Isolation of a Functional Vesicular Intermediate that Mediates ER to Golgi Transport in Yeast

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Abstract. We have used an in vitro assay that reconstitutes transport from the ER to the Golgi complex in yeast to identify a functional vesicular intermediate in transit to the Golgi apparatus. Permeabilized yeast cells, which serve as the donor in this assay, release a homogeneous population of vesicles that are biochemically distinct from the donor ER fraction. The isolated vesicles, containing a post-ER/pre-Golgi form of the marker protein pro-α-factor, were able to bind to and fuse with exogenously added Golgi membranes. The ability to isolate fusion competent vesicles provides direct evidence that ER to Golgi membrane transport is mediated by a discrete population of vesicular carriers.

CARRIER vesicles have been proposed to mediate the transport of macromolecules from the rough endoplasmic reticulum (ER) to the *cis*-Golgi complex (Jamieson and Palade, 1967; Palade, 1975). However, direct evidence for the existence of this vesicular compartment has not been obtained. Various approaches have been used to identify and isolate a potential intermediate in transit to the Golgi complex. Electron microscopic analysis and physiological studies have shown that low temperatures inhibit transport between the ER and Golgi apparatus in mammalian cells (Saraste and Kuismanen, 1984, Saraste et al., 1986; Tartakoff, 1986). At 15°C, the Golgi complex vesicularizes and proteins in transit to this compartment accumulate in pre-Golgi vacuoles. To enrich for vesicles that are likely to mediate transport at this stage of the pathway, methods such as the fractionation of membranes which have been labeled kinetically in vivo (Lodish et al., 1987) and preparative free-flow electrophoresis (Paulik et al., 1988) have also been used. Further studies or other approaches will be needed to obtain more highly enriched vesicle fractions.

Coated vesicles involved in intra-Golgi membrane traffic (Malhotra et al., 1989) and secretory vesicles that transport proteins from the *trans*-Golgi network (de Curtis and Simons, 1989) can be formed in vitro. This approach has permitted the identification and extensive purification of intermediate compartments that act at two distinct steps of the pathway. A genetic approach has also been employed in the yeast *Saccharomyces cerevisiae* to isolate post-Golgi secretory vesicles. Temperature-sensitive mutants defective for transport at this stage of the pathway accumulate vesicles that fail to deliver their contents to the plasma membrane (Novick et al., 1981). The yeast post-Golgi secretory mutants have made it feasible to trap an intermediate that is normally present in low amounts in the cell, facilitating the purification of secretory vesicles (Walworth and Novick, 1987; Holcomb et al., 1987). Although these approaches have led to the isolation of highly purified vesicle fractions, these vesicular compartments were not shown to be functional for transport and as such could not be used to address the mechanism of vesicle consumption.

In an effort to characterize the mechanism by which proteins are transported from the ER to the Golgi complex we have reconstituted these events in vitro (Ruohola et al., 1988). A radiolabeled precursor of the secreted pheromone α-factor is used as a marker protein in our assay. This 19-kD marker protein is translated in vitro with a yeast lysate and translocated into a donor compartment (the ER) retained within permeabilized yeast cells (PYC).[1] After translocation and the addition of N-linked oligosaccharides, pro-α-factor (26 kD) is transported to functional acceptor Golgi membranes, added exogenously to the cells, where it is further glycosylated to form a high molecular mass species. In this report we describe the use of this in vitro transport assay to isolated vesicles which have budded from the ER and are competent to fuse with the Golgi complex. The isolation of fusion competent vesicles provides direct evidence that carrier vesicles mediate transport at this stage of the pathway. This approach has enabled us to address the requirements for vesicle formation and utilization.

Materials and Methods

Growth Conditions and Strains

The yeast strains used in this study were: wild type, SFNY26-6A (MAT *a*, *his4-619*) and JRY 1239 (MAT *a*, *HMG1*, *HMG2*, *ura3-52*, *his3Δ200*, *ade2-101*, *lys2-801*, *met* + pJR59 (*HMG1 URA3* 2μ; YEp24 derivative).

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1. *Abbreviation used in this paper*: PYC, permeabilized yeast cells.

Cells used for the preparation of donor PYC, cytosol, and membrane fractions were grown overnight at 25°C in YPD medium (1% yeast extract, 2% Bactopeptone, and 2% glucose) to early log phase (OD_{599} = 1.5–4). JRY 1239 was grown in Wickerham's minimal medium (1946) supplemented with histidine, adenine, lysine, and methionine to an OD_{599} = 1.0. To change growth medium, cells were harvested in a clinical centrifuge at room temperature and then resuspended in new medium. Cell densities were measured in a 1-cm cuvette in a spectrophotometer (ultrospec plus; LKB Instruments, Gaithersburg, MD).

One-step In Vitro Transport Assay

All reagents used were obtained from Sigma Chemical Co., St. Louis, MO unless otherwise indicated. Prepro-α-factor was translated in vitro in a yeast translation lysate according to the protocol of Hansen et al. (1986) with minor modifications. Permeabilized yeast cells, donor cells, and S3 fraction were prepared as described before (Ruohola et al., 1988). The cytosolic fraction (also referred to as high speed supernatant or HSS) and the crude membrane fraction containing the functional acceptor compartment (also referred to as high speed pellet or HSP) were prepared from an S3 fraction centrifuged at 120,000 *g* for 1 h (36,000 rpm in a rotor; SW50; Beckman Instruments, Inc., Palo Alto, CA). Regenerated spheroplasts, used to prepare the components required for the assay, were used immediately or stored overnight on ice as a packed pellet. Protein was measured by the method of Bradford (1976) using ovalbumin as a protein standard.

The transport assay was usually performed in a total volume of 55–82 μl: 25 μl of donor PYC (60 μg of protein), 26–50 μl of an S3 fraction (1.0 mg of protein), 26–50 μl of a cytosolic fraction (0.7–0.8 mg of protein), 26–50 μl of a crude membrane fraction (0.2–0.4 mg of protein), 0.9 mM GDP-mannose, and an ATP-regenerating system (1 mM ATP, 0.1 mM GTP, 20 mM creatine phosphate, 0.2 mg/ml creatine phosphate kinase). Apyrase was used as described before (Ruohola et al., 1988). All components were incubated for varying amounts of time at 20°C. At the end of the assay, the donor PYC were centrifuged at room temperature for 23 s in a microfuge (Fisher Scientific Co., Pittsburgh, PA). The supernatant and pellet fractions were incubated with trypsin (0.47 mg/ml) for 20 min on ice. Trypsin inhibitor was added to each sample (1.88 mg/ml), the incubation was continued for 5 min, and the mix was heated at 100°C in the presence of 1% SDS.

