Distinct Biochemical Requirements for the Budding, Targeting, and Fusion of ER-derived Transport Vesicles

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Abstract. The transport of pro-alpha-factor from the ER to the Golgi apparatus in gently lysed yeast spheroplasts is mediated by diffusible vesicles. These transport vesicles contain core-glycosylated pro-alpha-factor and are physically separable from donor ER and target Golgi compartments. The formation of diffusible vesicles from the ER requires ATP, Sec12p, Sec23p, and GTP hydrolysis. The vesicles produced are functionally distinct from the ER: they transfer pro-alpha-factor to the Golgi apparatus faster and more efficiently than the ER, they do not require Sec12p or Sec23p to complete transfer, and transfer is resistant to GTPγS. Targeting of vesicles to the Golgi apparatus requires Yptp and Sec18p. Fusion of vesicles that have targeted requires calcium and ATP.

Transport of proteins between compartments of the secretory pathway is mediated by small vesicles which bud from a donor compartment and fuse with a target compartment (Palade, 1975). Transport vesicles that mediate ER to Golgi transport have been identified in vivo in mammalian cells (Jamieson and Palade, 1967) and yeast cells (Novick et al., 1981; Kaiser and Schekman, 1990). Membrane fractions with properties suggestive of an intermediate compartment between the ER and the Golgi have also been generated in vitro in mammalian (Paulik et al., 1988) and yeast lysates (Groesch et al., 1990).

10 sec Mutations block protein transport from the ER to the Golgi apparatus at 37°C (Novick et al., 1980). By electron microscopy, a subset of these mutants, the class II mutants (sec17, sec18, and sec22), accumulate 50-nm vesicles and enlarged ER structures at 37°C. A second subset, the class I mutants (sec12, sec13, sec16, and sec23), accumulate ER structures at 37°C and block the accumulation of vesicles when in double-mutant combination with any of the class II sec genes (Kaiser and Schekman, 1990). Thus, class I Sec proteins are thought to be required for transport vesicle budding and class II Sec proteins, for vesicle fusion. Yeast carrying a mutation in the ras-like gene YPT1 (Gallwitz, 1983) accumulate enlarged ER and Golgi structures at the nonpermissive temperatures (Segev et al., 1988; Schmitt et al., 1988).

GTPγS, a nonhydrolyzable analogue of GTP, inhibits protein transport from the ER to the Golgi (Baker et al., 1988; Ruohola et al., 1988; Beckers and Balch, 1989) and between mammalian Golgi cisternae (Melanoč et al., 1987). Electron microscopic analysis of intra-Golgi transport shows that GTPγS causes the accumulation of small 80 nm transport vesicles (Melanoč et al., 1987) that bear a non-clathrin protein coat (Malhotra et al., 1989). In addition to an effect on vesicle uncoating, GTPγS inhibits vesicle budding from the trans-Golgi network (Tooze and Huttner, 1990).

Treatment of membrane or cytosol proteins with NEM blocks ER-to-Golgi (Baker et al., 1988; Beckers et al., 1989), and intra-Golgi protein transport (Balch et al., 1984). In the latter, NEM treatment causes the accumulation of "uncoated" transport vesicles that are bound to Golgi membranes (Malhotra et al., 1988; Orci et al., 1989). Addition of NSF (N-ethylmaleimide-sensitive factor) restores transport to NEM-inhibited reactions (Glick and Rothman, 1987; Block et al., 1988; Beckers et al., 1989) and promotes vesicle fusion (Malhotra et al., 1988). Sec18p is the yeast homologue of NSF (Eagle et al., 1988; Wilson et al., 1989).

EGTA inhibits ER-to-Golgi, but not intra-Golgi protein transport (Baker et al., 1990; Beckers et al., 1989). Inhibition is reversed in both ER-to-Golgi transport reactions by addition of Ca2+ to nanomolar levels. The sequential application of inhibitors has demonstrated that Ca2+ is required at a late step in transport (Baker et al., 1990; Beckers et al., 1990).

In this report we have exploited the differential inhibition of transport caused by Sec mutant proteins and chemical inhibitors to identify diffusible vesicles that mediate protein transport from the ER to the Golgi apparatus in gently lysed yeast spheroplasts. We developed assays that independently measure vesicle budding, targeting, and fusion, and have examined the specific protein and nucleotide requirements for each stage.

1. Abbreviations used in this paper: gpaf, glycosylated pro-alpha-factor; HSP, high speed pellet; MSP, medium speed pellet; MSS, medium speed supernatant; NSF, N-ethylmaleimide-sensitive factor; ppaf, prepro-alpha-factor.
Preparation of Transport-competent Membranes and Cytosol

Cells were grown at 24°C in YPD medium to early log phase (∼4 OD600/ ml; 1 OD600 unit is ∼10^11 cells). Gently lysed yeast spheroplast membranes were prepared as described in Baker et al. (1988). To prepare cytosol fractions, ∼2,000 OD600 units of cells were harvested by centrifugation and washed twice by dilution in B88 (20 mM Hepes pH 6.8, 150 mM KOAc, 5 mM MgOAc, and 250 mM sorbitol). Cell pellets were resuspended in a Corex 30-ml glass tube, with 1 ml chilled 20 mM Hepes, pH 6.8, 5 mM MgOAc, 50 mM KOAc, 100 mM sorbitol, 1 mM ATP, 0.5 mM PMSF, and 1 mM DTT. Glass beads (4 g) were added and the cells were lysed by 8 × 30 s periods of agitation in a VWR vortexer (Scientific Industries, Inc., Bohemia, NY) at full speed. Samples were then mixed with 1.5 ml of 20 mM Hepes pH 6.8, 5 mM MgOAc, 2 M KCl, 400 mM sorbitol, 1 mM ATP, 0.5 mM PMSF, 1 mM DTT, and vortexed 4 × 30 s at full speed. The homogenate was clarified initially by centrifugation at 3,000 g for 5 min and the supernatant (3 ml) was further clarified by centrifugation at 100,000 g for 30 min. Cytosol was desalted by filtration in a Sephadex G25 column (15 ml, fine) that was equilibrated in B88 and 1 mM ATP. The eluted protein peak was pooled and distributed in 50–60 μl fractions which were frozen in liquid nitrogen for storage at −80°C. Protein concentration, which ranged from 12 to 16 mg/ml, was measured by the Bradford assay and compared to a BSA standard.

