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Reconstitution of *SEC* Gene Product–Dependent Intercompartmental Protein Transport

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Summary

Transport of α-factor precursor from the endoplasmic reticulum to the Golgi apparatus has been reconstituted in gently lysed yeast spheroplasts. Transport is measured through the coupled addition of outer-chain carbohydrate to [^{35}S]methionine-labeled α-factor precursor translocated into the endoplasmic reticulum of broken spheroplasts. The reaction is absolutely dependent on ATP, stimulated 6-fold by cytosol, and occurs between physically separable sealed compartments. Transport is inhibited by the guanine nucleotide analog GTPγS. *sec23* mutant cells have a temperature-sensitive defect in endoplasmic reticulum-to-Golgi transport in vivo. This defect has been reproduced in vitro using *sec23* membranes and cytosol. Transport at 30°C with *sec23* membranes requires addition of cytosol containing the *SEC23* (wild-type) gene product. This demonstrates that an in vitro interorganelle transport reaction depends on a factor required for transport in vivo. Complementation of *sec* mutations in vitro provides a functional assay for the purification of individual intercompartmental transport factors.

Introduction

Newly synthesized proteins are directed to their proper locations through rapid and precise intercompartmental transport reactions. Secretory and plasma membrane proteins traverse a series of membrane-enclosed compartments including the endoplasmic reticulum (ER) and the cisternae of the Golgi en route to the cell surface. While transport of proteins between these compartments has been extensively described in vivo (reviewed in Pfeffer and Rothman, 1987), the underlying mechanisms remain obscure. Reconstitution of intercompartmental transport in vitro is required to begin to study the enzymology of these reactions.

Several intercompartmental protein transport reactions have been reconstituted in mammalian systems. Reconstitution of transport between Golgi cisternae has led to the purification of at least one transport factor and identification of several novel transport intermediates (Melancon et al., 1987; Pfeffer and Rothman, 1987; J. Rothman, personal communication). Purification of transport factors has relied on specific inhibitors to inactivate single components followed by supplementation with untreated protein fractions. Direct fractionation has proved difficult, probably because of the large number of factors involved. Reconstitution of transport from the ER to the Golgi has recently been achieved using "semi-intact" Chinese hamster ovary cells, which have lost most of their cytosol but retain intact organelles (Beckers et al., 1987).

A large number of yeast secretory (*sec*) mutants have been isolated that have temperature-sensitive defects in protein transport (Novick et al., 1980). The motivation for developing an intercompartmental transport assay in yeast is the potential to purify and characterize individual transport factors through complementation of transport-defective mutant reactions. Among the several stages in the yeast secretory pathway, protein transport from the ER to the Golgi is particularly amenable to analysis. Eleven complementation groups have been identified whose products are required for ER–Golgi transport in vivo (Novick et al., 1981; Newman and Ferro-Novick, 1987). Furthermore, the addition of outer-chain carbohydrate to core oligosaccharides serves as a convenient diagnostic of arrival in the Golgi (Esmon et al., 1981) (see Figure 1). A yeast ER–Golgi in vitro transport reaction has been previously reported (Haselbeck and Schekman, 1986), but a lack of cytosol dependence and low transport efficiency (<2%) have precluded effective exploitation of the mutants.

Here we report an ER–Golgi in vitro transport reaction in which nearly one-third of the core glycosylated transport substrate receives outer-chain carbohydrate. Two innovations were essential: a method for preparing semi-intact yeast and a method for following transport in extracts prepared from wild-type cells. The reaction is absolutely dependent on ATP, stimulated 6-fold by cytosolic proteins, and occurs between physically separable compartments. The reaction is blocked by GTPγS and depends on the *SEC23* gene product.

Results

Preparation of Gently Lysed Yeast

We sought a method for preparing yeast lysates competent for ER-to-Golgi transport. Recently, two methods for preparing transport-competent, semi-intact mammalian cells were described (Simons and Virta, 1987; Beckers et al., 1987). These methods take advantage of the monolayer growth habit of the cells and cannot readily be generalized to cells such as yeast, which grow in liquid culture. We explored freeze-thaw lysis of spheroplasts as a means of cell breakage. Early studies indicated that the rate of freezing determined the extent of lysis. Slow freezing preserved spheroplasts intact; rapid freezing ruptured spheroplasts and internal organelles. We then varied the freezing rate, aiming for a gentle lysis procedure that released cytosolic proteins but left organelles intact.

This paper is dedicated to Arthur Kornberg on the occasion of his seventieth birthday.

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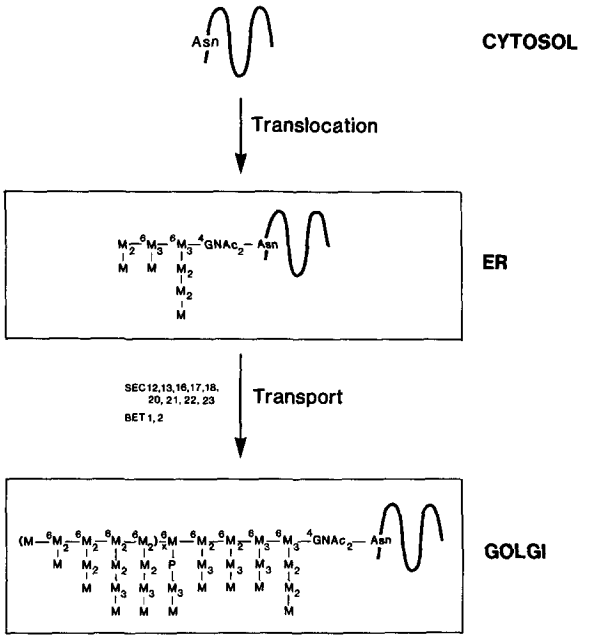

Figure 1. Glycosylation Coupled to Translocation of Proteins into the ER and Transport to the Golgi

Core oligosaccharides are added to proteins upon translocation into the ER. Transport to the Golgi is blocked in the *sec* and *bet* mutants listed. In the Golgi, core oligosaccharides are extended by addition of outer-chain carbohydrate. M, mannose; GNAc, N-acetylglucosamine; Asn, asparagine.

In brief, actively metabolizing spheroplasts were washed and resuspended in 400 mM sorbitol, 150 mM KOAc, 2 mM MgOAc, 0.5 mM EGTA, 20 mM HEPES (pH 6.8), frozen over liquid N_2, and stored at −85°C until needed. After thawing, about 50% of the spheroplasts were broken as judged by electron microscopy (data not shown). Examples of the morphology of broken spheroplasts are presented in Figures 2B–2D. Much of the cytoplasm was released (compare the density of ribosomes in Figure 2A to that in Figures 2B–2D), but major organelles such as nuclei and vacuoles were largely intact. Ribosome-studded membranes, likely to be endoplasmic reticulum, were observed (see Figure 2C, above the nucleus). An example of breaks in the plasma membrane through which most of the cytosol may have escaped is indicated in Figure 2B, upper right.

