

Reconstitution of Transport of Vesicular Stomatitis Virus G Protein from the Endoplasmic Reticulum to the Golgi Complex Using a Cell-free System

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Abstract. Transport of the vesicular stomatitis virus-encoded glycoprotein (G protein) between the endoplasmic reticulum (ER) and the *cis* Golgi compartment has been reconstituted in a cell-free system. Transfer is measured by the processing of the high mannose ($\text{man}_{8-9}\text{GlcNAc}_2$) ER form of G protein to the $\text{man}_5\text{GlcNAc}_2$ form by the *cis* Golgi enzyme α -mannosidase I. G protein is rapidly and efficiently transported to the Golgi complex by a process resembling that observed *in vivo*. G protein is trimmed from the high mannose form to the $\text{man}_5\text{GlcNAc}_2$ form without the appearance of the intermediate $\text{man}_{6-7}\text{GlcNAc}_2$ oligosaccharide species, as is observed *in vivo*. G protein is found in a sealed membrane-bound compart-

ment before and after incubation. Processing *in vitro* is sensitive to detergent, and the Golgi α -mannosidase I inhibitor 1-deoxymannojirimycin. Transport between the ER and Golgi complex *in vitro* requires the addition of a high speed supernatant (cytosol) of cell homogenates, and requires energy in the form of ATP. Efficient reconstitution of export of protein from the ER requires the preparation of homogenates from mitotic cell populations in which the nuclear envelope, ER, and Golgi compartments have been physiologically disassembled before cell homogenization. These results suggest that the high efficiency of transport observed here may require reassembly of functional organelles *in vitro*.

AN understanding of the biochemical mechanisms that control the vesicular transport of protein between compartments of the secretory pathway of eucaryotic cells will require successful reconstitution of individual segments of the pathway using novel cell-free systems. Transport of membrane glycoprotein between the *cis* and *medial* compartments of the Golgi complex has been successfully reconstituted *in vitro* (3, 13, 21, 22). Studies in this system are providing, for the first time, new insight into the biochemical mechanisms involved in intercompartmental transfer in this organelle (5, 49, 73, 74). Other stages in the exocytic pathway remain to be reconstituted *in vitro* with high efficiency. An important step in the delivery of protein to the cell surface is the selective export of proteins from their site of synthesis in the cellular endoplasmic reticulum (ER).¹ The biochemical basis for synthesis (72), insertion (75), and core-glycosylation (55) of protein in the rough endoplasmic reticulum (RER) has been the subject of intense investigation. In contrast, little is known of the subsequent biochemical events that regulate export of protein from the ER to the next compartment of the exocytic pathway, the Golgi complex.

1. *Abbreviations used in this paper:* CP, creatine phosphate; CPK, rabbit muscle creatine phosphokinase; DMM, 1-deoxymannojirimycin; ER, endoplasmic reticulum; NEM, N-ethylmaleimide; RER, rough endoplasmic reticulum.

Isolation of yeast temperature-sensitive mutants that are pleiotropically defective in export of both membrane-bound and secreted protein from the ER has established that a common biochemical basis regulates trafficking from this organelle (48). Comparative studies defining differences in the kinetics of transport of a variety of membrane-bound and secreted proteins (23, 40, 61, 65, 76), the altered secretion of genetically engineered mutant proteins (19, 24, 56, 57), and the requirement for cofactors (53) or quaternary interactions (36, 37, 46, 50, 64) for export, suggest that signals encoded in the primary sequence of a protein provide important information, facilitating an initial step in interaction with this common transport machinery. Transport is vesicular; proteins are delivered between compartments through the budding and specific fusion of carrier vesicles (51). The enzymological basis for these events in the exocytic pathway remains to be defined.