Forms of pro-α-factor were precipitated with 2 μl of anti-α-factor antibody or by binding to Con A-Sepharose. To precipitate pro-α-factor with antibody, samples were diluted with 1 ml of PBS-Triton (12.5 mM sodium phosphate, 0.2 M NaCl, 2% Triton X-100) containing 1× protease inhibitor cocktail (Waters and Blobel, 1986) and incubated for 2 h at room temperature or overnight on ice in the presence of 2 μl of anti-α-factor antibody. The antigen-antibody complexes were precipitated by binding to protein A-Sepharose as described before (Newman and Ferro-Novick, 1987). The beads were washed two times with 1 ml of urea wash buffer (2 M urea, 200 mM NaCl, 1% Triton X-100, 100 mM Tris, pH 7.6) and two times with 1 ml of 1% β-mercaptoethanol. Pro-α-factor was solubilized from the beads in 70 μl of sample buffer. When Con A-Sepharose was used, the SDS-treated sample was diluted with 1 ml of high salt wash (500 mM NaCl, 1% Triton X-100, 20 mM Tris, pH 7.5) and then incubated for 2 h at room temperature with 90 μl of a 20% (vol/vol) solution of Con A-Sepharose. The beads were washed as described before (Bacon et al., 1989) except that each wash buffer was used only once. Forms of pro-α-factor were solubilized from the beads by heating in 70 μl of sample buffer. Solubilized samples were always analyzed in two ways: by electrophoresis in a 12.5% SDS-polyacrylamide slab gel and by liquid scintillation counting using a scintillation counter (LS 5000TD; Beckman Instruments, Inc.).

Anti-outer chain antibody was used to quantitate conversion of the 26-kD species to the high molecular mass form of pro-α-factor. This antibody was raised by injecting heat-killed wild-type yeast cells (X2180-1A and SFNY26-6A) into New Zealand white rabbits as described before (Ballou, 1970). Characterization of this antibody indicated that it specifically recognized the high molecular mass Golgi form of pro-α-factor (see Fig. 1 *A*) and not the 26-kD species.

To precipitate the high molecular mass Golgi form of pro-α-factor, 20 μl of the sample treated with either Con A-Sepharose or anti-α-factor antibody, was diluted (1:10) with PBS-Triton and incubated for 2 h at room temperature in the presence of 6 μl of anti-outer chain antibody. At the end of this incubation, samples were incubated with protein A-Sepharose and washed as before. Pro-α-factor was solubilized from the beads with 70 μl of sample buffer. The cpm reported in all figures were based on the counts obtained from 20 μl of a solubilized sample. The zero time point (which contained 10% or less of the total counts) was subtracted from all samples analyzed.

Two-step In Vitro Transport Assay

When the assay was performed in two steps, vesicles were released from cells during a 30- or 60-min incubation containing donor PYC, cytosol or a crude membrane fraction, and an ATP-regenerating system at 20°C. The supernatant fraction, containing vesicles, was separated from the donor compartment by centrifugation (23 s) in a microfuge (Fisher Scientific Co.) and was subsequently incubated with a crude membrane fraction during a second incubation (60–90 min) at 20°C. Samples were then processed as described above.

Gel Filtration of Vesicles Released from Donor Cells

A total of 10–11 assays (donor PYC, cytosol, and an ATP-regenerating system) were performed simultaneously at 20°C for 60 min. Afterwards, the donor cells were pelleted, and the vesicles released into the soluble fraction were separated from cytosol on a 13 ml Sephacryl S-300 column (Pharmacia LKB Biotechnology, Inc., Piscataway, NJ) that was equilibrated with transport buffer (115 mM potassium acetate, 2.5 mM magnesium chloride, 0.2 M sorbitol, 1× protease inhibitor [described by Waters and Blobel, 1986], and 1/40 vol of 1 M Hepes, pH 7.2). The column was calibrated using the following markers: blue dextran (2,000,000 D); BSA (66 kD); cytochrome c (12.4 kD); *p*-nitrophenol (139 D). Blue dextran eluted in the void volume (fraction 10), while BSA and cytochrome c were in fraction 17 and *p*-nitrophenol was in fractions 34 and 35. The vesicles eluted in the void volume (fractions 10 and 11) and were cleanly separated from the soluble markers. To approximate the ratio of vesicles to acceptor normally maintained in the reaction, the vesicle fractions were concentrated (1.25 times greater than the original volume) by centrifugation in a concentrator (centricon-10; Amicon Corp., Danvers, MA) for 1 h at 5,000 *g*. Approximately 40–50% of the vesicles were recovered after concentration.

Results

Vesicles Containing the 26 kD Pro-α-Factor Precursor Are Released from Donor PYC

When donor PYC are incubated with cytosol, ATP, and a crude membrane fraction that contains the acceptor compartment, the 26-kD pro-α-factor precursor exits the ER and is transported to the Golgi apparatus where it is terminally glycosylated. During this incubation, a fraction of the 26-kD species is released from the donor cells (Ruohola et al., 1988). If the pro-α-factor precursor that is released from the cells is contained within vesicles that are derived from the ER, it should pellet during high speed centrifugation and be resistant to digestion by protease. To address these points, donor PYC, cytosol, and ATP were incubated and then briefly sedimented at 11,000 *g*. The pro-α-factor remaining in the supernatant was subjected to centrifugation at ~120,000 *g* for 1 h. Aliquots of the resulting supernatant and pellet were treated with or without trypsin and then precipitated with the solid phase lectin adsorbant Con A-Sepharose. The data in Fig. 1 indicate that most of the Con A-precipitable counts were pelletable and resistant to digestion by trypsin in the absence of detergent. The small fraction of Con A-precipitable counts that remained in the supernatant were largely sensitive to protease digestion. These findings indicate that the majority of pro-α-factor released from cells during the transport reaction is protected by a sealed membrane.

