processes. The characterization of such components will be facilitated by an analysis of these events in an organism that is amenable to both genetic and biochemical manipulation. In this report, we describe a new assay that efficiently reconstitutes transport

from the ER to the Golgi complex in yeast. This assay and the existence of a large collection of mutants that are temperature-sensitive for the transport of proteins between these organelles (Novick et al., 1980; Newman and Ferro-Novick, 1987) should expedite an analysis of this stage of the secretory pathway. We report here that the acceptor compartment is defective in one of these mutants, *sec23.*

Requirements for Transport

A unique feature of our in vitro system is the coupled use of translocation across the ER membrane and transport to the Golgi complex using the pheromone α-factor as a marker protein. By in vitro translating prepro-α-factor in a yeast lysate, we can easily obtain a radiolabeled protein of high specific radioactivity. Radiolabeled prepro-α-factor is translocated directly into microsomes or into the ER lumen retained within permeabilized yeast cells. Protein sequestered within the lumen is then transported to the Golgi apparatus in the presence of ATP and an S3 fraction, that contains soluble and membrane-bound components (Fig. 3, lanes *4* and *5*). The assay we developed differs from the mammalian ER to Golgi in vitro transport assay described by Beckers et al. (1987) in that we introduce radiolabeled marker protein into the lumen of the ER in vitro during translocation rather than in vivo during virus infection. Our assay also differs from the mammalian system (Beckers et al., 1987; Simons and Virta, 1987) in that the acceptor Golgi compartment must always be provided exogenously to the permeabilized cells.

Transport to the Golgi complex in vitro requires the addition of acceptor compartments supplied by the S3 fraction. This was demonstrated by the failure of the *mnn9* S3 fraction to support outer chain elongation (Fig. 8, lane *2*). Therefore, if acceptor membranes are present in the permeabilized cells they are not used. The S3 fraction and the permeabilized cells are prepared differently (see Materials and Methods). Thus, the conditions used to prepare permeabilized yeast cells may lead to inactivation or loss of.the acceptor compartments. Although we have shown that permeabilized cells serve as an efficient donor in this reaction, they are not essential. When an S3 fraction and ATP are added to yeast microsomes, containing the 26-kD species of α-factor, the high molecular weight Golgi form is produced.

We have shown that GTPγS can inhibit ER to Golgi transport in vitro (Fig. 9). Inhibition of transport by GTPγS has been used by others (Melancon et al., 1987) to implicate the involvement of a GTP-binding protein in inter-Golgi transport. One proposal is that this protein functions in a cycle in which the GTP-bound form of the protein binds to a site on a transport vesicle. This complex is recognized by an effector on the acceptor compartment, triggering membrane fusion. Release of the GTP-binding protein is coupled to hydrolysis of GTP. GTPγS disrupts the system because it cannot be hydrolyzed. Recently it has been shown that mutations in the GTP-binding protein *ypt1* can affect ER to Golgi transport in yeast (Segev et al., 1988; Schmitt et al., 1988). Further experiments will be needed before one can conclude if Ypt1 or another GTP-binding protein mediates the effects of GTPγS.

The addition of outer chain carbohydrate to core glycosylated invertase has previously been reported to occur in yeast homogenates at a efficiency of 2–5% (Haselbeck and Schekman, 1986). This reaction was attributed to transport from

the ER to the Golgi complex and showed an absolute dependence on manganese chloride (5 mM), but did not require the addition of a soluble factor(s). Our present reaction conditions require a soluble component(s) but does not require manganese chloride. If our assay is performed in the presence of 5 mM manganese chloride, the addition of outer chain carbohydrate to the ER form of α-factor is not dependent on the presence of ATP. Since an energy requirement for transport from the ER to the Golgi complex has been demonstrated in vivo (Jamieson and Palade, 1968; Novick et al., 1981; Balch et al., 1986), the reaction in the presence of high concentrations of manganese chloride is unlikely to be authentically reconstituting in vivo events.

Intercompartmental Transport

Two possibilities could account for the formation of the high molecular weight form of α-factor. It could be the result of authentic intercompartmental transport from the ER to the Golgi complex. Alternatively, translocation of the 19-kD form of α-factor into a fused compartment in the S3 fraction or fusion of the Golgi complex with the ER after translocation could occur. Four lines of evidence establish that we are studying intercompartmental transport. First, differential centrifugation and fractionation on a sucrose density gradient have shown that the ER and Golgi forms of α-factor are enriched in separable compartments which are therefore unlikely to be fused. Second, GTPγS does not inhibit the formation of the 26-kD species but does inhibit conversion to the high molecular weight species. This result is inconsistent with the possibility that the 19-kD species is translocated into a fused compartment in the S3 fraction. Third, permeabilized cells, prepared from one of the mutants that block transport from the ER to the Golgi complex in vivo, contain defective donor membranes and fail to convert the 26-kD form of α-factor to the high molecular weight species when assayed in the presence of an active wild-type S3 fraction. This indicates that, in the absence of functional permeabilized cells, the wild-type S3 fraction is not capable of coupled translocation, core glycosylation, and outer chain addition of the residual 19-kD form of α-factor retained in the cells. Because formation of the high molecular weight form is dependent on the nature of the permeabilized cells, the 26-kD ER form of α-factor associated with the permeabilized cells is a necessary intermediate in transport to the Golgi complex in vitro. Fourth, the quantitation of the data in Fig. 3 indicates that conversion to the high molecular weight form of α-factor formed in the second stage of the reaction is largely due to the depletion of 26-kD species of α-factor formed in the first stage. This indicates that most of the α-factor which is converted to the high molecular weight form resided in the ER before transport to the Golgi apparatus.

Analysis of a Secretory Mutant in the In Vitro Assay

An analysis of the *sec23* mutant has shown that a defect in the Sec23 protein inactivates the acceptor compartment (Fig. 11, compare lanes *2* and *3*). The acceptor fraction, isolated from permissively grown *sec23* mutant cells is defective for transport when assayed at 20°C. Therefore, it is possible that the Golgi complex may be partially defective in vivo even at the permissive temperature. This defect may be rate limiting in vitro at 20°C, but may not be limiting in vivo. While our results do not necessarily imply that the *SEC23* gene product is a component of the Golgi complex, loss of Sec23 protein activity leads to failure of acceptor function either directly, through interaction with the Golgi complex, or through a series of biochemical reactions. Because the in vitro delivery of prepro-α-factor to the Golgi complex is *SEC* dependent, it is likely that transport is occurring in a manner that reflects normal vectorial flow.

Future Uses of the In Vitro Assay

Because our assay allows us to separate the donor, acceptor, and soluble fractions we have been able to demonstrate a defect in the acceptor compartment of the *sec23* mutant. The analysis we have done with this mutant can be extended to the other *sec* and *bet* mutants that disrupt transit at this stage of the pathway. This will enable us to determine which fraction is defective in each of the secretory mutants. A combined genetic and biochemical approach will lead to the identification of proteins that play a role in this process. Some of these components, however, may not be represented among the known *SEC* or *BET* gene products. The in vitro transport assay may permit the purification and characterization of proteins involved in this event and the genetic flexibility of yeast will enable us to address their role in vivo. In addition, since the processing of prepro-α-factor to the mature form is thought to begin in a late Golgi compartment, with further optimization this assay may be extended to study intra-Golgi transport in yeast.

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