To begin to understand the first stage of transport, export of newly synthesized protein from the ER, we are using a model system based on the G protein of vesicular stomatitis virus (VSV). The synthesis (31, 41), posttranslational processing (34, 35, 42, 66, 68), and transport of G protein from the ER to the cell surface (7, 8) has been extensively studied *in vivo*, and is indistinguishable from normal cellular membrane-bound glycoproteins, providing a sound experimental

system to explore new avenues of understanding of the secretory pathway. During synthesis, G protein acquires two high mannose core-oligosaccharides (42). Transfer of G protein from the ER to the *cis* compartment of the Golgi in vivo results in the G protein oligosaccharides being trimmed from the $\text{man}_{8-9}\text{GlcNAc}_2$ forms to the $\text{man}_5\text{GlcNAc}_2$ species as a result of the early Golgi oligosaccharide-processing enzyme α -mannosidase I (1, 4, 34, 35, 67). Trimming of G protein can be followed directly in the mutant Chinese hamster ovary (CHO) cell line clone 15B, which is missing the *medial* Golgi processing enzyme *N*-acetylglucosamine transferase I (16, 26). As a consequence, further processing of the $\text{man}_5\text{GlcNAc}_2$ oligosaccharide form to the complex structure by sequentially acting α -mannosidase II and glycosyltransferases in later compartments of the Golgi is blocked (15, 18, 34). G protein is delivered to the cell surface in the $\text{man}_5\text{GlcNAc}_2$ form, since processing, not transport, is the sole defect in clone 15B (62).

Export of G protein from the ER in vivo can be readily distinguished from later transport steps between Golgi compartments through use of the temperature-sensitive VSV mutant strain ts045, in which G protein is found exclusively in the ER when cells are incubated at the restrictive temperature (39.5°C) during infection (32, 33, 39, 44, 63, 77). Shift of cells from the restrictive to the permissive temperature (32°C) in vivo results in the synchronous release of G protein to the Golgi complex as shown both morphologically (7, 8) and biochemically through trimming to the $\text{man}_5\text{GlcNAc}_2$ form (4). Transport of ts045 G protein between the ER and the Golgi apparatus in vivo has been shown to occur in two biochemically distinct steps: an early temperature-sensitive, ATP-dependent export (budding) step, followed by a temperature- and ATP-insensitive step providing for delivery (fusion) of G protein to the *cis* Golgi compartment containing α -mannosidase I (1, 4).

In this paper, using the transport of G protein as a model system, we provide evidence that we have successfully reconstituted a new stage of the exocytic pathway in vitro, transport of protein from the ER to a *cis* Golgi compartment containing α -mannosidase I. Transport is highly efficient and proceeds with kinetics similar to those observed in vivo. Transport occurs between sealed membrane-bound compartments, requires energy in the form of ATP, and is dependent upon the addition of a cytosol fraction containing soluble components prepared from a high speed supernatant of centrifuged cell homogenates.

Materials and Methods

Materials

A CHO cell line clone, 15B (26); a mouse cell line clone, 6 (25); VSV (Indiana serotype); and the mutant strain ts045 were obtained from J. E. Rothman, Stanford University, Stanford, CA. L-[³⁵S]methionine (>800 Ci/mmol) was purchased from Amersham Corp., Arlington Heights, IL. [³H]mannose (25 Ci/mmol) was purchased from New England Nuclear, Boston, MA. 1-deoxymannojirimycin (DMM) was purchased from Genzyme, Boston, MA; swainsonine was purchased from Calbiochem, La Jolla, CA; rabbit muscle creatine phosphokinase and nocodazole were obtained from Sigma Chemical Co., St. Louis, MO.

Methods

Growth and Synchronization of Cells. The CHO cell lines were grown in monolayer (10-cm dishes) in minimal essential medium (α -MEM) (Gibco,

Grand Island, NY) supplemented with 10% fetal calf serum. The mouse L cell clone 6 was grown in DME supplemented with 15% fetal calf serum and 0.1% sodium pyruvate.

For preparation of synchronized mitotic cell populations of clone 15B the following protocol was used. Confluent cells (1×10^7 cells/10-cm LUX tissue culture dish [Miles Laboratories, Elkhart, IN]) were trypsin-released and seeded into new tissue culture dishes at a cell density of 2.5×10^6 cells/10-cm dish. After 24 h, the medium was replaced with fresh medium and the cells were incubated for an additional 24 h. At this time, closely packed cells (2×10^7 cells/10-cm dish) were trypsin-released and seeded into new dishes at a cell density of 6.7×10^6 cells/10-cm dish in the presence of 2.5 mM thymidine. After 14 h, cells were washed twice with 2 ml of TD buffer (138 mM NaCl, 5 mM KCl, 25 mM TRIS base, 0.4 mM Na_2HPO_4 ; pH 7.4), and reincubated in the presence of 5 ml of α -MEM for 5.5 h. At 5.5 h a stock solution of nocodazole dissolved at 50 $\mu\text{g}/\text{ml}$ in DMSO was added to each dish with mixing to a final concentration of 0.1 $\mu\text{g}/\text{ml}$. After an additional 3-h incubation cells were harvested by the mitotic shake procedure (69). With this procedure, 50–70% of the total cell population in each dish was arrested in mitosis. The mitotic index of harvested cells ranged from 80 to 95% for different preparations. The growth of cells to high density in the first step of the synchronization procedure is essential for the high yields of mitotic 15B cells.