To determine whether pro-α-factor released from donor cells is contained within a homogeneous population of membranes, the putative transport vesicles were examined using sucrose gradients. The appearance of heterogeneous membrane species containing 26-kD pro-α-factor might suggest that the vesicles are contaminated with artifactually vesicularized ER membranes released from donor cells.

The vesicles were first analyzed on an equilibrium sucrose

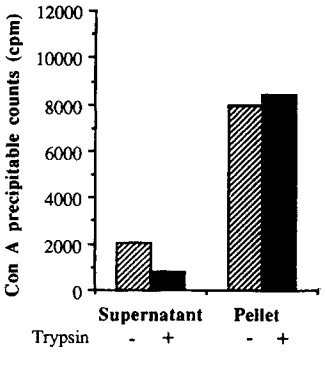

Figure 1. The 26-kD pro-α-factor precursor released from the donor PYC is contained within a sealed membrane compartment. Donor PYC were incubated with cytosol and ATP at 20°C. Afterwards, the cells were centrifuged in a microfuge (23 s) and the pro-α-factor remaining in the supernatant was recentrifuged at 120,000 *g* for 1 h. Pro-α-factor in the supernatant and in the pellet was treated with or without trypsin as described in Materials and Methods and then precipitated with Con A–Sepharose.

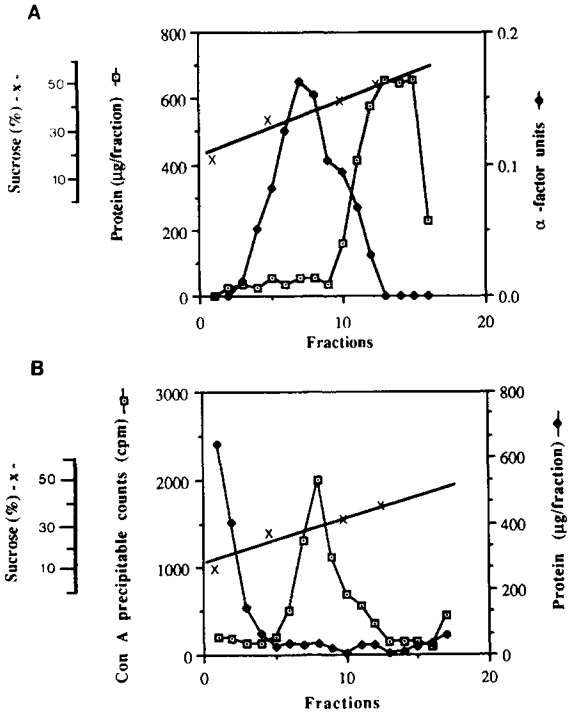

Figure 2. Sedimentation analysis of the vesicles released from donor PYC. (*A*) A total of 11 (55-μl each) transport reactions (donor PYC, cytosol, and an ATP-regenerating system) were performed simultaneously at 20°C for 20 min. At the end of the transport assay, the PYC were pelleted and the supernatant (350 μl) was resuspended in 1.2 ml of 60% (wt/wt) sucrose buffered with 10 mM Hepes, pH 7.2. The sample was placed at the bottom of a 5-ml ultraclear centrifuge tube (Beckman Instruments, Inc.) and overlaid with the following solutions of sucrose: 1 ml 40% (wt/wt), 1 ml 30% (wt/wt), and 1 ml 20% (wt/wt). The gradient was centrifuged in a SW50.1 rotor for 15 h at 120,000 *g* and then fractionated from the top (fraction volume ~300 μl). A portion of each fraction (50 μl) was treated with trypsin (10 μg for 20 min at 0°C) and then with trypsin inhibitor (40 μg for 5 min at 0°C). Samples were heated at 100°C in 1% SDS. Pro-α-factor was precipitated from each fraction with anti–α-factor antibody and quantitated by densitometric scanning of autoradiograms. Protein was measured by the method of Bradford (1976). The density of sucrose in each fraction was measured by refractometry. (*B*) Vesicles were formed with the conditions described in *A*. A portion of the supernatant (200 μl) was layered on top of a 10–50% (wt/wt) linear 5-ml sucrose gradient prepared in 10 mM Hepes, pH 7.2. The gradient was centrifuged in a SW50.1 rotor at 40,000 rpm for 140 min and fractionated (fraction volume ~300 μl) from the top. The location of vesicles in each fraction was determined as described in *A* except that Con A–Sepharose was used to precipitate the 26-kD species from each fraction.

gradient. The migration of vesicles in this gradient was assessed by assaying fractions for trypsin-resistant pro-α-factor (26 kD). Vesicles were resuspended in 60% sucrose, placed at the bottom of a centrifuge tube, and overlaid with sucrose solutions of decreasing concentration: 40, 30, and 20%. After a 15-h centrifugation at ~120,000 *g*, the vesicles had migrated as a single peak at the same density as 40% sucrose (1.1764 g/cm³) (Fig. 2 *A*). We noted that the vesicles released from the PYC during the assay are less dense than the yeast ER membrane, which pellets in 40% sucrose (Ruohola and Ferro-Novick, 1987). When the vesicles were subjected to centrifugation (~150,000 *g* for 140 min) through a 10–50% linear sucrose gradient, they sedimented at 28–30% sucrose (Fig. 2 *B*). Thus, under different gradient and centrifugation conditions, the vesicles still migrate as a single population. These findings suggest that only one membrane species, which contains the 26-kD form of pro-α-factor, is released from donor cells during the assay.

Vesicles Containing the 26-kD Precursor Form of Pro-α-Factor Are Intermediates in Transport

We hypothesized that the 26-kD precursor of pro-α-factor may be contained within a vesicular compartment that mediates transport from the ER to the Golgi complex. Accordingly, vesicles containing the 26-kD species must be released from the permeabilized cells before transport to the Golgi complex can occur. To test this proposal, the kinetics of pro-α-factor transport were monitored. Donor PYC were incubated with cytosol and the acceptor compartment for various times at 20°C. Afterwards, the permeabilized cells were removed by centrifugation and the supernatant was assayed for both the 26-kD and high molecular mass Golgi species by precipitation with anti-α-factor antibody. Antibody directed against outer chain carbohydrate, which is added to yeast glycoproteins only in the Golgi complex (Kukuruzinska et al., 1987), was used in a second immunoprecipitation. This antibody precipitates the high molecular mass species, but not the 26-kD form of pro-α-factor (see Fig. 3 *A*). The results in Fig. 3 *B* indicate that the time course of outer chain addition to pro-α-factor can be divided into three phases: a lag period (~10 min), a linear phase, and a plateau phase (Fig. 3 *B*). Most of the anti-α-factor precipitable counts were released rapidly from the donor PYC before completion of the lag in outer chain addition, and release was not dependent upon the presence of functional acceptor membranes (compare Fig. 3, *B* and *C*). These findings indicate that the 26-kD form of pro-α-factor is released from donor PYC before the high molecular mass Golgi species is detected.