Design of Transport Assay

Glycosylation serves as a convenient diagnostic of protein translocation into the ER and transport to the Golgi. Core oligosaccharides are added to proteins in the ER and then elongated in the Golgi by addition of outer-chain carbohydrate (Kukuruzinska et al., 1987) (Figure 1). Carbohydrate addition may be detected by any of the following: a shift in mobility on an SDS–polyacrylamide gel; precipitation with concanavalin A (ConA), a lectin that recognizes mannose-containing oligosaccharides; or immunoprecipitation with an antiserum specific for α1→6-linked mannose (α1→6-Man), which recognizes only outer-chain epitopes (Esmon et al., 1981).

Initial attempts at establishing an ER–Golgi in vitro reaction in gently lysed yeast were modeled on the previously published yeast ER–Golgi assay (Haselbeck and Schekman, 1986). Invertase was accumulated in spheroplasts of an ER-blocked *sec* mutant at the nonpermissive temperature. Spheroplasts were frozen and thawed, and membranes were incubated with ATP and cytosol at the permissive temperature and monitored for addition of outer-chain carbohydrate to invertase. These attempts met with little success.

We were concerned that mutant defects that were conditional in vivo might be accentuated in vitro and potentially be irreversible. Therefore, a transport assay using components prepared from wild-type cells was developed. The low steady-state level of proteins in transit through the ER of wild-type cells precluded use of an endogenous substrate in an in vitro transport assay. We reasoned that the lack of substrate could be overcome by introduction of an exogenous, radiolabeled protein via translocation into the ER of broken spheroplasts. This approach had two further advantages: first, intact spheroplasts surviving the lysis procedure would produce no background in the reaction (the labeled substrate could not penetrate); and second, since the substrate was the only labeled species in the reaction, it could be followed easily.

We chose prepro-α-factor, the precursor of the secreted mating pheremone α-factor, as a substrate. Transport in vivo is rapid and has been extensively characterized (Julius et al., 1984; Fuller et al., 1988). Prepro-α-factor is converted to 30 kd core glycosylated pro-α-factor in the ER and then processed to mature pheromone after addition of outer-chain carbohydrate in the Golgi. Most important, prepro-α-factor is efficiently translocated posttranslationally into yeast microsomes (Rothblatt and Meyer, 1986; Waters and Blobel, 1986; Hansen et al., 1986). Thus we could prepare [^{35}S]methionine-labeled prepro-α-factor translation product in advance and avoid the complications of translation in the presence of freeze-thaw lysates (e.g., endogenous mRNA background, [^{35}S]methionine incorporation by unbroken spheroplasts).

Prepro-α-Factor Added to Gently Lysed Yeast Is Converted to the 30 kd ER Form and to a More Slowly Migrating Form Immunoprecipitable with Anti-α1→6-Man Serum

[^{35}S]methionine-labeled prepro-α-factor was synthesized in a yeast lysate, and the translation reaction was desalted to remove unincorporated [^{35}S]methionine and glycerol. Translation product, washed membranes, yeast cytosol, an ATP-regenerating system, and GDP-mannose were incubated together at 20°C for 45 min. As seen in Figure 3, lane 2, roughly half of the prepro-α-factor added to the incubation was converted to the 30 kd ER form. Furthermore, about 25% of the pro-α-factor was converted to forms of heterogeneous mobility that migrated more slowly than the ER form and that were not present when SDS was added at the beginning of the incubation (Figure 3, lane 1).