Infection of Mitotic Cells with VSV. Protein synthesis is required just before entry of cells into mitosis (70). Since virus infection rapidly inhibits host protein synthesis, the timing of virus infection is critical to insure a maximal yield of infected, mitotic cells. For infection of clone 15B with ts045, one-third of the total synchronized cells in each experiment were infected after a 5.5-h period of incubation in the absence of thymidine. Each dish was incubated with 10–20 plaque forming units of virus per cell in 1 ml of an infection medium containing α -MEM, 25 mM HEPES (pH 7.4), 0.1 $\mu\text{g}/\text{ml}$ nocodazole per 10-cm dish for 30 min at 32°C with gentle rocking. Each dish was subsequently supplemented with an additional 4 ml of α -MEM containing 10% fetal calf serum and 0.1 $\mu\text{g}/\text{ml}$ nocodazole, and incubated at 37°C for 2.0 h before harvest of cells by mitotic shake. With this procedure, 15–30% of the total cell population in each dish was arrested in mitosis. The mitotic index of harvested cells ranged from 60 to 80% for different preparations. A mitotic cell population infected with wild-type VSV was obtained using an identical protocol, with the exception that all incubations were at 37°C.

Infection of Interphase Cells. An asynchronous interphase cell population of either the 15B or clone 6 cell lines was infected with ts045 virus in a fashion identical to that described for mitotic cells, with the exception that nocodazole was not included in the infection media. After binding of virus to cells for 30 min at 32°C, cells were incubated in the presence of α -MEM containing 10% fetal calf serum for 3.5–4.5 h at the restrictive temperature (39.5°C). Infection of cells with wild-type virus was carried out at 37°C.

Labeling of Cells. Mitotic or interphase cell populations infected with ts045 were washed three times with 5-ml portions of a labeling medium (Joklik's minimal essential medium) containing nonessential amino acids (Gibco) and 20 mM HEPES-NaOH, pH 7.4, at 39.5°C. For labeling of mitotic cells, the labeling medium contained, in addition, 0.1 $\mu\text{g}/\text{ml}$ of nocodazole. For experiments with [³⁵S]methionine the labeling medium lacked methionine. Cells were incubated for 5 min at 39.5°C (the restrictive temperature) before the addition of [³⁵S]methionine (25–50 $\mu\text{Ci}/\text{ml}$). After a 10-min pulse, unlabeled methionine was added to a final concentration of 2.5 mM, and the incubation continued for 3 min at 39.5°C before transfer of cells to a 10 \times volume excess of ice-cold labeling medium to rapidly chill cells and inhibit transport. The infected, labeled mitotic cells were pooled with the uninfected mitotic cells harvested from the remaining two-thirds of the cells used in each experiment.

For labeling of wild-type virus-infected cells, cells were washed in labeling medium lacking methionine as described above, preincubated for 5 min at 37°C, and labeled for 3 min in the presence of [³⁵S]methionine. After a 3-min pulse, unlabeled methionine was added to a final concentration of 2.5 mM and cells immediately transferred to a 10 \times excess volume of ice-cold medium. Nocodazole was included additionally in the medium for labeling of infected mitotic cells.