In Vitro Transport

Stage I: Translocation. For each experiment an aliquot of frozen membranes (from 60 OD600 units of cell equivalents) was thawed and washed three times by resuspension in B88 and brief (∼10 s) centrifugation in a microfuge (Fischer Scientific Co.). Membranes were then resuspended in an SI0 yeast lysate that contained [35S]methionine-labeled prepro-alpha-factor (Baker et al., 1988) and an ATP-regenerating system in final volumes of either 160 or 80 μl. The final concentration of components in a 25-μl translocation reaction was 375-500 pg membranes (measured by the method of Lowry (1951), modified to contain SDS), 90 μg of yeast SI00 lysate, 50 μM GDP-mannose, 1 mM ATP, 40 mM creatine phosphate and 200 μg/ml creatine phosphokinase, all dissolved in B88. The mix was incubated for 15 min at 10°C to allow posttranslational translocation, cooled to 4°C, and washed with 1 ml B88 and centrifuged in a microfuge. Membranes were then resuspended in 1 ml B88 and mixed by rotation at 4°C for 7 min. A final pellet fraction was resuspended in B88 at a concentration of 150 to 200 μg per ml.

Stage II: Transport. Stage II incubations (25 μl) contained 10 μl of stage I membranes, 90 μg of cytosol (preincubated for 5 min at 29°C), 50 μM GDP-mannose, 1 mM ATP, 40 mM CP, 200 μg/ml CPK, all in B88. The amount/concentration of other chemicals added per reaction was: 1 μg anti-Yptlp Fα fragments, 0.66 μg of yeast Yptlp isolated from Escherichia coli (Baker et al., 1990), 20 μM GTPyS ± 1 mM GTP; 10 mM NEM ± 20 mM DTT; 5 mM EGTA/600 μM MnCl2 ± 250 μM CaCl2. The concentration of free ions in this EGTA buffer was estimated to be ~63 mM calcium and ~10 mM manganese using a modified version of the computer program described by Robertson and Potter (1985).

For all time courses, a 200 μl stage II mix was prepared and aliquoted in 25 μl portions into 0.5 ml microfuge tubes. The tubes were incubated at either 0, 20, or 29°C for the indicated times. Each tube represented one time point. Reactions were terminated by placing each tube at 0°C until the last time point was taken. After the completion of the time course each tube was fractionated by centrifugation for 37 s in a microfuge (Scientific Industrial Co.). A high-speed supernatant (MSP) fraction was taken and the remaining MSS was aspirated. The medium speed pellet (MSP) fractions were resuspended to 25 μl and 15 μl was processed. Each fraction was treated with 250 μg/ml trypsin for 10 min at 4°C and then with 250 μg/ml trypsin inhibitor for ≥5 min at 4°C. SDS was added to a final concentration of 1% and the tubes were heated at 95°C for 7 min. Equal aliquots from each tube were treated with either Con A-Sepharose or protein A-Sepharose coupled with anti-α6 antibody as described by Baker et al. (1988). Pro-alpha-factor constitutes the only radiolabeled protein. The radioactive immunoprecipitates were heated to 95°C in 1% SDS for 7 min and dissolved in Universal ES scintillation fluid (ICN Biomedicals, Irvine, CA) for quantitation in a scintillation counter.

Stage III: Transport Intermediate Chases. Yptlp Fα and Ca2+-requiring intermediates were generated at 20°C in large (200 μl) stage II incubations using wild-type or mutant lysates as indicated. At the end of stage II incubations, the reactions were fractionated by centrifugation in a microfuge (Scientific Industrial Co.) for 1 min at 4°C. A 150-μl MSS fraction was taken from the meniscus. Each 150 μl MSS fraction was further centrifuged at 88,000 g for 15 min at 4°C to generate a high speed pellet (HSP). The HSP fractions were resuspended in B88 in the case of Yptlp Fα intermediates or in EGTA/Mn2+ buffer in the case of Ca2+-requiring intermediates. An ATP-regenerating system, cytosol, and fresh membranes were supplied to the Yptlp Fα intermediate to the same concentrations as in a stage II incubation. Unless otherwise indicated, ATP and calcium (250 μM) were added to the chase incubations that contained the Ca2+-requiring intermediate. Chemical inhibitors were used at the same amount/concentration as described for stage II incubations. Complete stage III incubations were chilled on ice, mixed with SDS to 1%, and heated to 95°C for 7 min. Equal aliquots from each tube were processed as described for stage II reactions.