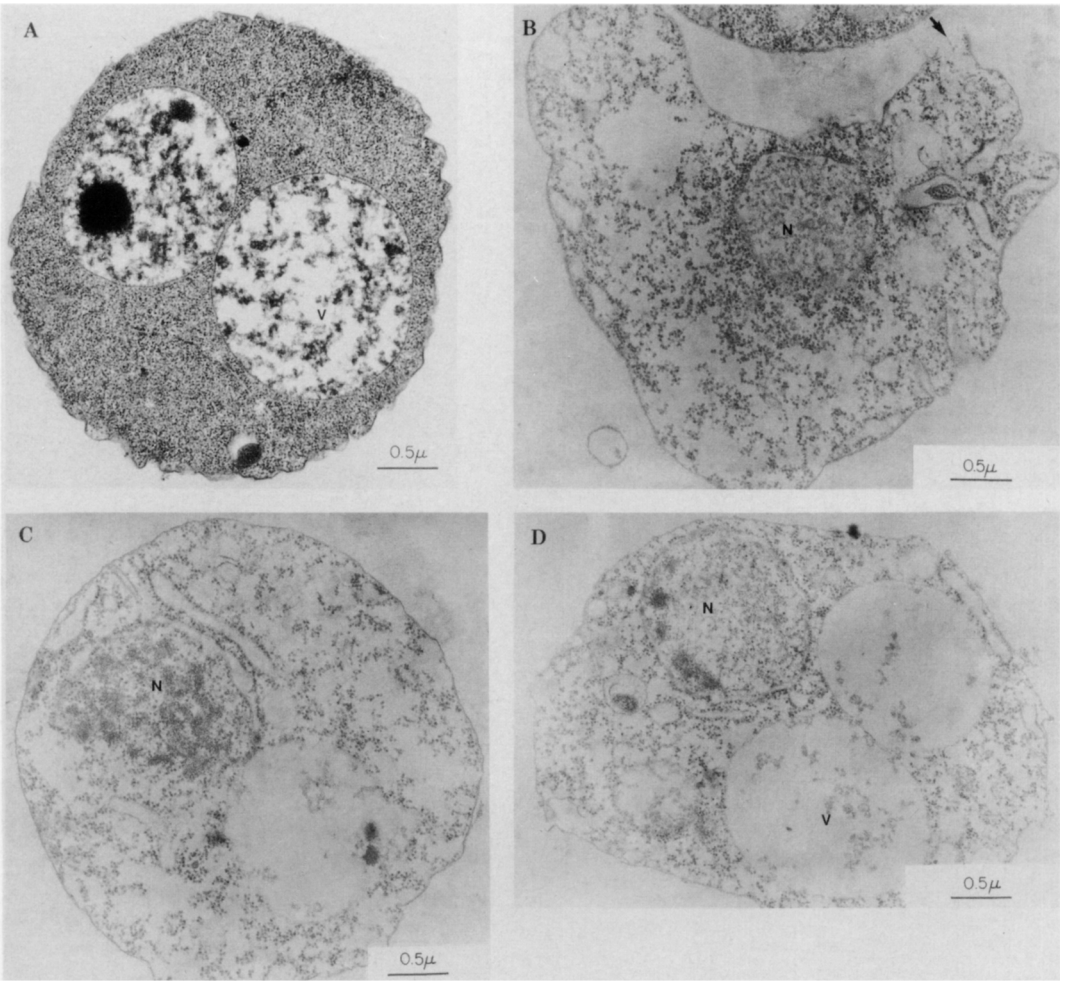

Figure 2. Morphology of Gently Lysed Spheroplasts

Spheroplasts were processed for electron microscopy before (A) and after (B–D) freeze-thawing. The arrow in (B) marks a break in the plasma membrane. N, nucleus; V, vacuole.

In vivo, the Golgi species of pro-α-factor migrates as a high molecular weight, heterogeneous collection of outer-chain glycosylated forms (Julius et al., 1984; Fuller et al., 1988). To determine whether the heterogeneous forms observed in vitro were due to mannose addition by Golgi mannosyltransferases, we used an antibody specific for α1→6-Man. The antibody precipitated the species in a broad region beginning slightly above the 30 kd ER form (Figure 3, lane 4); forms between this region and core glycosylated precursor probably lack sufficient α1→6-Man to be precipitated. The antibody reacted specifically with α1→6-Man added during the incubation: no precipitation was observed when SDS was added at the beginning of the incubation (Figure 3, lane 3) or when competitor α1→6-Man oligosaccharide (100 μM) was present during the immunoprecipitation. α1→2- and α1→3-linked mannose oligosaccharides at millimolar concentrations did not inhibit immunoprecipitation (data not shown).

To avoid the necessity of running SDS–polyacrylamide gels after each experiment and to obtain more quantitative data, the amount of α-factor precursor receiving carbohydrate modification was determined by precipitation with anti-α1→6-Man serum or ConA, followed by scintillation counting. ConA precipitation measured the total amount of prepro-α-factor translocated during an incubation, since ConA reacted with all glycosylated species (Figure 3, lane 6). The ratio of anti-α1→6-Man precipitable pro-α-factor to ConA precipitable pro-α-factor, the "transport efficiency," was typically around 0.25, with some variation between different preparations and experiments. The anti-α1→6-Man serum gave a slight underestimate of the amount of α-factor receiving outer-chain modification, since it did not precipitate the portion of heterogeneous material just above the ER form.

Low-Temperature Incubations Separate Transport from Translocation

Further characterization of the transport reaction required temporal separation of translocation and transport. ER-to-Golgi transport is blocked at low temperatures in other eukaryotic cells (Balch et al., 1986; Tartakoff, 1986). Therefore, we explored the effect of low-temperature incubations

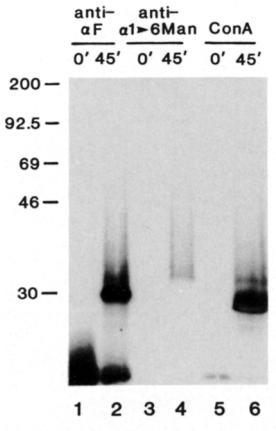

Figure 3. Prepro-α-Factor Incubated with Gently Lysed Yeast Is Converted to the 30 kd ER Form and to a More Slowly Migrating Form That Is Immunoprecipitable with Anti-α1→6-Man Serum

Gently lysed yeast and [^{35}S]methionine-labeled prepro-α-factor were incubated together in the presence of GDP-mannose and an ATP-regenerating system. Laemmli sample buffer was added at the times indicated, and the quenched reactions were heated for 5 min at 95°C. Portions (20 μl) of each reaction were precipitated with anti-α-factor serum, anti-α1→6-Man serum, or ConA. The immunoprecipitates were electrophoresed on an 11.25% polyacrylamide gel. The gel was incubated with Amplify, dried, and exposed to X-ray film for 48 hr. Sizes in kd are marked at left.

on the production of core glycosylated and outer-chain glycosylated precursor. As seen in Figure 4, translocation and α1→6-Man addition were separated when the reaction was conducted at low temperature. At 10°C, translocation occurred readily but outer-chain glycosylation was reduced to 10% of the value at 20°C. Further characterization of the reaction supported the view that transport, not outer-chain glycosylation per se, was the temperature-dependent step (see below).

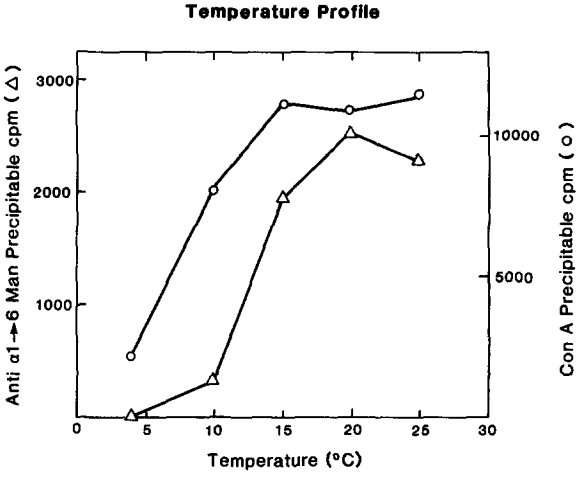

Figure 4. Temperature Dependence of Transport

[^{35}S]methionine-labeled prepro-α-factor, gently lysed yeast, an ATP-regenerating system, and GDP-mannose sufficient for seven one-stage reactions were mixed in one tube at 4°C. Aliquots (25 μl) were incubated for 45 min at the indicated temperatures. Reactions were terminated by addition of 40 μl of Laemmli sample buffer and heating at 95°C. Reaction products were precipitated with anti-α1→6-Man serum or ConA, and the precipitates were analyzed by scintillation counting.

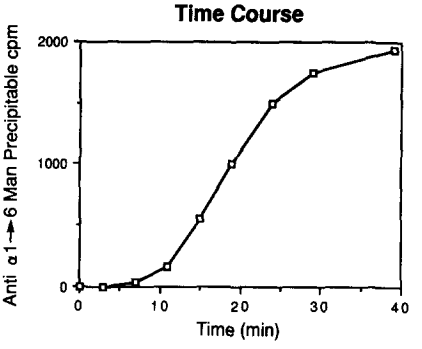

Figure 5. Time Course of Transport

Aliquots were terminated at intervals after the beginning of the second stage of a two-stage reaction and analyzed by immunoprecipitation and scintillation counting. The zero-time background (120 cpm) was subtracted to obtain the values shown. ConA-precipitable pro-α-factor averaged 4739 cpm per point.