We reported in an earlier study (Bacon et al., 1989) that the 26-kD form of pro-α-factor is processed to a 28-kD species before it is converted to the high molecular mass form. Conversion to 28-kD pro-α-factor requires the presence of the acceptor fraction (Bacon et al., 1989), suggesting that the 28-kD species resides in a post-ER compartment that

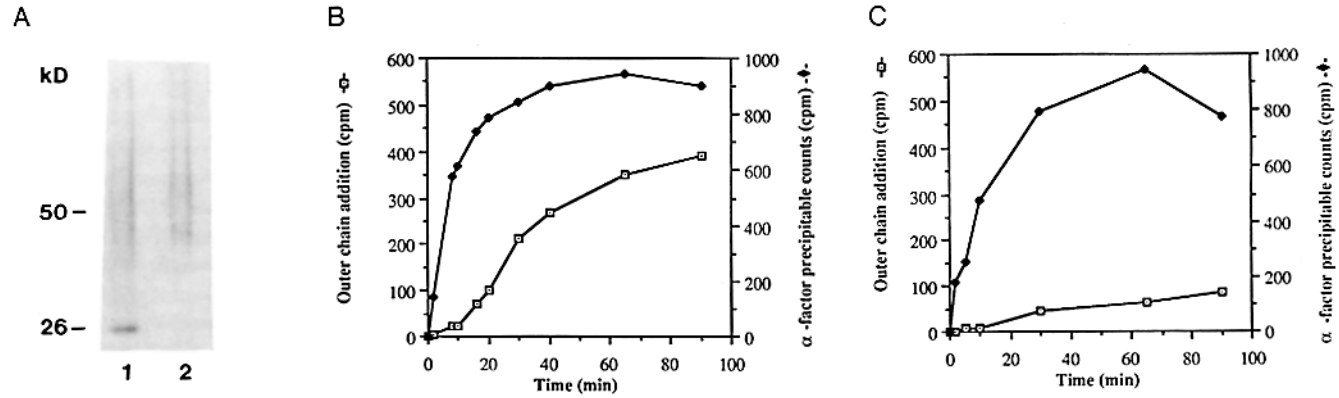

Figure 3. Vesicles are released from the donor PYC before the high molecular mass Golgi species is detected. (*A*) The donor PYC were incubated for 90 min at 20°C in the presence of a supernatant of a yeast lysate centrifuged at 3,000 *g* (S3 fraction). At the end of the incubation, the cells were pelleted in a microfuge and the supernatant was treated with Con A–Sepharose (lane *1*). The Con A–precipitable material was then reprecipitated with anti–outer-chain antibody as described in Materials and Methods (lane 2). Anti–outer-chain antibody precipitated the highest molecular mass forms of pro-α-factor and not the 26-kD form. (*B*) The donor compartment was incubated with an S3 fraction for various times at 20°C. At each time point, the donor PYC were pelleted and the supernatant was treated with trypsin (0.47 mg/ml) during a 20-min incubation on ice. Trypsin inhibitor (1.88 mg/ml) was added and after 5 min the sample was boiled in the presence of 1% SDS. Trypsin-resistant pro-α-factor that remained in the supernatant was first precipitated with anti–α-factor antibody and then reprecipitated with anti–outer-chain antibody. The cpm reported were based on counts obtained from 20 μl of a solubilized sample that was quantitated by liquid scintillation counting. The findings shown in *B* and *C* were averaged from three experiments. The zero time point was subtracted from all samples analyzed; generally this sample contained 10% or less of the total counts. Outer-chain addition denotes the counts precipitated with anti–outer-chain antibody and α-factor-precipitable counts indicates the counts precipitated with anti–α-factor antibody. (*C*) The same as in *B* except that the donor PYC were incubated with cytosol (no functional acceptor compartment present) in the presence of an ATP-regenerating system.

may be an early subcompartment of the Golgi complex. A time course of this processing event is shown in Fig. 4. Initially, only 26-kD pro-α-factor and partially glycosylated forms of pro-α-factor (described in Ruohola et al., 1988) are seen outside the cells (Fig. 4, lane *1*). However, by 5 min (and before completion of the lag), the 28-kD species also appears (Fig. 4, lane *2*). Thus, 26-kD pro-α-factor is released from donor PYC before the appearance of the 28-kD form.

Requirements for Vesicle Formation and Release

The requirements for ER-derived transport vesicle formation were assessed quantitatively by monitoring release of pro-α-factor from donor-permeabilized yeast cells. Pro-α-factor, contained within a sealed membrane compartment, was released from cells when the donor compartment was incubated with cytosol and ATP at 20°C (Fig. 3 *C* and Fig. 5, lane *A*). Vesicle release was dependent upon the concentration of cytosol used in the experiment, increasing severalfold as the cytosol concentration was raised from 0.05 to 0.8 mg protein per reaction. If the cytosolic fraction was heated to 95°C for 15 min before its use in the assay, vesicle release was dramatically reduced (Fig. 5, lane *B*). Vesicle release failed to occur when the incubation was performed on ice (Newman, A., M. Groesch and S. Ferro-Novick, unpublished results). These findings imply that proteins in the cytosol are responsible for forming and releasing vesicles from donor-permeabilized cells in vitro. ATP was required for this event since apyrase, an enzyme that hydrolyzes ATP to ADP and P_i, blocked this process (Fig. 5, lane *C*). Therefore, vesicle release from donor-permeabilized cells requires