Fractionation in Sucrose Gradients

MSS fractions (150 μl) were obtained from large incubations. Each MSS fraction was mixed with either 50 μl of B88 (−salt) or 50 μl of B88 plus 3.6 M KCl (+salt) and further incubated for 5 min at 20°C. The mix was then cooled and loaded on top of a sucrose gradient with a log-linear distribution of sucrose from 15 to 45% (wt/wt) in B88. The gradients were then centrifuged at 32,000 rpm for 2 h at 4°C in a rotor (model SW50.1; Beckman Instruments, Inc., Palo Alto, CA). Gradient fractions were collected from the top into 36 × 150 μl fractions. The densities of every other fraction were measured in a Zeiss refractometer and expressed as percent (wt/wt) sucrose. The [35S]gpαf content of every other fraction was determined by Con A and anti-α6 mannose/protein A precipitation as before (Baker et al., 1988).

The log-linear sucrose gradients were prepared by overlaying at room temperature 0.4 ml of 55% (wt/vol) sucrose, 0.5 ml of 40% sucrose, 0.5 ml of 30% sucrose, 1.2 ml of 25% sucrose, 1.2 ml of 20% sucrose, and 1.2 ml of 15% sucrose in a 5.3 ml Ultraclear Beckman thin wall tube (Beckman Instruments, Inc.). This step gradient was then centrifuged at 3 h at 32,000 rpm in a rotor (SW 50.1; Beckman Instruments Inc.) at 4°C to create a smooth sucrose gradient.

Quantitative Translocation Assay

The fractions tested for translocation activity were generated in a "mock" stage II reaction that lacked radioactive precursor. Samples representing each time point were fractionated in a Fischer microfuge (Fischer Scientific, Pittsburgh, PA) as described for stage II incubations to generate 15 μl MSP and MSS fractions. A 5-μl aliquot of each MSS and MSP fraction was mixed with 5 μl of an SI00 lysate containing [35S]methionine-labeled prepro-alpha-factor, an ATP-regenerating system, and B88 in a final volume of 25 μl. The mix was then incubated for 30 min at 20°C to allow translocation. The completed reactions were cooled, SDS was added to 1%, and the samples were heated at 95°C for 7 min. Translocated core-gpαf was quantified by Con A precipitation and scintillation counting. This translocation assay is linear in the range of membrane concentrations used.

Results

Review of In Vitro Reaction

Protein transport from the ER was reconstituted in gently lysed yeast spheroplasts (membranes) using radiolabeled [35S]pre-pro-alpha-factor (pporf) as a marker secretory protein (Baker et al., 1988). The marker is posttranslationally translocated into the lumen of the ER during a 15 min incubation at 10°C (stage I). Once in the ER lumen, pro-alpha-factor...
is glycosylated with three N-linked core-carbohydrate chains. Membranes are separated from untranslocated precursor by centrifugation, then incubated at 20°C with a cytosol fraction, an ATP-regenerating system, and GDP-mannose (stage II). During this stage, core-glycosylated pro-alpha-factor (core-gpaf) is transported to the Golgi apparatus where it is further modified with “outer-chain” mannose residues in α1,6 linkage. All glycosylated forms of pro-alpha-factor (gpaf) bind to the plant lectin Con A whereas all outer-chain modified forms of gpaf bind to antibodies specific for α1,6 mannose linkages (Franzusoff and Schekman, 1989). Transport efficiency is expressed as the ratio of radiolabeled gpaf precipitated with outer-chain antibodies to total gpaf precipitated with Con A. The zero time point of the MSS (always <10% of maximum signal) was subtracted as background from the other MSS fractions to obtain the values shown.

Pro-alpha-factor is packaged into a Post-ER Compartment

Given the difference in sedimentation of the ER and Golgi compartments, we examined slowly sedimenting membrane fractions generated early in a transport incubation for evidence of a vesicular intermediate that contained core-gpaf. The production of slowly-sedimenting membranes containing gpaf (Fig. 1 A, top), and depletion from the pellet fraction (bottom), was monitored during a stage II incubation. To ensure that only membrane-enclosed gpaf was quantified, fractions were treated with trypsin to degrade material released by membrane rupture. gpaf was not inherently trypsin resistant because it was degraded when detergent was included to lyse membranes. Of the total gpaf, 45% was released to the supernatant fraction and of this, 40% received outer-chain carbohydrate. The appearance of gpaf in the supernatant fraction occurred with no lag whereas outer-chain gpaf began to accumulate only after 15 min. Thus, the two forms of gpaf appeared in the supernatant fraction with different kinetics, suggesting that a vesicle intermediate containing core-gpaf was released from the rapidly sedimentable membrane en route to the Golgi apparatus, located mostly in the supernatant fraction. SDS-PAGE and autoradiography of the material released early showed it to consist strictly of core-glycosylated gpaf (not shown).

The membrane-enclosed gpaf that was released was not derived by nonspecific fragmentation of the ER. A mock incubation lacking 35S-gpaf was fractionated at intervals and membranes in the supernatant and pellet fractions evaluated for the ability to form new gpaf (Fig. 1 B). Essentially all (>95%) of the translocation activity, a marker of ER, was preserved and continued to sediment in the pellet fraction. Furthermore, release of membrane-enclosed gpaf into the supernatant fraction required cytosol and ATP at an optimum temperature range between 20 and 29°C (Fig. 2) indicative of a specific cellular process.

Fig. 3 is a cartoon of the in vitro transport reaction. The
Figure 2. Requirements for the release of pro-alpha-factor in a slowly sedimenting compartment. After stage I, membranes were washed twice and aliquoted into tubes that contained the indicated components. To the tubes containing no ATP, apyrase (0.25 U) was added to hydrolyse residual ATP; these tubes were supplemented with 50 μM GDP-mannose, normally added in the ATP regeneration mix. The reaction volumes were adjusted to 25 μl with reaction buffer. After 75-min incubations at different temperatures, each sample was fractionated, protease-treated and processed as described in Fig. 1A.