The selective block of transport at 10°C allowed study of transport independent of translocation through the use of two-stage incubations. In the first stage, prepro-α-factor translation product, washed membranes, an ATP-regenerating system, and GDP-mannose were incubated together for 15 min at 10°C. The membranes were then washed and warmed to 20°C with various additions. ConA precipitable cpm remained constant during the 20°C incubation, indicating that no translocation-competent precursor remained after the washes. In a typical experiment, membranes containing translocated precursor were subjected to various conditions in a second stage to determine the requirements of transport.

Properties of Transport

The kinetics of transport were investigated using a two-stage reaction. Aliquots of the second-stage incubation were stopped at invervals, and in each case anti-α1→6-Man precipitable cpm was measured (Figure 5). A pronounced lag of about 10 min was followed by a 20 min linear phase that leveled off at 40 min. The lag period, which varied from 7 to 12 min between different experiments and preparations, may represent a rate-limiting early step in transport such as protein folding or vesicle budding.

ATP is required for transport from the ER to the Golgi in vivo (Novick et al., 1981; Balch et al., 1986). To determine whether ATP was required for the in vitro transport reaction, a two-stage reaction was performed in which either an ATP-regenerating system or apyrase (phosphoanhydride hydrolase) was added at the beginning of the second stage. ATP depletion by treatment with apyrase (Figure 6), or glycerol with glycerol kinase (data not shown), completely blocked α1→6-Man addition.

GDP-mannose is the sugar donor for outer-chain addition (Kukuruzinska et al., 1987). The in vitro reaction was stimulated by, but did not require, GDP-mannose (Figure 6), perhaps because of endogenous pools in the Golgi or de novo synthesis during the incubation.

Organelle integrity was required for and maintained during transport. Permeabilization of organelles by addition

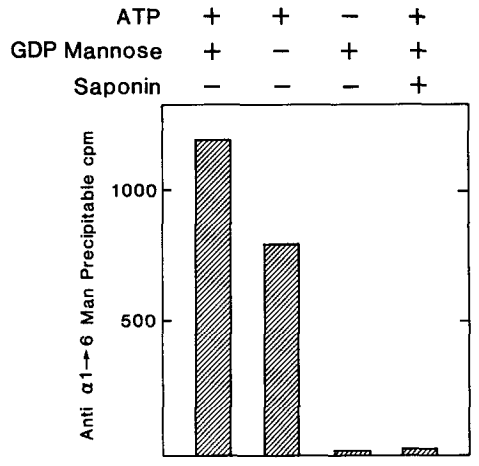

Figure 6. Energy Dependence, GDP-Mannose Stimulation, and Detergent Inhibition of Transport

At the end of the first stage (15 min, 10°C incubation), membranes containing translocated pro-α-factor were washed twice and aliquoted to tubes containing 50 μM GDP-mannose, an ATP-regenerating system, and 0.2% saponin, as indicated. The tube lacking ATP contained 2.5 U of apyrase. Samples were incubated for 45 min at 20°C and then immunoprecipitated and counted. The background at the beginning of the second stage was 125 cpm and was subtracted to obtain the values shown. ConA precipitable pro-α-factor averaged 5825 cpm per point.

Table 2. Cytosolic Protein Is Required for Transport

Addition	Anti-α1→6-Man Precipitable cpm (% maximum)
Untreated cytosol (80 μg)	100
Reaction buffer	16
BSA (80 μg)	14
Treated cytosol (80 μg):	
+ Trypsin $\xrightarrow{30\ min,\ 4°C}$ + trypsin inhibitor	15
+ Trypsin + trypsin inhibitor $\xrightarrow{30\ min,\ 4°C}$	86
+ NEM $\xrightarrow{30\ min,\ 4°C}$ + DTT	13
+ DTT + NEM $\xrightarrow{30\ min,\ 4°C}$	83
15 min, 95°C	20

A 225 μl two-stage reaction mix was incubated for 15 min at 10°C, and the membranes were washed and resuspended to a final volume of 180 μl in reaction buffer containing GDP-mannose and an ATP-regenerating system. Portions (20 μl) were aliquoted to tubes containing 5 μl of the indicated protein solution. After 45 min at 20°C, 40 μl of Laemmli sample buffer was added, and reaction products were analyzed by immunoprecipitation followed by scintillation counting. BSA was dissolved in reaction buffer, and the cytosol was desalted by gel filtration in reaction buffer. For cytosol treatments, 250 μg/ml trypsin or 10 mM NEM was incubated with cytosol for 30 min at 40°C and quenched by addition of 500 μg/ml soybean trypsin inhibitor or 200 mM DTT. In control treatments, the trypsin inhibitor or DTT was added at the beginning of the incubation. For heat treatment, cytosol was incubated for 15 min at 95°C and then centrifuged for 1 min at 12,000 × g to remove aggregates of denatured protein.

of the detergent saponin at the beginning of the second-stage incubation completely blocked α1→6-Man addition (Figure 6). Both the ER form and the heterogeneously glycosylated form of the α-factor precursor resided within sealed compartments at the end of a transport reaction: both sedimented (Table 1A) and were protected from proteinase K (data not shown) in the absence but not the presence of Triton X-100. The concentration of detergent required to release both α-factor precursor forms did not affect the sedimentation of an integral ER membrane protein, NADPH–cytochrome c reductase (Table 1A), indicating that they were soluble within the lumen of their respective organelles.

Cytosolic proteins were required for transport. Transport was stimulated 6-fold by addition of cytosol in the second-stage incubation (Table 2). Stimulation by cytosol reached saturation at 80 μg protein per reaction (data not shown). Stimulation was not due to a nonspecific increase in protein concentration; 80 μg of BSA was without effect (Table 2). The residual transport occurring in the absence of added cytosol may be mediated by factors remaining associated with washed membranes.