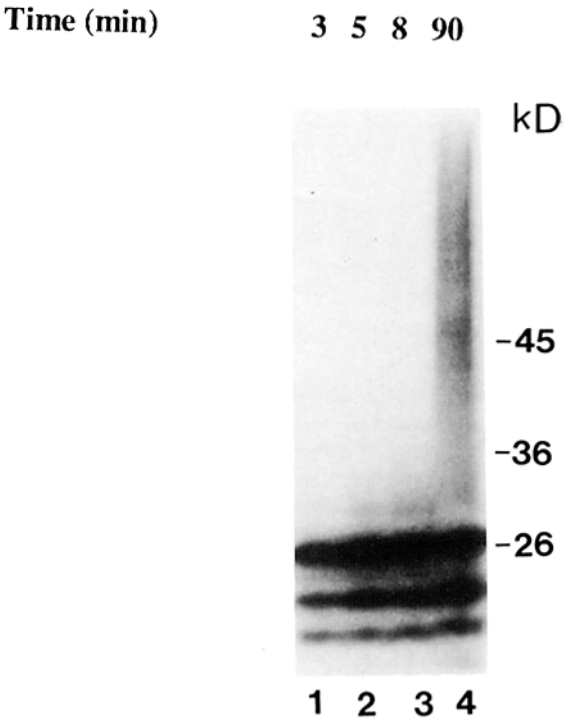

Figure 4. The 28-kD form of pro-α-factor appears during the first 10 min of the transport assay. Donor PYC were incubated at 20°C with cytosol, a crude membrane fraction that contains the acceptor compartment, and ATP for the indicated times. At each time point the cells were pelleted and the supernatant and pellets were treated as described in Materials and Methods. The gel of the supernatant is shown.

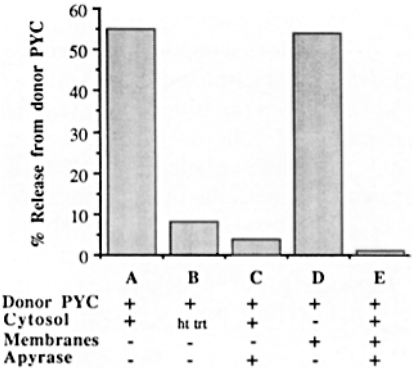

Figure 5. Requirements for vesicle formation and release. Donor-permeabilized cells were incubated for 90 min with cytosol and ATP (lane *A*), with heat-treated cytosol (15 min at 95°C and then centrifuged for 1 min in a microfuge [Fisher Scientific Co.]) and ATP (lane *B*), with cytosol and apyrase to deplete ATP (lane *C*), with a crude membrane fraction (lane *D*), or with cytosol, crude membranes, and apyrase (lane *E*). At the end of the assay, the donor PYC were pelleted and samples were treated as described in Materials and Methods. Vesicle release was measured as described below. The values presented in this figure are representative. The protein concentration of cytosol, as determined by the Bradford assay, was approximately 0.7–0.75 mg per reaction. % release = (Con A-precipitable counts in the supernatant − Con A-precipitable counts of the zero time point)/(total Con A-precipitable counts in the supernatant and pellet).

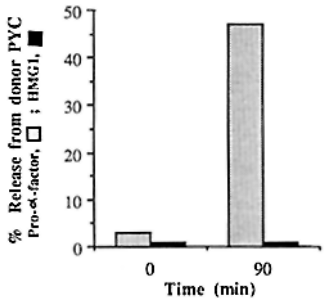

Figure 6. HMG-CoA reductase (HMG1) is not released into the vesicle fraction during the reaction. Parallel in vitro transport assays were performed using either radiolabeled or unlabeled pro-α-factor translocated into the ER of donor PYC prepared from JRY 1239, a strain that overproduces HMG1. Donor PYC containing radiolabeled pro-α-factor were incubated with an S3 fraction and ATP and vesicle release was measured as described for Fig. 4. The donor PYC translocated with unlabeled pro-α-factor were incubated with an S3 fraction (prepared from SFNY 26-6A) that does not contain detectable amounts of HMG1. The amount of HMG1 released from these donor cells was determined by Western blot analysis. The PYC were pelleted and the supernatant fraction was centrifuged at 120,000 *g* for 1 h, conditions that efficiently pellet vesicles. HMG1 was solubilized from this pellet and from the donor PYC during a 15-min incubation at 65–70°C in sample buffer containing protease inhibitors. Samples were then boiled and subjected to SDS-polyacrylamide gel electrophoresis (7.5%) and electrophoretically transferred onto nitrocellulose. Anti-HMG1 antiserum was used at a dilution of 1:1,000 and the nitrocellulose filters were dried and exposed to Kodak XAR-5 film at −80°C. The graph shows the percent of pro-α-factor and HMG1 released into the supernatant at equivalent times during the parallel assays. No detectable amounts of HMG1 were released into the supernatant fraction.

a donor compartment, ATP, and proteins present in the cytosol.

Donor cells also released vesicles when they were assayed in the presence of a crude membrane fraction (Fig. 5, lane *D*) and an ATP-regenerating system. These membranes retained their vesicle-forming activity even after residual contaminating cytosol was removed from this fraction. Therefore, proteins required for this event can also be supplied from a concentrated crude membrane fraction. Preliminary findings suggest that the factors required for vesicle budding may be released from membranes in a soluble form (Groesch, M., and S. Ferro-Novick, unpublished results). One explanation for this observation is that budding factors may be peripheral membrane proteins that do not partition completely to the cytosol during the fractionation procedure. Donor PYC were unable to release vesicles when they were incubated with a crude membrane fraction and apyrase in the presence (Fig. 5, lane *E*) or absence of cytosol. In addition, there was no vesicle release when the cells were incubated with an ATP-regenerating system in the absence of both cytosol and crude membranes (not shown). This indicates that membranes residing in the donor fraction are not sufficient for efficient vesicle release. It is possible that the active components on these membranes are released during preparation of the permeabilized donor cells. Alternatively, the membranes may not be present in sufficient quantities to support vesicle release.

An ER Marker Protein Is Not Released from Donor PYC during Vesicle Formation and Release

The movement of proteins along the secretory pathway is a dynamic process. Resident proteins of the ER must be retained during membrane flow, while secreted proteins are sorted into vesicles destined to exit this organelle. We have shown above that pro-α-factor released from the cells is contained within a homogeneous membrane species. If these membranes are transport vesicles, rather than artifactually vesicularized ER, then the yeast ER membrane protein HMG-CoA reductase (Wright et al., 1988) should be retained in donor cells during vesicle formation. To address this question, parallel transport assays were performed at 20°C. Vesicle forming activity was measured in the standard assay, while the distribution of an isozyme of HMG-CoA reductase (HMG1) was assessed from an assay in which unlabeled pro-α-factor was translocated into the donor compartment. At the end of the assay, the presence of HMG1 was determined in both the supernatant and pellet fractions by Western blot analysis. We observed that HMG1 resided in the pelleted donor PYC and no detectable amounts were released from cells during the assay (Fig. 6). Titration experiments demonstrated that 8% or more of the total HMG1 released into the supernatant fraction could be detected by this method of analysis (not shown). Based on these findings we can conclude that 0–8% of the total HMG1 was released from donor cells, whereas 47% of the pro-α-factor was released from donor cells. Thus, pro-α-factor was sorted into vesicles while a resident protein was retained in the donor fraction.