ER remains associated with the broken cells and sediments rapidly (MSP) in a microcentrifuge while ER-derived vesicles are released from the cells before they target and fuse with the Golgi apparatus which also fractionates mostly in the supernatant (MSS) fraction. To establish the authenticity of this model temperature-sensitive Sec proteins and chemical inhibitors were tested for their ability to block differentially these reactions. For convenience, the results are summarized in the diagram.

**Distinct Requirements for Vesicle Budding and Fusion**

To distinguish the processes that generated slowly sedimenting membranes that contained core- and outer-chain forms of gpαf, we evaluated their appearance in lysates of sec mutant strains that are defective in the production or consumption of ER-derived transport vesicles (Kaiser and Schekman, 1990). The kinetics of release of membrane-enclosed gpαf (Fig. 4, circles) and formation of outer-chain-gpαf (Fig. 4, triangles) was monitored at 20 and 29°C, temperatures judged to be permissive and restrictive, respectively, for sec12, sec23, and sec18 lysates. The formation of outer-chain-gpαf proceeded more slowly at 29°C than at 20°C in wild-type lysates (Fig. 4A, right). In sec12 and sec23 lysates this transport was reduced by 75%, and in sec18 by 50% (Fig. 4, B–D, right). Although higher temperatures further reduced the extent of transport in mutant lysates, reactions with wild-type components also were less efficient.

Release of membrane-enclosed gpαf into the supernatant fraction was also more rapid at 29°C than at 20°C in wild-type lysates (Fig. 4A, left). No defect in the release was detected in incubations containing sec18 membranes and cytosol (Fig. 4D, left). sec12 and sec23 lysates showed a considerable reduction, 75% and 60% respectively, in the final extent of release of gpαf at 29°C relative to 20°C (Fig. 4, B and C, left). Some of the gpαf released early in sec12 incubations was returned to a rapidly sedimenting form. The initial rate of gpαf release in sec12 and sec23 lysates was not reduced, perhaps reflecting a lag in the inactivation of the mutant protein. Unfortunately, preincubation of mutant or wild-type membranes at 29°C under conditions of no budding (e.g., no ATP) caused membranes to lose transport activity. Taken together, these results suggest that Sec12p and Sec23p are required to generate a slowly sedimenting membrane that contains core-gpαf whereas Sec18p is required for the transfer of this species to the compartment that assembles outer-chain carbohydrate.

Differential sedimentation analysis was also used to examine the effect of chemical inhibitors of transport on the generation and consumption of the slowly sedimenting membranes that contained core-gpαf. Formation of outer-chain gpαf was inhibited by Fab fragments of an antibody raised against Ypt1p (Baker et al., 1990), by chelation of Ca²⁺ with EGTA, by treatment of membranes and cytosol with NEM, and by incubation in the presence of GTPγS (Fig. 5, right panels). In each case, inhibition was prevented by a specific antidote (Fig. 5A, excess Ypt1p; B, Ca²⁺; C, DTT; D, GTP). The generation of slowly sedimenting membranes containing gpαf was not affected by the first three inhibitors. GTPγS, however, reduced by 94% the initial rate of production of this slowly sedimenting species, but reduced the final extent of production by only 40%. Taken together, these results suggest that Ypt1p, Ca²⁺, and an NEM-sensitive protein perform roles in the consumption but not formation of a putative vesicular intermediate in protein transport from the ER. GTP hydrolysis, however, seems to be important both in the formation and consumption of diffusible vesicles.

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Transport Blocks Cause the Accumulation of ER-derived Vesicles

A further distinction among the various "fusion" transport blocks was revealed by velocity sedimentation in sucrose gradients of membranes in the supernatant fractions from inhibited reactions. Gradient separation was designed to test whether the membranes that contained core-gpaf were attached or separate from the membranes that contained outer-chain gpaf. Membranes were distinguished by their content of core-gpaf (Con A-precipitable $[^{35}\text{S}]$-gpaf corrected by subtraction of outer-chain antibody precipitable $[^{35}\text{S}]$-gpaf) or outer-chain gpaf. Membranes that contained outer-chain gpaf represent a compartment of the Golgi apparatus (see review of in vitro reaction).

Membranes collected in the supernatant fraction of a 20°C incubation of wild-type components sedimented fast in a 15–45% (wt/wt) sucrose gradient, approaching an equilibrium density in fraction 30 (Fig. 6 A). The pattern of sedimentation of core- (Fig. 6 A, open circles) and outer-chain gpaf (closed triangles) were superimposed with a slightly greater fraction of the core-gpaf in the more slowly sedimenting fractions. Calcium chelation, which blocked formation of outer-chain gpaf, nevertheless produced core-gpaf-containing membranes that sedimented to the position of the Golgi compartment (Fig. 6 B).

A different spectrum of membranes was produced with other inhibitors. Membranes collected in the supernatant fraction when transport was blocked with anti-Ypt1p Fas, sedimented slowly, centered about a peak at fraction 17 (Fig. 6 C). A similar pattern (peak fraction at 19) was seen with NEM-treated membranes and cytosol (Fig. 6 E). In this case, residual outer-chain gpaf produced in spite of the NEM treatment sedimented near the bottom of the gradient as in a normal reaction.

The production of slowly sedimenting membranes that contained core-gpaf was delayed but not dramatically reduced by incubation with GTPyS (Fig. 5). After a 90-min
incubation, the released material sedimented on a sucrose gradient to the same position as untreated membranes (data not shown). However, treatment of membranes with 0.9 M KCl for 5 min at 20°C before centrifugation caused the membranes to sediment more slowly as a uniform peak centered around fraction 17 (Fig. 6D). GTPγS-inhibited membranes may accumulate as aggregates that are dispersed by increased ionic strength, or as vesicles bound to the Golgi apparatus through electrostatic interactions. The pattern of sedimentation of membranes generated in uninhibited reactions or in Ca2+-depleted reactions was not affected by incubation with 0.9 M KCL before centrifugation (data not shown).