Cytosol stimulation was due to proteins. Cytosol treated with trypsin or with the alkylating agent N-ethylmaleimide (NEM) failed to stimulate transport. Control treatments with trypsin inhibitor and trypsin or DTT and NEM had lit-

Table 1. Fractionation of Compartments Containing Core Glycosylated and Outer-Chain Glycosylated Pro-α-Factor

Fraction	Protein (μg)	NADPH–Cytochrome c Reductase (% total)	Core Glycosylated α-Factor (% total)	Anti-α1→6-Man Precipitable α-Factor (% total)
A Total	557	100	100	100
− Triton pellet	361	88	77	85
+ Triton pellet	213	61	9	3
B LSP	183	57	26	7
MSP	54	24	17	12
HSP	55	9	15	41

A large one-stage reaction was fractionated as described in Experimental Procedures. (A) Two aliquots were centrifuged at 100,000 × g for 10 min after no treatment (− Triton pellet) or after addition of 0.1% Triton X-100 (+ Triton pellet). (B) A separate portion was fractionated into an LSP (300 × g, 10 min), an MSP (7600 × g, 5 min) and an HSP (100,000 × g, 10 min) by differential centrifugation. An unfractionated sample and each of the pellet samples were analyzed as described in Experimental Procedures. The values presented for core glycosylated pro-α-factor were obtained by subtracting the anti-α1→6-Man precipitable cpm from the ConA precipitable cpm. This represents an overestimate of core glycosylated material because not all outer-chain glycosylated pro-α-factor was precipitated by the anti-α1→6-Man serum. Material not accounted for in the pellet fractions may have been released into the soluble fraction during incubation at 20°C or during fractionation. Technical problems precluded quantitation of this material. Total values were 49,052 cpm of core glycosylated pro-α-factor and 15,424 cpm of anti-α1→6-Man precipitable pro-α-factor.

tle effect on stimulation. Heat treatment also destroyed the stimulatory activity of cytosol (Table 2).

Anti-α1→6-Man Precipitable α-Factor Is Enriched in a Compartment That Is Depleted of an ER Marker

The in vitro conversion of the ER form of the α-factor precursor to the anti-α1→6-Man precipitable form displayed the properties expected of an intercompartmental transport reaction. To address the intercompartmental nature of the reaction more directly, we turned to subcellular fractionation. The yeast ER sediments more rapidly than the Golgi, and the two organelles can be partially resolved by differential centrifugation (Esmon, 1986). We therefore investigated the sedimentation behavior of the anti-α1→6-Man precipitable pro-α-factor relative to that of the majority of the ConA precipitable pro-α-factor.

A one-stage reaction was incubated for 45 min at 20°C and then fractionated into an LSP (low-speed pellet; 300 × g, 10 min), MSP (medium-speed pellet; 7600 × g, 5 min), and HSP (high-speed pellet; 100,000 × g, 10 min) by differential centrifugation. Intact and broken spheroplasts sedimented in the LSP while the MSP and HSP contained organelles that were released by the broken spheroplasts before or during the 20°C incubation. As can be seen in Table 1B, the LSP and MSP together contained 90% of the recovered NADPH–cytochrome c reductase activity and 75% of the sedimentable core glycosylated pro-α-factor. In contrast, the anti-α1→6-Man precipitable pro-α-factor was recovered primarily in the HSP. We presume that this fraction contains Golgi membranes, but the small scale of this reaction precluded direct assay of mannosyltransferase activity. The fractionation behavior of the highly glycosylated pro-α-factor was a property of the compartment that contained it: no sedimentation was observed when organelles were first permeabilized by addition of detergent (Table 1A).

Transport Is Blocked by GTPγS

The G protein activators GTPγS and AlF_4^- have been shown to inhibit the mammalian intra-Golgi in vitro transport reaction and cause the accumulation of coated transport vesicles (Melancon et al., 1987). As seen in Figure 7, GTPγS also inhibited the yeast ER–Golgi transport reaction. Half-maximal inhibition occurred at about 5 μM. ATPγS and GMP-PNP had little inhibitory effect at concentrations up to 100 μM. No inhibition was observed when 400 μM GTP was added with the GTPγS. The combination of 4 mM F^- and 50 μM Al^{3+} reduced transport to 35% of control levels, while neither compound alone had a significant inhibitory effect.

sec Mutations Block Transport In Vitro

The *sec* mutants provide both a test of the authenticity of the in vitro reaction and a means to identify and purify individual transport factors. The analysis focused on the three ER-blocked *sec* mutants (*sec12, sec18,* and *sec23*) whose genes have been sequenced and gene products localized. We first examined the effects of imposing the *sec* mutant block in vivo. *sec* mutant cell lysates were prepared as described in Experimental Procedures except

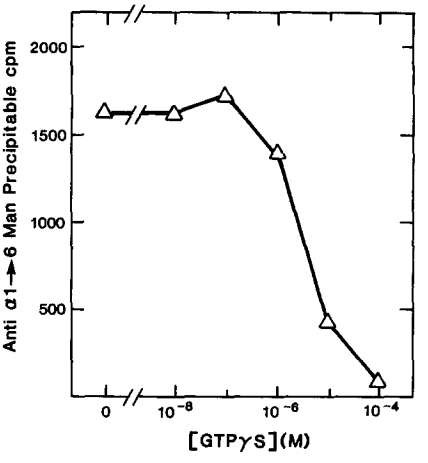

Figure 7. Inhibition of Transport by GTPγS

Increasing concentrations of GTPγS were added to aliquots of complete reaction mix at the beginning of the second stage of a standard two-stage reaction. Samples were terminated after 45 min at 20°C and analyzed by immunoprecipitation and scintillation counting. ConA precipitable pro-α-factor averaged 6900 cpm per point.

that spheroplasts were shifted to 37°C (the nonpermissive temperature) during the last 15 min of the regeneration step. While this treatment had no effect on the transport capacity of wild-type lysates, it completely blocked pro-α-factor transport in the mutant lysates (data not shown). Morphological alterations take place within *sec* mutants even after brief incubation at the nonpermissive temperature (C. Kaiser and R. S., unpublished results); thus, defects revealed by an in vivo preshift may be indirect. To evaluate directly the role of *sec* gene products in the in vitro reaction, we analyzed the transport activity of mutant lysates prepared without exposure to the nonpermissive temperature.

The temperature-sensitive transport defect of *sec23* cells was reproduced in vitro. *sec23* lysates were prepared from cells grown at the permissive temperature, and [^{35}S]methionine-labeled prepro-α-factor was synthesized in a *sec23* translation extract. A two-stage reaction was carried out using *sec23* components in the first stage. Cytosol added in the second stage was prepared either from *sec23* cells or from *sec23* cells containing a plasmid bearing the *SEC23* gene. Aliquots of each reaction were incubated at 15°C, 25°C, or 30°C in the second stage. As seen in Figure 8, reactions that contained *SEC23* gene product proceeded efficiently at all temperatures. The reactions supplemented with *sec23* cytosol were markedly temperature sensitive. Transport at 30°C was reduced 5-fold relative to transport at 15°C. We could not test higher temperatures because the efficiency of the wild-type reaction decreased rapidly above 30°C. *sec23* cytosol did not inhibit reactions containing the *SEC23* gene product (data not shown). Although it is possible that there was a general temperature-sensitive defect in *sec23* cytosol, the simplest explanation of these results is that transport at 30°C depended on the presence of the *SEC23* gene product.