Vesicles Are Not Derived from an Inactive Post-ER Compartment Retained in Donor Cells

To further characterize the vesicles released from the donor PYC, we determined if they could be derived from Golgi

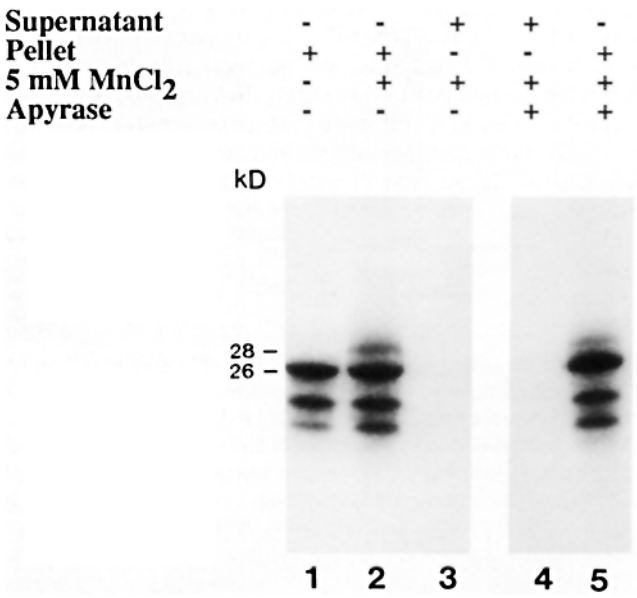

Figure 7. Donor PYC contain the compartment that converts the 26-kD species to the 28-kD form of pro-α-factor. Donor PYC were incubated with 5 mM manganese chloride in the absence of cytosol and exogenously added membranes for 90 min at 20°C. Afterwards, the cells were centrifuged and forms of pro-α-factor released into the supernatant (lanes *3* and *4*) and residing in the pellet (lanes *2* and *5*) were examined. The 28-kD species was produced only when the assay was performed in the presence of manganese chloride (lane *2* vs. lane *1*). The formation of the 28-kD species also was observed when apyrase was added to the incubation (lane *5*).

membranes within the cells. While previous work has shown that the donor PYC used in our assay do not contain the functional acceptor (Ruohola et al., 1988), it did not preclude the possibility that inactive Golgi membranes could be present. By Western blot analysis we have shown that Kex2, a protein presumed to reside in a late compartment of the Golgi complex (Julius et al., 1984; Fuller et al., 1988), is retained in the donor fraction (Groesch and Ferro-Novick, unpublished results). Together these findings suggest that inactive Golgi membranes are likely to be present in donor cells. The Golgi membranes could be inactive either with regard to transport or with regard to the enzymes necessary to process the 26-kD form of pro-α-factor to a higher molecular mass species. These membranes may be inactivated when the cells are permeabilized or during translocation of prepro-α-factor into the donor fraction.

We have made an observation which indicates that the PYC do contain the modification activities necessary to process 26-kD pro-α-factor to a higher molecular mass form. When donor cells, containing the 26-kD and partially glycosylated forms of pro-α-factor (Fig. 7, lane *1*), are incubated with high concentrations of $MnCl_2$ (5 mM) in the absence of cytosol and ATP (Fig. 7, lane *2*), 26-kD pro-α-factor is processed to the 28-kD species. The presence of high concentrations of divalent cations during this incubation may induce artifactual fusion between the ER and other membranes retained in the cells, allowing formation of 28-kD pro-α-factor. This artifactually induced fusion does not require energy (Fig. 7, lane *5*), and as expected, the 28-kD species is not released from the PYC during this incubation (lanes *3* and *4* vs. lanes *2* and *5*). Therefore it appears that endogenous Golgi membranes defective for transport may reside in the PYC. Since the 28-kD species is not observed in vesicles released from the permeabilized cells during our normal reaction, we can conclude that these vesicles have not fused with post-ER membranes within the cells. Furthermore, isolated vesicles containing 26-kD pro-α-factor are unable to process pro-α-factor to the 28-kD species by the addition of $MnCl_2$ (not shown), supporting our conclusion that the vesicular intermediate is not derived from a post-ER compartment.

The Vesicles Formed In Vitro Are Functionally Active for Transport

The data presented thus far would suggest that the vesicles formed in vitro are an intermediate in transport. Carrier vesicles that mediate transit between the ER and Golgi should bind to and fuse with acceptor Golgi membranes to complete this stage of transport. To determine if these vesicles are functional, vesicles released from the PYC were separated from the donor compartment and incubated with cytosol and acceptor membranes in the presence of an ATP-regenerating system. Transport was achieved during this incubation and

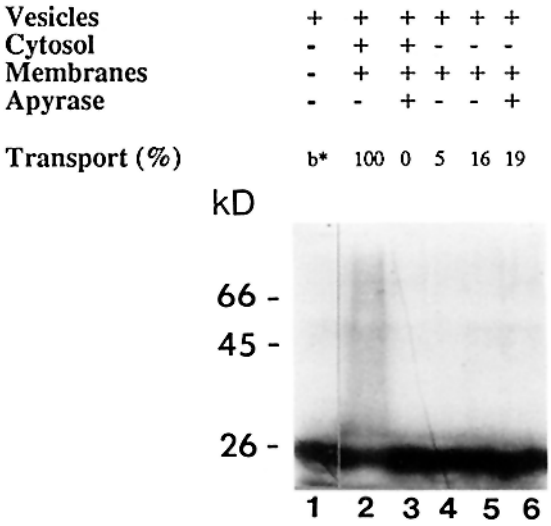

Figure 8. Cytosol is required for late transport events. The transport assay was carried out in two steps. In the first step, transport vesicles were formed during an incubation of donor PYC with cytosol and an ATP-regenerating system. After a 60-min incubation at 20°C, the donor PYC were pelleted and the supernatant containing vesicles and cytosol was applied to a Sephacryl S-300 column. The vesicles eluted from the column were concentrated as described in Materials and Methods. Vesicles (25 μl) were incubated with buffer alone (lane *1*; *b** = background) or with the acceptor compartment (lanes *2–6*) for 60 min at 20°C. Incubations with the acceptor compartment were performed with cytosol (0.8 mg) in the presence (lane *2*) or absence of ATP (lane *3*) or without cytosol in the presence (lanes *4* and *5*) or absence of ATP (lane *6*). Samples were treated with Con A–Sepharose and electrophoresed in a 12.5% SDS–polyacrylamide gel. The Con A–precipitable material was then reprecipitated with anti–outer-chain antibody to determine the percent of transport.