Membranes and cytosol prepared from sec18 were incubated at 29°C and compared to wild-type fractions treated similarly. Under these conditions the mutant reaction was only 50% blocked (Fig. 4D), however a large difference in the fraction of core-gpaF sedimenting slowly in the sucrose gradient was observed (Fig. 6, F and G). A peak of core-gpaF, centered at fraction 15, accumulated at 29°C in incubations of the mutant lysate. Outer-chain-gpaF, produced because the mutant block was not completely restrictive, sedimented at the normal position near the bottom of the gradient.

The results of sedimentation analysis showed that a slowly sedimenting vesicle, distinct from the Golgi compartment in which outer-chain is assembled, accumulates when the function of Ypt1p, Sec18p, or an NEM-sensitive component(s) is inactivated. Although the rate of production of this species is greatly reduced by incubation in the presence of GTPγS, a vesicle that is distinct from the Golgi compartment also accumulates. Ca2+ is required at a later step, after the vesicle intermediate adheres to the Golgi compartment.

**Consumption of Transport Intermediates**

Completion of transport, measured by formation of outer-chain-gpaF, was detected when the core-gpaF-containing membranes accumulated in the presence of Ypt1p antibody Fα, fragments or EGTA were incubated with fresh components. Membranes in the supernatant fraction of reactions performed in the presence of these inhibitors were centrifuged at 88,000 g for 15 min to create high speed pellet fractions devoid of soluble components. Membrane pellets were then
Figure 6. ER-derived vesicles and the Golgi apparatus are resolved by velocity sedimentation in sucrose gradients. Scaled-up MSS fractions obtained from stage II reactions that were blocked in vesicle fusion were analyzed by velocity sedimentation in sucrose gradients. The MSS fractions were treated with salt (0.9 M KCl final concentration) or not before sedimentation (see text). Fractions were collected from the top. The density and the gpαf content of every other fraction were quantified as described in Materials and Methods. Open circles represent core-gpαf. Closed triangles represent outer-chain modified gpαf. The black dots represent percent (wt/wt) sucrose. Cytosolic proteins did not enter the gradient and remained in the top three fractions.

resuspended in reaction buffer and incubated with various additions (stage III incubation). Optimum stage III transport from the Yptlp-Fab intermediate was achieved at 20°C in the presence of fresh cytosol and membranes, and required ATP (not shown). Formation of outer-chain-gpαf was more rapid and efficient (34% vs. 26%) starting from this intermediate than in a typical stage II incubation starting from the ER/10°C intermediate (Fig. 7).

Transport from the Ca²⁺-requiring intermediate required ATP, and was optimal at temperatures between 20 and 29°C in the presence of Ca²⁺. Transport was not stimulated by the addition of fresh membranes or cytosol (not shown). Consumption of this intermediate at 20°C was fast and reached 40% efficiency (Fig. 7). The typical transport lag phase seen in stage II incubations was absent in stage III incubations (Fig. 7).

Consumption of the transport intermediates was also tested for Sec protein dependence and sensitivity to chemical inhibitors. Completion of transport from the Yptlp-Fab intermediate was blocked by fresh addition of antibody frag-
Figure 8. Chase of Yptlp-Fβ (A and B) and Ca²⁺-requiring (C and D) transport intermediates, in the presence of chemical inhibitors or Sec mutant proteins. (A) A Yptlp-Fβ intermediate/HSP fraction generated in a wild-type lysate at 20°C was resuspended with ATP, cytosol, and fresh membranes and incubated in the presence of various chemical inhibitors or treated with NEM before the chase period. The concentration of inhibitors and the NEM treatment are the same as described for Fig. 5. The reactions were terminated and processed as described in Fig. 7. The results are plotted as percent of maximum transport. Maximum transport was obtained in a parallel chase without inhibition (not shown). Background transport from stage II was subtracted to obtain the values plotted. (B) Yptlp-Fβ intermediate/HSP fractions were generated in wild-type and mutant lysates at 20°C and were mixed with ATP, cytosol, and fresh membranes from each mutant, respectively. The required components were pretreated for 10 min at 20 or 29°C before mixing with the HSP fractions. Pretreatment of the HSP fractions before mixing with the required components yielded the same results. The reactions were performed at 20°C for 65 min and processed as described in A. Transport at 20°C represents maximum transport. Background transport was corrected as above. (C) A Ca²⁺-requiring intermediate/HSP fraction generated in a wild-type lysate at 20°C was resuspended in EGTA/Mn²⁺ buffer and aliquoted to tubes containing ATP, calcium (250 μM), and the various inhibitors. Calcium was omitted where indicated. The concentration of inhibitors and NEM treatment are the same as described in Fig. 5. The chase incubations were performed at 20°C during 75 min and processed as described in A. Maximum transport was obtained in a parallel chase without inhibition (not shown). Background transport was corrected as above. (D) Ca²⁺-requiring intermediate/HSP fractions were generated in wild-type and mutant lysates at 20°C and resuspended with EGTA/Mn²⁺ buffer containing ATP. The tubes were incubated at 20 or 29°C for 20 min and then calcium (250 μM) was added and the reactions were further incubated at 20 or 29°C for 55 min. Transport at 20°C represents maximum transport. Background was corrected as above.