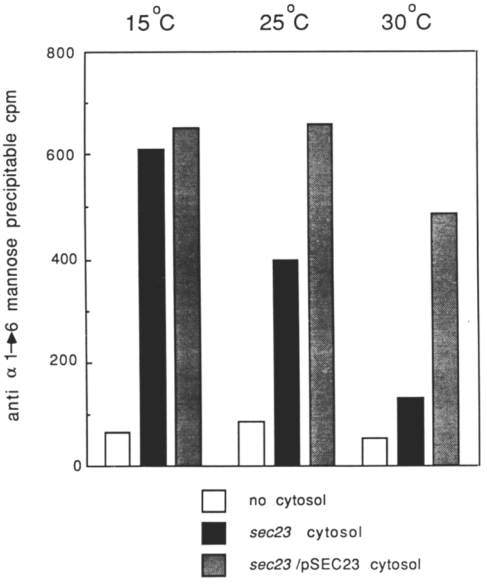

Figure 8. Temperature Sensitivity of In Vitro Transport with Mutant *sec23* Components

Broken spheroplasts prepared from a *sec23* strain were incubated with [^{35}S]prepro-α-factor made in a *sec23* translation extract during the first stage of a two-stage reaction. For the second stage, washed membranes were mixed with cytosol prepared from either a *sec23* strain or the same *sec23* strain carrying a *SEC23* gene on a single-copy plasmid (pSEC23). Transport was allowed to proceed at 15°C or 25°C for 45 min, or at 30°C for 25 min. ConA precipitable pro-α-factor averaged 4482 cpm per point.

We also examined transport in *sec12* and *sec18* lysates. *sec12* lysates were defective in transport at all temperatures, even when supplemented with wild-type cytosol. The lack of rescue was consistent with results from cell fractionation and sequence analysis showing that the *SEC12* gene product is an integral membrane protein (A. Nakano and R. S., submitted). *sec18* lysates were also defective at all temperatures. Supplementation with cytosol containing the *SEC18* gene product gave a 2-fold stimulation of transport (data not shown).

Discussion

We have developed a new yeast ER-to-Golgi in vitro transport assay. Prepro-α-factor, the precursor of the yeast mating pheromone α-factor, is synthesized in a yeast translation extract in the presence of [^{35}S]methionine and then translocated into the ER of gently lysed spheroplasts. Transport to the Golgi is measured through addition of outer-chain carbohydrate. The amount of [^{35}S]methionine-labeled prepro-α-factor receiving outer chain is quantified by immunoprecipitation with an α1→6-Man–specific antibody. Twenty-five percent of the α-factor precursor that enters the ER is transported to the Golgi during a typical incubation.

The in vitro reaction has properties expected of intercompartmental protein transport. The reaction is dependent on energy, as is ER-to-Golgi transport in vivo, and addition of a soluble protein fraction stimulates the reaction 6-fold. Outer-chain addition is accompanied by movement of the α-factor precursor from membranes that sediment rapidly to membranes that sediment more slowly and which are depleted of an ER marker enzyme. Transport is abolished by detergent, presumably because of a requirement for sealed compartments.

Artifactual signals may result from α1→6-Man addition by a mannosyltransferase located in the ER or by nonspecific fusion between the ER and the Golgi. We consider these possibilities unlikely. First, secretory proteins may accumulate in the ER for over 1 hr in vivo without any detectable α1→6-Man addition (Esmon et al., 1981). Second, the requirement for ATP and stimulation by cytosol are difficult to reconcile with either glycosylation in the ER or nonspecific fusion. Third, addition of α1→6-Man in the ER and nonspecific fusion cannot easily account for the observed differences in the sedimentation behavior of the modified and unmodified forms of the α-factor precursor. Finally, *sec* mutations that block transport in vivo block the in vitro reaction.

Role of GTP-Binding Proteins in ER–Golgi Transport

YPT1, a gene encoding a GTP-binding protein, is required for transport of the secretory protein invertase from the ER to the Golgi in vivo (Segev et al., 1988; Schmitt et al., 1988). To investigate the role of YPT1 or other GTP-binding proteins in transport, we have used the appoach of Melancon et al. (1987), who showed that the G protein activator GTPγS inhibits the mammalian intra-Golgi in vitro transport reaction. We find also that GTPγS specifically inhibits the ER–Golgi in vitro transport reaction. The inhibited component could function as a G protein–like signal transducer. Alternatively, the GTPγS-sensitive factor may play a role more analogous to that of the elongation factor Ef-Tu in protein synthesis (Kaziro, 1978). Targeting factors, which must recycle to direct repeated rounds of transport, may use the energy of GTP hydrolysis to cycle between two conformations, as do many well-characterized GTP-binding proteins. More specifically, a GTP-binding protein could guide transport vesicles to their destination in much the same way that Ef-Tu directs aminoacyl-tRNAs to the ribosome.

SEC Protein–Dependent Transport In Vitro

In vitro reconstitution has become the method of choice for studying the molecular mechanisms of interorganelle transport. Although reconstituted transport reactions exhibit many of the properties of transport in vivo, there has been no direct demonstration that these in vitro reactions use any of the factors required for transport in vivo. In vitro analysis of the effects of *sec* mutations that block ER-to-Golgi transport in vivo provides an opportunity to authenticate the reaction.

A temperature-sensitive defect in ER-to-Golgi transport caused by the *sec23* mutation has been reproduced in vitro. Transport at 30°C in *sec23* membranes supplemented with *sec23* cytosol is reduced 5-fold relative to transport at 15°C. Supplementation with cytosol containing the *SEC23* gene product restores transport at 30°C.

Hence, the *SEC23* gene product is involved in transport in vitro as well as in vivo.