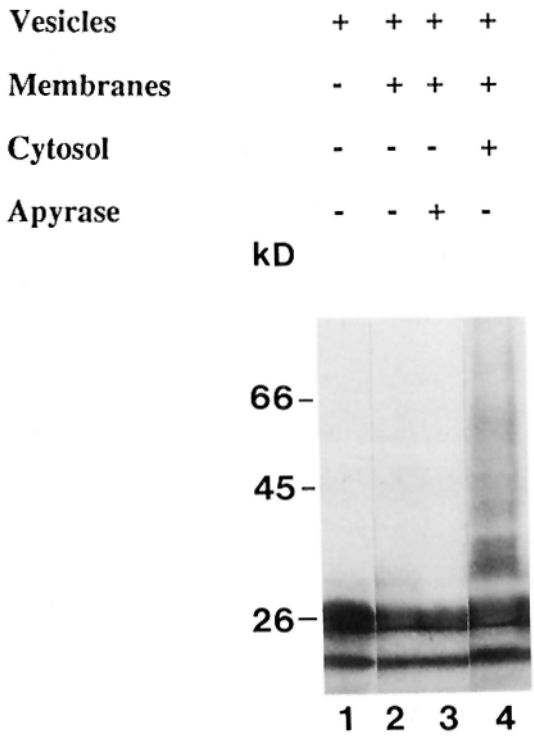

Figure 9. Vesicles formed from a crude membrane fraction can complete the transport reaction. The transport assay was executed in two steps. Vesicles were formed during a 30-min incubation of donor PYC, crude membranes, and an ATP-regenerating system at 20°C. Afterwards, the donor cells were centrifuged, and the vesicles remaining in the supernatant were removed (lane *1*) and incubated for an additional 90 min in the presence of a crude membrane fraction containing functional Golgi membranes. This second incubation was performed in the presence (lane *2*) or absence (lane *3*) of ATP or with added cytosol and ATP (lane *4*). When the assay was performed in one step, a 2× concentration of crude membranes did not inhibit transport.

the 26-kD species, initially contained within carrier vesicles, was converted to the high molecular mass Golgi form (Fig. 8, lane *2*).

We determined whether cytosol is required for the binding and fusion of vesicles with the acceptor membrane and subsequent transport of pro-α-factor to a site of extensive glycosylation in the Golgi complex. Vesicles formed during an incubation of donor PYC with cytosol were separated from the cytosol by passage through a Sephacryl S-300 column. Calibration of the Sephacryl S-300 column demonstrated that the vesicles, eluted in the void volume, were cleanly separated from two soluble protein markers, BSA (66 kD) and cytochrome c (12.4 kD). Protease protection experiments indicated that the column-eluted vesicles were sealed and contained pro-α-factor (not shown). Gel-filtered vesicles were incubated with various components during a 60-min incubation. Conversion of the 26-kD form to the high molecular mass species was assessed by precipitation with anti–outer-chain antibody and SDS–polyacrylamide gel electrophoresis (Figure 8). The control, gel-filtered vesicles incubated with buffer alone, did not demonstrate the high molecular mass Golgi form of pro-α-factor. The precipitable counts obtained from this sample were subtracted as background (Fig. 8, lane *b**) from all other samples (Fig. 8, lane *1*). Column-eluted vesicles could go on to complete transport only when they were incubated with the acceptor membrane in the presence of both ATP and cytosol (Fig. 8, lane *2*). Formation of the high molecular mass species was dramatically reduced when cytosol was eliminated from the assay (Fig. 8, lanes *4* and *5*) or when ATP (lane *3*) or both cytosol and ATP (lane *6*) were eliminated from the assay. The appearance of the high molecular mass form of pro-α-factor was dependent on the addition of functional acceptor acceptor membranes (not shown). These findings indicate that vesicles, cytosol, ATP and acceptor Golgi membranes are required to complete this reaction.

We have shown that vesicles are released from donor cells when they are incubated with a crude membrane fraction in the presence of an ATP-regenerating system. Are the vesicles formed in this way also competent to fuse with the Golgi complex? To address this question, vesicles were first formed during an incubation of donor cells with crude membranes (Fig. 9, lane *1*). Afterwards, the cells were centrifuged and the vesicles were incubated with additional crude membranes containing functional Golgi membranes. This incubation also included cytosol and ATP (Fig. 9, lane *4*), ATP alone (lane *2*), or apyrase to deplete ATP (lane *3*). The data in Fig. 9 indicate that transport was completed only when the second incubation was performed in the presence of cytosol and ATP (Fig. 9, lane *4*). If the cytosolic fraction was heated to 95°C for 15 min before use in the assay, it could not support conversion of the 26-kD form of pro-α-factor to the high molecular mass species (not shown). Therefore, crude membranes can supply the factors required to form vesicles, but they cannot provide all the proteins required to transport