Preparation of Cell Homogenates. Cells in ice-cold labeling medium were pelleted for 3 min at 800 g, and washed once with a TEA buffer containing 10 mM triethanolamine and 150 mM KCl (pH 7.4). Cells were pelleted and resuspended in a H/KCl buffer containing 50 mM HEPES, 90 mM KCl (pH 7.2) at a ratio of 100–200 μl pelleted cells per 1 ml of H/KCl buffer, and homogenized using a stainless steel ball-bearing homogenizer as described previously (2). A postchromosomal supernatant (PCS) was prepared where indicated by centrifugation of the homogenate for 5 min at 800 g. Cell homogenates (5–15 mg/ml protein with $\sim 40\%$ of

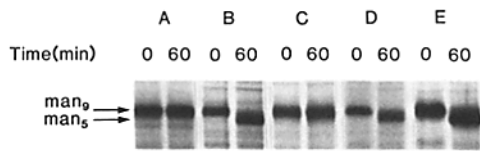


Figure 1. Trimming of G protein in vitro. In vitro incubation of cell homogenates prepared from ts045-infected interphase 15B cells (A), ts045-infected mitotic 15B cells (B), ts045-infected interphase 15B cells incubated with nocodazole for 3.5 h before preparation of cell homogenates (C), and wild-type virus-infected mitotic 15B (D). G protein was labeled with [³⁵S]methionine at either the restrictive temperature (ts045), or for 3 min at 37°C (wild-type; see Materials and Methods) to localize G protein to the endoplasmic reticulum before cell homogenization. Homogenates were incubated on ice (0°C), 32°C (ts045), or 37°C (wild-type) for 60 min as described in Materials and Methods. (E) Extent of trimming of G protein observed in vivo in ts045-infected 15B cells labeled at the restrictive temperature (39.5°C) and transferred directly to ice (0 min), or incubated at the permissive temperature (32°C) for 60 min. For each incubation condition, transport was terminated and the extent of G protein processing determined as described in Materials and Methods.

total protein being membrane protein) were used immediately for assay, or transferred in 125- μ l portions to 500- μ l microcentrifuge tubes and rapidly frozen in liquid nitrogen and stored at -80°C. For assay, frozen fractions were thawed by a brief incubation at 37°C, and transferred to ice. Frozen, thawed fractions yielded results upon incubation in vitro identical to those obtained using fresh cell homogenates. Frozen crude cell homogenate is stable for at least 1 mo under these conditions.

To prepare a membrane fraction devoid of soluble cytosolic proteins, 125 μ l of the cell homogenate was diluted into 5 ml of H/KCl buffer containing 1 mM EDTA, and centrifuged in a rotor (SW 50.1; Beckman Instruments, Inc., Palo Alto, CA) for 20 min at 30,000 rpm. The pellet was gently resuspended in 125 μ l of H/KCl buffer and used for incubation in vitro as indicated in Results. A cytosol fraction was prepared by centrifugation of cell homogenates in a SW 50.1 for 60 min at 49,000 rpm and the supernatant carefully removed to avoid disturbing the pellet. Where indicated the cytosol was depleted of low molecular components by gel filtration on a Sephadex G-25 column equilibrated with H/KCl buffer. Protein was determined by the method of Lowry (45).

Incubation Conditions to Achieve Transport In Vitro. In addition to cell homogenate (10 to 50 μ g per incubation), each assay contained in a total volume of 50 μ l (final concentrations): 50 mM Hepes-KOH (pH 7.2), 90 mM KCl, 2.5 mM magnesium chloride, 1 mM ATP, 5 mM creatine phosphate (CP), and 0.2 IU of rabbit muscle creatine phosphokinase (CPK). The ATP-regenerating system was made fresh daily by mixing 20 μ l CPK (2,000 IU/ml, stored at -80°C), 100 μ l of 200 mM CP, 100 μ l of 40 mM ATP (Na form, neutralized with NaOH); and added as a concentrate to the assay to obtain the indicated final concentrations. Additional modifications of the standard incubation conditions are indicated in Results. Each assay was initiated by incubation for indicated time and temperature as described in Results. Transport was terminated by transfer to ice, followed by the addition of 15 μ l of a 5 \times concentrate of a gel sample buffer (38) containing 12.5 ml of a 1 M Tris (pH 6.8) buffer, 20 ml glycerol, 0.5 g dithiothreitol (DTT), 4 g SDS, 4 mg bromophenol blue, in a final volume of 40 ml H₂O.