Discussion

We have exploited a yeast cell-free protein transport reaction to define a vesicular intermediate that carries core-gpβf from the ER to the Golgi apparatus. The transport vesicles sediment distinctly from the ER and Golgi membranes, and are devoid of protein translocation or outer-chain carbohydrate modification activities which mark the ER and the Golgi membranes, respectively. We find that the formation of diffusive vesicles from the ER requires ATP, GTP hydrolysis, and cytosol, but does not require Ca²⁺ or an NEM-sensitive protein. Delivery of vesicle contents to the Golgi apparatus also requires ATP, cytosol, but in addition requires an NEM-sensitive protein(s) and Ca²⁺. Specific protein requirements were revealed by inhibition of Sec protein function. Sec2p, an integral ER membrane glycoprotein (Nakano et al., 1988), and Sec23p, a peripheral membrane protein (Hicke and Schekman, 1989), are required for vesicle budding; Sec1p, a cytosolic and peripheral membrane protein (Eakle et al., 1988), and Yptlp, cytosolic and on the surface of the Golgi apparatus (Molenaar et al., 1988; Segev et al., 1988), are required for vesicle targeting.

A summary of the findings from the cell-free reaction is shown in Fig. 3. This work demonstrates biochemically the existence of an intermediate compartment between the ER and the Golgi apparatus, assigns a novel role for Yptlp and Sec1p in vesicle targeting, and presents evidence for the role of multiple GTP-binding proteins in one round of vesicular transport.

Diffusible Transport Vesicles

Transport vesicles that accumulate in sec18 incubations or in the presence of inhibitors (Yptlp antibody Fβ fragments, GTPY/S, NEM treatment) sediment more slowly (>18S) than ER and Golgi membranes (>30S), marked respectively by translocation activity and content of outer-chain-gpβf. Could these vesicles represent a subcompartment of the ER devoid of translocation activity or a cis-compartment of the Golgi, rather than a transitional vesicle? Three arguments favor the transitional vesicle assignment. First, sec18 cells accumulate 50-nm vesicles in addition to ER tubules at the restrictive temperature (Kaiser and Schekman, 1990). These vesicles are intermediates in protein transport because their
appearance in vivo depends upon prior action of SEC genes required for vesicle formation. Incubation of sec18 lysates at the restrictive temperature produces slowly sedimenting membranes that contain core-gpaf and are devoid of translocation activity. Because the reaction conditions are not completely restrictive, some outer-chain gpaf is produced and accumulates within a compartment that sediments more rapidly (Fig. 6 G). Thus, the slowly sedimenting vesicle species that accumulates is not bound to the Golgi compartment defined by its content of outer-chain gpaf. Likewise, reactions inhibited by NEM treatment produce slowly sedimenting membranes as well as membranes that sediment fast and contain outer-chain gpaf. Second, it is unlikely that the vesicles represent a subcompartment of the ER because completion of transport from the slowly sedimenting intermediate does not require the function of Sec12p and Sec23p (Fig. 8) which are required for vesicle formation from the ER in vivo (Kaiser and Schekman, 1990) and in vitro (this work). Third, completion of transport from the vesicle intermediate proceeds efficiently in the presence of GTPyS. If the vesicle intermediate was a specialized region of the ER or a cis-compartment of the Golgi, completion of transport to the site of outer-chain addition would involve a complete cycle of vesicle budding and fusion and would likely be inhibited by GTPyS, an inhibitor of many reconstituted vesicle-mediated transport events (reviewed by Balch, 1989).

We suggest that Sec18 and Yptlp proteins function to promote or maintain the attachment of transport vesicles to the Golgi membrane since inhibition of either one results in the accumulation of ER-derived vesicles not firmly bound to the Golgi membranes. Given the selective localization of the members of the Yptl/Sec4 family of proteins (Chavrier et al., 1990) it is possible that Yptlp forms part of a specific targeting signal whereas Sec18p forms part of a more general membrane attachment machinery.

The conclusion that Sec18p functions in vesicle attachment differs somewhat from the role in membrane fusion proposed for its mammalian homologue, NSF (Malhotra et al., 1988). Malhotra et al. found that inactivation of NSF by NEM treatment leads to the accumulation of uncoated Golgi vesicles firmly bound to target Golgi cisternae. This is interpreted as an intermediate stage in vesicle fusion. However, unlike the situation we find with diffusible transport vesicles derived from the ER, vesicles that mediate transport within the Golgi complex seem always to be bound to a donor or acceptor cisterna. If one assumes that some cytosolic structure assures the local retention of Golgi-derived vesicles, then NSF may perform the docking function we propose for Sec18p.

Experiments designed to reveal the sequence of requirements for consumption of the vesicle intermediate show that the Ca²⁺-dependent step is late, after the Yptl/Sec18 step (Fig. 8). Reactions blocked by chelation of Ca²⁺ accumulate core-gpaf in a compartment that sediments to the same position as Golgi membranes (Fig. 6 B). Ca²⁺ chelation may cause the accumulation of vesicles firmly docked on the Golgi membrane, thus implicating Ca²⁺ in vesicle fusion but not targeting. Alternatively, Ca²⁺ chelation may prevent outer-chain glycosylation of core-gpaf in the lumen of the Golgi apparatus. The second possibility is difficult to reconcile with the observation that formation of outer-chain gpaf from the Ca²⁺-requiring intermediate requires ATP and is inhibited by NEM.