The *sec* mutants will facilitate purification and characterization of the proteins that mediate intercompartmental transport. Supplementation of mutant reactions with wild-type cytosol provides a functional assay for the purification of *SEC* gene products. Molecular cloning techniques may be used independently to investigate the structure and location of SEC proteins. For example, the *SEC23* gene has been sequenced, and the gene product has been localized using an antibody. The *SEC23* gene product is a relatively abundant cytoplasmic protein. In gently lysed yeast spheroplasts, a large portion of the protein is loosely associated with the cytoplasmic surface of a membrane that sediments rapidly, although most of the protein is soluble under the conditions used to prepare cytosol for the in vitro reaction (Hicke and Schekman, unpublished results). Knowledge of the fractionation behavior of the *SEC23* gene product will be useful in developing models of the role the protein plays in transport and, together with the antibody, will aid in purification of the functional *SEC23* gene product.

Further Applications of Protein Import into Gently Lysed Cells

Yeast spheroplasts can be broken by freeze-thawing to produce ghosts that have lost most of their cytosol but retain intact organelles. These ghosts may support a variety of complex reactions in addition to the ones described here. The method is rapid and convenient: once frozen, the spheroplasts can be stored indefinitely at −85°C until needed. Since the procedure does not require adhesion of a monolayer of cells to a surface, it may be extended to a variety of cell types that grow in liquid culture.

The system we have developed may be amenable to reconstitution of later steps in protein transport. Intra-Golgi transport may be investigated by a simple extension of the ER–Golgi assay. The first step in the proteolytic maturation of the α-factor precursor, cleavage by the KEX2 endopeptidase, is thought to take place in a late Golgi compartment (Fuller et al., 1988). Under standard reaction conditions, we have detected the production of an α-factor–related species that comigrates with mature α-factor on a 15% polyacrylamide gel, but thus far we have been unable to demonstrate ATP and cytosol dependence (A. Capriotti, D. B., and R. S., unpublished results).

The use of a transport substrate synthesized in vitro allows considerable flexibility. In previous intercompartmental transport assays, the substrate has been accumulated in the donor compartment in vivo prior to lysis. This approach is limited to rapidly synthesized proteins that can be caught in transit through the donor compartment, proteins with conditional defects in transport, and, in yeast, *sec* mutant cells. Our approach is free of these restrictions. Any step along the secretory pathway may potentially be reconstituted by introducing an appropriate radiolabeled substrate into the ER of lysed spheroplasts. For example, the precursor to the yeast vacuolar protease carboxypeptidase Y may be used to follow transport to the vacuole. Furthermore, substrates that vary by the presence or absence of a sorting determinant may be introduced into lysed spheroplasts to investigate the molecular mechanisms of sorting.

Experimental Procedures

Materials

The yeast strains used in this study were GPY59 (*leu2-3,112 ura3-52 trp1-289 prb1 pep4::URA3 gal2 MATa*), GPY60 (*leu2-3,112 ura3-52 his4-579 trp1-289 prb1 pep4::URA3 gal2 MATα*), DBY5-3A (*sec23 leu2-3,112 ura3-52 pep4::URA3 MATα*), and LHY3-8C (*sec23 leu2-3,112 ura3-52 his3 MATα*). pDJ100 was obtained from D. Julius and J. Thorner. pSEC23 contains the *SEC23* gene on a 5.5 kb PvuII fragment inserted into the centromere vector YCp50.

Casamino acids medium is 6.7 g/l yeast nitrogen base without amino acids (Difco Laboratories Inc.), 1% vitamin assay Casamino acids (Difco Laboratories Inc.), 0.01% adenine, 0.01% histidine, 0.01% tryptophan, 0.01% methionine, and 2% glucose, with or without 0.002% uracil. YP medium contained 1% Bacto-Yeast Extract and 2% Bacto-Peptone (Difco Laboratories Inc.). Cultures were grown in YP containing 5% glucose (YPD) to early log phase as described by Deshaies and Schekman (1987). *SEC* strains were grown at 30°C, *sec* strains at 24°C. One OD_{600} unit was approximately 10^7 cells.

Anti-α1→6-Man serum was produced in response to *mnn1 mnn2* cells as described by Ballou (1970). α1→6-, α1→2-, and α1→3-Man oligosaccharides were provided by Clint Ballou (this department). Lyticase was prepared as described by Scott and Schekman (1980). ConA–Sepharose, apyrase (grade VII), and GDP-mannose were obtained from Sigma. Protein A–Sepharose was from Pharmacia. Other reagents were obtained as described by Kepes and Schekman (1988).

Preparation of Transport-Competent Membranes

Cells were grown to 2–4 OD_{600} U/ml in YPD, harvested by centrifugation (1000 × g, 5 min, 24°C), and resuspended at 50 OD_{600} U/ml in 10 mM DTT, 100 mM Tris–HCl (pH 9.4). After 5 min at 24°C, cells were harvested by centrifugation, resuspended at 50 OD_{600} U/ml in 0.7 M sorbitol, 0.75× YP, 0.5% glucose, 10 mM Tris–HCl (pH 7.5), 20 U lyticase per OD_{600} U of cells, and incubated at 30°C until the OD_{600} of a 1:100 dilution in H_2O dropped to less than 10% of the initial value. Spheroplasts were harvested by centrifugation, resuspended at 5 OD_{600} U/ml in 0.7 M sorbitol, 0.75× YP, 1% glucose, and incubated with gentle shaking for 20 min at 30°C. Spheroplasts, which had resumed metabolism, were harvested by centrifugation (1000 × g, 5 min, 4°C), washed, and resuspended at 300 OD_{600} U/ml in lysis buffer (400 mM sorbitol, 20 mM HEPES [pH 6.8], 150 mM KOAc, 2 mM MgOAc, 0.5 mM EGTA) at 4°C. Aliquots (200 μl) of the spheroplast suspension were transferred to Eppendorf tubes and frozen in the vapors above liquid N_2 in a sealed ice bucket. After 45 min the tubes were transferred to a −85°C freezer. No loss in transport activity was detected during 2 months of storage.

When *sec* mutant strains were used, the temperature during spheroplast formation and regeneration was reduced to 24°C.

Preparation of [^{35}S]Methionine-Labeled Prepro-α-Factor

Transcription of the plasmid pDJ100 and translation of the mRNA in a yeast extract were according to standard procedures (Hansen et al., 1986; Moldave and Gasior, 1983) except that the translation extract was prepared by glass-bead agitation of cells (Deshaies, 1988). Translation reactions (2–5 ml) were desalted by gel filtration on a Sephadex G-25 column (5 ml resin per ml translation reaction) equilibrated in reaction buffer (20 mM HEPES [pH 6.8], 150 mM KOAc, 250 mM sorbitol, 5 mM MgOAc). Peak fractions were pooled, and aliquots were frozen in liquid N_2 and stored at −85°C. No significant loss of transport competence was detected over 1 month of storage.