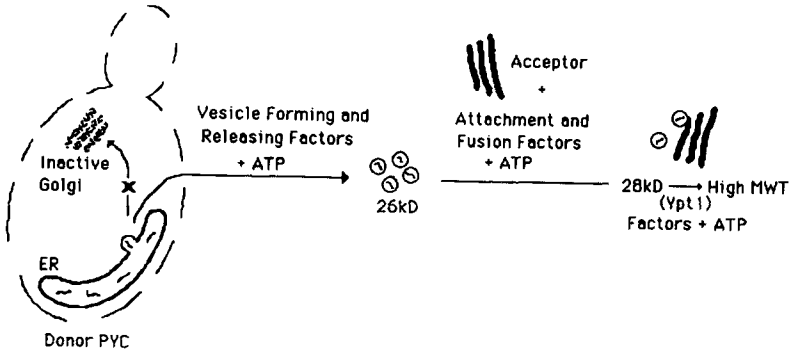

Figure 10. A model for pro-α-factor transport in vitro. The transit of pro-α-factor in vitro is summarized in this model. Vesicles, containing the 26-kD form of pro-α-factor, are released from donor PYC when they are incubated with either cytosol or a crude membrane fraction in the presence of an ATP-regenerating system. Carrier vesicles bind to and fuse with the Golgi complex to produce the 28-kD form of pro-α-factor. This species is then converted to a heterogeneous high molecular mass species. Conversion of the 26-kD species to the high molecular mass (*High MWT*) Golgi form requires the presence of cytosol, an active Golgi fraction, and an ATP-regenerating system.

pro-α-factor to a site of extensive glycosylation in the Golgi complex. Since the formation and utilization of vesicles require different factors, these two processes are likely to be biochemically distinct events.

Discussion

We have used an in vitro assay to isolate vesicles that are functional intermediates in the transport of proteins from the ER to the Golgi complex. This work provides strong support for the hypothesis that vesicles are responsible for carrying proteins between the ER and Golgi complex (Palade, 1975; for a recent review see Lodish, 1988). We have used several criteria to establish that the vesicles formed in our assay constitute a distinct intermediate in ER to Golgi transport. Kinetic studies demonstrate that vesicles are released from permeabilized donor cells before pro-α-factor receives Golgi complex-specific modifications. In addition, vesicle formation and release requires donor membranes, cytosolic proteins, and ATP. These are requirements anticipated for the formation of carrier vesicles since both cytosol and ATP are required for the vesicle-mediated movement of proteins from one cisternae of the Golgi complex to another (Balch et al., 1984; Wattenberg et al., 1986). ATP is also needed for protein transport in vivo (Jamieson and Palade, 1968; Novick et al., 1981). The vesicles containing pro-α-factor are a homogeneous population that contain no detectable amounts of the ER marker protein, HMG1. Since these vesicles contain only 26-kD pro-α-factor, we can conclude that the carrier vesicles have not fused with any post-ER compartment retained within donor cells. Together, these results suggest that pro-α-factor is released from the cells in discrete carrier vesicles derived from donor ER membrane.

The kinetics of transport-coupled outer chain addition show an initial lag. This lag is not due to a temporal requirement for the formation and release of vesicles from donor cells since vesicle release begins before the lag is completed. Consistent with this observation we find that the lag is not eliminated when vesicles are added to Golgi membranes at the beginning of the time course. The 28-kD species first appears during the lag in outer chain addition. This form of pro-α-factor is not precipitated with our anti-outer-chain antibody, possibly because it lacks sufficient carbohydrate determinants. Therefore, if the 28-kD species resides in the Golgi complex, pro-α-factor may reach the acceptor compartment during the lag.

Based on previous findings (Ruohola et al., 1988; Bacon et al., 1989) and results presented here, we have proposed a model for pro-α-factor transport in vitro that is diagrammed in Figure 10. Vesicle formation and release is dependent on the presence of donor membranes, ATP, and proteins from a yeast lysate. Upon the addition of cytosolic proteins, ATP, and a crude membrane fraction containing the functional acceptor, these vesicles bind to and fuse with a compartment capable of processing 26-kD pro-α-factor to a 28-kD species. The requirements for the processing of pro-α-factor from the 26- to the 28-kD species suggest that the 28-kD form resides in a compartment that is distinct from the vesicular intermediate. The 28-kD species is then processed to higher molecular mass Golgi forms, again suggesting transport from one compartment to another. In support of this model we have shown that 26-kD pro-α-factor is processed to the 28-kD species when vesicles are incubated with low concentrations of cytosolic proteins in the presence of the acceptor fraction and ATP. If this incubation is performed with high concentrations of cytosolic proteins, 26-kD pro-α-factor is converted to the high molecular mass form (Bacon, R., and S. Ferro-Novick, unpublished results). High concentrations of cytosol may be required to achieve transport through successive cisternae of the Golgi apparatus. The vesicle formation and releasing factors can be supplied either by cytosol or a crude membrane fraction; this is in contrast to the vesicle attachment and fusion factors that can be supplied only by cytosol. Since the activities required to form and utilize vesicles have different fractionation characteristics, at least a subset of these factors is likely to be different.

Recently, we have shown that a mutation in the GTP-binding protein, Ypt1, blocks transport in vitro and results in the partial accumulation of 28-kD pro-α-factor (Bacon et al., 1989). This and other observations have led to the proposal that Ypt1 is required for conversion of 28-kD pro-α-factor to the high molecular mass form and thus may function in Golgi membrane transport (Bacon et al., 1989; Segev et al., 1988). In addition to this role, genetic studies suggest that Ypt1 could play a role in ER to Golgi membrane transport (Segev et al., 1988; Schmitt et al., 1988; Bacon et al., 1989).

Our in vitro assay offers a powerful advantage for studying ER to Golgi protein transport in that we can physically separate transport into two steps. This aspect of the assay has allowed us to isolate functional intermediate vesicles and will facilitate the purification and analysis of this transient compartment. In studying these carrier vesicles, we will learn more about the mechanism of vesicle-mediated protein transport. Toward this end we are currently determining the protein composition of the vesicles. Antibodies prepared against the yeast gene products, implicated in ER to Golgi transport, will be useful in this evaluation. Since post-Golgi secretory vesicles do not contain a coat (Walworth and Novick, 1987; de Curtis and Simons, 1989) and purified intra-Golgi transport vesicles contain a nonclathrin coat (Malhotra et al., 1989), it will be interesting to determine if yeast ER to Golgi vesicles are coated. If a coat exists we can assess its composition. This analysis awaits the purification of carrier vesicles.

At present, we can separate transport from the endoplasmic reticulum through the Golgi complex into two steps, the release of vesicles and their utilization. However, the second step of our assay is undoubtedly composed of many more stages. These stages include the targeting and attachment of vesicles to the acceptor and fusion with this membrane to permit the mixing of contents. Further refinement of our assay should allow us to dissect the process of ER to Golgi transport into these individual events.

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