**GTP Binding Proteins**

At least two stages, vesicle release and targeting, require GTP hydrolysis or a small GTP binding protein in the cell-free transport reaction described here. The nonhydrolyzable analogue GTPyS introduces a 20–30-min lag phase in vesicle release (Fig. 5 D). This delay may reflect an inhibition of vesicle budding or may result from the production of vesicle aggregates which dissociate with time. Alternatively, GTPyS may slow the rate of packaging of core-gpaf into vesicles. Tooze and Huttner (1990) also reported that vesicle budding or release from the trans-Golgi network is inhibited by GTPyS.

Longer incubations in the presence of GTPyS result in significant vesicle release. Vesicles formed in the presence of the analogue are defective in delivering core-gpaf to the Golgi for outer-chain addition even after removal of GTPyS and addition of fresh components and GTP (not shown). These vesicles may be improperly formed and lack components necessary for fusion. Alternatively, GTP hydrolysis by a distinct GTP-binding protein may be required for vesicles to "mature" in preparation for fusion. GTPyS bound to this "maturation" protein would prevent subsequent fusion of the vesicle.

GTPyS was first shown to inhibit vesicular traffic in the Golgi apparatus (Malençon et al., 1987). A quantitative EM assay was used to show that GTPyS inhibits vesicle fusion. Inhibited reactions display numerous nonclathrin-coated vesicles which have completed budding and are loosely bound to target Golgi cisternae (Malhotra et al., 1989). Subsequent fusion requires uncoating before the action of NSF/Sec18p (Orci et al., 1989). These results were used to conclude that GTP hydrolysis is required only for vesicle uncoating. However, an inhibition or delay of budding by GTPyS may have been missed because of the difficulty of measuring bud formation at very early time points in the incubation.

Three different small GTP binding proteins have been implicated in protein transport from the ER in yeast. These are Yptlp (Segev et al., 1988; Bacon et al., 1989; Baker et al., 1990), Sarlp (Nakano and Muramatsu, 1989), and possibly Arflp (Stearns et al., 1990). Phenotypic analysis of mutant alleles of the three genes has not allowed assignment of roles in vesicular traffic for the members of this group.

A logical target of GTPyS in the budding reaction is Sarlp, a ras-like GTP binding protein whose gene was cloned as a multicopy suppressor of the sec12-1 mutation (Nakano and Muramatsu, 1989). Recent experiments suggest that Sarlp interacts directly with Sec12p (D'Enfert et al., 1991). As Sec12p is implicated in budding it seems possible that GTP binding or hydrolysis by Sarlp may facilitate some aspect of Sec12p function in the budding reaction. A process such as the recruitment of subunits of a coat structure could be facilitated by cycles of Sarlp-mediated GTP hydrolysis. GTP hydrolysis may accelerate, but not be required for the assembly of such a coat structure.

Another ras-like GTP-binding protein, Yptlp, acts at a distinct later step to promote targeting of vesicles to the Golgi membrane. Yptlp antibody F₆ fragments (Figs. 5 A and 6
Sec Protein Requirements for Vesicle Budding

Vesicle budding requires only a subset of the proteins necessary for an entire round of transport from the ER. Genetic and cytological characterization led to the proposal that an interacting group of gene products, Sec12p, Sec13p, Sec16p, and Sec23p are required for vesicle budding or some step before budding, and that Sec7p, Sec18p, and Sec22p perform a role in vesicle fusion (Kaiser and Schekman, 1990). We tested each of these mutants in vitro at 29°C, the highest temperature that sustains full transport with wild-type components. Only a subset of these mutants were sufficiently defective at this temperature to allow an evaluation of the nature of the block. Incubations containing sec12 or sec23 components show clear but delayed inhibition of the budding reaction (Fig. 4, B and C). This delay may simply reflect the time it takes for the mutant protein to become inactive at 29°C.

Sec12 mutant membranes display an unusual pattern of budding at 29°C. After an initial normal 20-min period of budding, rather than leveling off as observed in sec23 incubations, slowly sedimenting core-gpaf appears to be converted to a rapidly sedimenting form. This could mean that defective vesicles are produced during the first minutes of incubation that are then reassembled by the ER, or aggregate, when mutant Sec12p becomes inactive. sec12 mutant membranes did not lyse more readily than wild-type membranes that defectivvesicles are produced during the first minutes of incubation (Fig. 7: Fig. 8, A and B). Unlike the overall reaction, the reversal of the Yptlp antibody block is not affected by GTP-γ-S (Fig. 8 A). This implies that GTP hydrolysis by Yptlp is not required to complete at least one cycle of targeting mediated by this protein. In contrast to this conclusion, mutations that inactivate GTP hydrolysis by Yptlp are required for transport in vivo. The Golgi apparatus is defective in yptl mutant cells (Baker et al., 1988). Reconstitution of SEC gene product-dependent intercompartmental protein transport. Cell. 54:335-344.

References

Bacon, R. A. and Schekman, R. 1989. Calcium and GTP-essential components in vesicular trafficking between the endoplasmic reticulum and the Golgi apparatus. J. Cell Biol. 108:1245-1256.

Baker, D., M. Block, B. Glick, J. Rothman, and W. Balch. 1989. Vesicular transport between the endoplasmic reticulum and the Golgi stack requires the NEM-sensitive fusion protein. Nature (London). 339:397-398.

Block, M., B. Glick, C. Wilcox, F. Wieland, and J. Rothman. 1988. Purification of an N-ethylmaleimide-sensitive protein catalyzing vesicular transport. Proc. Natl. Acad. Sci. USA. 85:7852-7856.

Chavrier, P., R. Parton, H. Hauri, K. Simons, and M. Zerial. 1990. Localization of low molecular weight GTP binding proteins to exocytic and endocytic compartments. Cell. 62:317-329.

D’Enfert, C., L. Wuestebube, and R. Schekman. 1991. Sec2p-dependent membrane binding of the small GTP-binding protein Sar1p promotes formation of transport vesicles from the ER. J. Cell Biol. In press.