Preparation of Cytosol

Wild-type cells (4000 OD_{600} U) grown at 30°C in YPD or *sec* mutant cells grown at 24°C in Casamino acids medium were harvested by centrifugation and washed in reaction buffer at 4°C. Cells were resuspended in 2 ml of reaction buffer containing 1 mM DTT and 0.5 mM PMSF, 4 g of glass beads was added, and the cells were lysed by ten 30 sec periods of agitation on a VWR Vortexer 2 at full speed. The

homogenate was clarified by centrifugation at 3000 × g for 5 min and the supernatant fraction further centrifuged at 100,000 × g for 30 min. The resultant S100 fraction was either desalted by filtration on Sephadex G-25 equilibrated in reaction buffer and then frozen in aliquots in liquid N_2, or was aliquoted and frozen immediately. Filtered and unfiltered cytosol fractions stimulated transport to the same extent and were stable over several months of storage at −85°C. Protein concentration ranged from 20 to 30 mg/ml.

In Vitro Transport Reaction

For each experiment an aliquot of gently lysed yeast was thawed by immersion in a 25°C water bath, and the required amount of membranes (20 μl per reaction) was washed three times by brief (~10 sec) centrifugation in a Fisher microcentrifuge and resuspension in 1 ml of reaction buffer at 4°C.

One-stage reactions contained 5 μl of prepro-α-factor translation product (150,000 trichloroacetic acid precipitable cpm), 60 μg of additional cytosol, 50 μM GDP-mannose, 1 mM ATP, 40 mM creatine phosphate (CP), 200 μg/ml creatine phosphokinase (CPK), and 20 μl (original volume) of membranes washed as above and resuspended in reaction buffer to bring the reaction volume to 25 μl. The GDP-mannose, ATP, CP, and CPK were added from a 10× stock solution prepared in reaction buffer and stored in small aliquots at −85°C. After 45 min at 20°C, reactions were terminated by addition of 40 μl of Laemmli (1970) sample buffer and heated for 5 min at 95°C.

The first stage of two-stage reactions contained 8 μl of prepro-α-factor translation product, 50 μM GDP-mannose, 1 mM ATP, 40 mM CP, 200 μg/ml CPK, and 20 μl (original volume) of membranes washed as above and resuspended in reaction buffer to bring the reaction volume to 25 μl. The reaction mix was incubated for 15 min at 10°C, and the membranes were washed two times by centrifugation and resuspension in reaction buffer at 4°C. A complete second-stage incubation, in a final volume of 25 μl, contained the following: washed pro-α-factor–containing membranes, 80 μg of cytosol, 1 mM ATP, 50 μM GDP-mannose, 40 mM CP, and 200 μg/ml CPK. Typically, eight to ten reactions were combined in one tube during the first stage, and the washed pro-α-factor–containing membranes were aliquoted to individual tubes with various additions at the beginning of the second-stage incubation. After 45 min at 20°C, the reactions were terminated by addition of Laemmli sample buffer and heated for 5 min at 95°C.

Immunoprecipitation

A 20 μl portion of a terminated reaction was mixed with 35 μl of a 20% (vol/vol) suspension of Protein A–Sepharose, 8–10 μl of anti-α1→6-Man serum or 8 μl of anti-α-factor serum, and 1 ml of IP buffer (150 mM NaCl, 1% Triton X-100, 0.1% SDS, 15 mM Tris–HCl [pH 7.5]) in an Eppendorf tube and rotated for 2 hr at room temperature or overnight at 4°C. The immunoprecipitates were collected by centrifugation and washed twice with 1 ml of IP buffer; twice with 1 ml of 2 M urea, 200 mM NaCl, 1% Triton X-100, 100 mM Tris–HCl (pH 7.5); once with 1 ml of 500 mM NaCl, 1% Triton X-100, 20 mM Tris–HCl (pH 7.5); and once with 1 ml of 50 mM NaCl, 10 mM Tris–HCl (pH 7.5).

The washed immunoprecipitates were analyzed either by electrophoresis and autoradiography or directly by scintillation counting. Immunoprecipitates to be analyzed by electrophoresis were heated at 95°C in Laemmli sample buffer and electrophoresed on an 11.25% SDS–polyacrylamide gel (Laemmli, 1970). The gel was treated with Amplify after fixation, dried, and exposed to X-ray film. Immunoprecipitates to be analyzed by scintillation counting were heated for 5 min at 95°C in 150 μl of 2% SDS, then transferred to 8 ml scintillation vials. Five milliliters of Aquasol (New England Nuclear) was added to each vial, and the samples were counted in a scintillation counter. The background (typically around 100 cpm) was determined by counting immunoprecipitates of complete reactions that had been stopped by addition of Laemmli sample buffer at the start of the incubation, and was subtracted from all reported values.

For ConA precipitations, 10 μl of a terminated reaction and 30 μl of a 20% (vol/vol) suspension of ConA–Sepharose were added to 1 ml of 500 mM NaCl, 1% Triton-X-100, 20 mM Tris–HCl (pH 7.5), and samples were rotated for 2 hr at room temperature or overnight at 4°C. The washes and subsequent analysis were as described for antibody precipitations. Background, determined as above, was typically around 300 cpm.

Fractionation

A one-stage reaction scaled up 8-fold and a parallel mock reaction with desalted cytosol substituted for the prepro-α-factor translation product were incubated for 45 min at 20°C and then each processed as follows: A 25 μl aliquot treated with 0.1% Triton X-100 and an untreated 25 μl aliquot were diluted to 200 μl with lysis buffer and centrifuged at 100,000 × g for 10 min. One hundred microliters of the remaining reaction mix was diluted to 1 ml with lysis buffer and centrifuged for 10 min at 300 × g. The LSP was saved and the supernatant fraction centrifuged for 5 min at 7600 × g. The MSP was saved and the supernatant fraction centrifuged for 10 min at 100,000 × g to give the HSP. An unfractionated sample and each of the pellet fractions were analyzed by anti-α1→6-Man or ConA precipitation following solubilization in Laemmli sample buffer (complete reaction). Fractions from the mock reaction were used to measure protein (Markwell et al., 1978) and NADPH–cytochrome c reductase (Kubota et al., 1977).

Electron Microscopy

Samples were fixed in a mixture of 0.2% paraformaldehyde, 2.5% glutaraldehyde, and 0.1% picric acid at pH 7.2 for 32 hr, followed by 0.9% NaCl washes and fixation in uranyl acetate and then 2% OsO_4. Dehydration was followed by Medcast (Pelco) embedding. Silver sections stained 1 min in lead citrate were photographed in a Philips 301 electron microscope.

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