Eakle, K., M. Bernstein, and S. Emr. 1988. Characterization of a component of the yeast secretory machinery: identification of the SEC18 gene product. Mol. Cell. Biol. 8:4098-4109.

Franzusoff, A., and R. Schekman. 1989. Functional compartments of the yeast Golgi apparatus are defined by the sec7 mutation. EMBO (Eur. Mol. Biol. Organ.) J. 8:2695-2702.

Gallwitz, D., C. Donath, and C. Sander. 1983. A yeast gene encoding a protein homologous to the human C- has/bas proto-oncogene product. Nature (London). 306:704-707.

Gieresch, M., H. Huhola, R. Bacon, G. Rossi, and S. Ferro-Novick. 1990. Isolation of a functional vesicular intermediate that mediates ER to Golgi transport in yeast. J. Cell Biol. 111:45-53.

Hickman, L., and R. Schekman. 1989. Yeast Sec23p acts in the cytoplasm to promote protein transport from the endoplasmic reticulum to the Golgi complex in vivo and in vitro. EMBO (Eur. Mol. Biol. Organ.) J., 8,(6), 1677-1684.

Jamieson, J., and G. Palade. 1967. Intracellular transport of secretory proteins in the pancreas exocrine cell. J. Cell Biol. 34:577-596.

Kaiser, C., and R. Schekman. 1990. Distinct set of SEC genes govern transport vesicle formation and fusion early in the secretory pathway. Cell. 61:723-737.

Lowry, O. H., N. J. Rosebrough, A. L. Farr, and R. J. Randall. 1952. Protein measurement with the Folin phenol reagent. J. Biol. Chem. 193:265-275.

Malhotra, V., L. Orci, B. Glick, M. Block, and J. Rothman. 1988. Role of an N-ethylmaleimide-sensitive transport component in promoting fusion of transport vesicles with cisternae of the Golgi stack. Cell. 54:221-227.

Malhotra, V., T. Serafini, L. Orci, J. Shepherd, and J. Rothman. 1989. Purification of a novel class of coated vesicles mediating biosynthetic protein transport through the Golgi stack. Cell. 58:329-336.

Melaon, P., B. Glick, V. Malhotra, D. Weidmann, T. Serafini, M. Gleason, L. Orci, and J. Rothman. 1987. Involvement of GTP-binding "G" proteins in transport through the Golgi stack. Cell. 51:1053-1062.

Moberg, F., R. Prange, and D. Gallwitz, 1985. A carboxyl-terminal cysteine residue is required for palmitic acid binding and biological activity of the ras-related yeast YPT1 protein. EMBO (Eur. Mol. Biol. Organ.) J. 7:971-976.

Nakano, A., and M. Muramatsu. 1989. A novel GTP-binding protein, Sar1p, is involved in protein transport from the endoplasmic reticulum to the Golgi apparatus. J. Cell Biol. 109:2677-2691.

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tation groups required for post-translational events in the yeast secretory pathway. Cell. 21:205–215.
Novick, P., S. Ferro, and R. Schekman. 1981. Order of events in the yeast secretory pathway. Cell. 25:461–469.
Orci, L., V. Malhotra, M. Amherdt, T. Serafini, and J. Rothman. 1989. Dissection of a single round of vesicular transport: sequential intermediates for intercisernal movement in the Golgi stack. Cell. 56:357–368.
Palade, G. 1975. Intracellular aspects of the process of protein secretion. Science (Wash. DC). 189:347–358.
Paulik, M., D. D. Nowack, and D. J. Morré. 1988. Isolation of a vesicular intermediate in cell-free transfer of membrane from transitional elements of the endoplasmic reticulum to Golgi apparatus cisternae of rat liver. J. Biol. Chem. 263:17738–17748.
Robertson, S., and J. Potter. 1984. The regulation of free Ca$^{2+}$ ion concentration by metal chelators. Methods Pharmacol. 5:63–71.
Ruohola, H., A. Kabacem, and S. Ferro-Novick. 1988. Reconstitution of protein transport from the endoplasmic reticulum to the Golgi complex in yeast: the acceptor Golgi compartment is defective in the sec23 mutant. J. Cell Biol. 107:1465–1476.
Schmitt, H., P. Wagner, E. Pfaff, and D. Gallwitz. 1986. The ras-related YPT1 gene product in yeast: a GTP-binding protein that might be involved in microtubule organization. Cell. 47:401–412.
Schmitt, H., M. Puzicha, and D. Gallwitz. 1988. Study of a temperature sensitive mutant of the ras-related YPT1 gene product in yeast suggests a role in the regulation of intracellular calcium. Cell. 53:635–647.
Segev, N., J. Mulholland, and D. Botstein. 1988. The yeast GTP-binding YPT1 protein and a mammalian counterpart are associated with the secretory machinery. Cell. 52:915–924.
Stearns, T., M. Willingham, D. Botstein, and R. Kahn. 1990. ADP-ribosylation factor is functionally and physically associated with the Golgi complex. Proc. Natl. Acad. Sci. USA. 87:1238–1242.
Tooze, S. A., U. Weiss, and W. B. Huttner. 1990. Requirement for GTP hydrolysis in the formation of secretory vesicles. Nature (Lond.). 347:207–208.
Wilson, D., C. Wilcox, G. Flynn, E. Chen, W. Kuang, W. Hanzel, M. Block, A. Ullrich, and J. Rothman. 1989. A fusion protein required for vesicle-mediated transport in both mammalian cells and yeast. Nature (Lond.). 339:355–359.