Quantitation of Transport. Samples were processed for SDS gel electrophoresis using 10% polyacrylamide gels (38), treated for autoradiographic enhancement by incubation for 30 min in 100 ml of a 30% methanol solution containing 0.125 M salicylic acid (Na form; pH 7.0), dried, and autoradiographed for 1-3 d (XAR-5 film, Kodak). The fraction of G protein processed to the man₅GlcNAc₂ form was determined by densitometry of the exposed autoradiogram using a transmission scanning densitometer (GS300; Hoefer Instruments, San Francisco, CA) connected to an IBM-XT with the GS350 integrating software (Hoefer Instruments). The quantitative relationship between appearance of the faster migrating band using SDS gel electrophoresis, and processing to the man₅GlcNAc₂ oligosaccharide form has been established previously (4).

Oligosaccharide Size Class Determination. Clone 15B mitotic cells infected with ts045 were labeled for 15 min at 39.5°C with [³H]mannose in

labeling medium containing methionine, but with one-tenth the normal glucose level. A cell homogenate was prepared and incubated in vitro as described in Results. Samples were processed for SDS PAGE, autoradiographed, and the G protein band excised by comparison of the dried gel to the exposed autoradiogram. The excised gel was resuspended in a buffer containing 100 mM Tris-HCl (pH 8), 10 mM CaCl₂, 1 mM Na azide, 1.2 mg/ml pronase, and digested for 36 h at 56°C with additional pronase (1.2 mg/ml) added at 12 and 24 h of incubation. The glycopeptides were digested with 0.02 U/ml of endoglycosidase H (Miles Laboratories Inc.) in 0.15 M citrate buffer, pH 5.5 for 24 h at 37°C. The released oligosaccharides were separated into component size classes by high resolution Biogel P-4 gel filtration chromatography using a 1 \times 120-cm column equilibrated with 0.1 M NaHCO₃, pH 8.0. Oligosaccharide standards were generously provided by M. Snider, Carnegie Institute of Washington, Baltimore, MD.

Results

Preparation of Active Crude Cell Homogenates

Transport of G protein from the ER to the Golgi complex can be directly measured in vivo by the activity of the *cis* Golgi enzyme α -mannosidase I, which trims G protein oligosaccharides from the man₈₋₉GlcNAc₂ species to the man₅GlcNAc₂ form (1, 4). A cell homogenate was prepared from a VSV-infected 15B interphase cell population in which [³⁵S]methionine-labeled ts045 G protein was localized to the ER by labeling at the restrictive temperature (39.5°C) before homogenization. No trimming of G protein in vitro was observed with incubation conditions that support inter-Golgi transport (Fig. 1 A) (3). These results raise the possibility that the transport function of one or both of these early compartments was disrupted during cell homogenization.

The ER is a single, reticular network of tubules and cisternae in continuity with the nuclear envelope and found throughout the cytoplasm of the eukaryotic cell. During cell homogenization the ER is sheared into microsomes. Microsomes are resealed ER vesicles, which, in the case of the RER, contain bound ribosomes and retain the capacity for co-translational insertion and core-glycosylation of nascent protein in vitro (72). The structure of the Golgi apparatus in vivo is controversial. While serial reconstructions in Sertoli cells support a model in which it is a single, continuous ribbon-like structure in which functionally distinct compartments are juxtaposed (52), the presence of several distinct Golgi stacks in other cell lines remains to be ruled out. The Golgi complex shears during homogenization to release single or stacked cisternal fragments similar to those observed in vivo (5, 6), which are active in inter-Golgi transfer reactions in vitro (3).

One way to circumvent the problem of vesiculation during homogenization, which may disrupt the transport properties of these organelles for the ER to Golgi stage of the secretory pathway in vitro, is to obtain homogenates from cells undergoing mitosis. In this stage of the cell cycle both the nuclear envelope (14) and the Golgi complex (54) are disassembled. The extent of disassembly of the ER is less well understood. Since the ER is an extension of the outer nuclear envelope, it seems likely that the extensive reticular network of the nonnuclear rough and smooth ER membranes may be similarly disassembled during mitosis. Analysis of serial thin sections prepared from mitotic cells arrested at metaphase suggests that this is the case for CHO cells (Hermanowsky, A., and W. E. Balch, manuscript in preparation). These physiologically disassembled structures found in mitotic

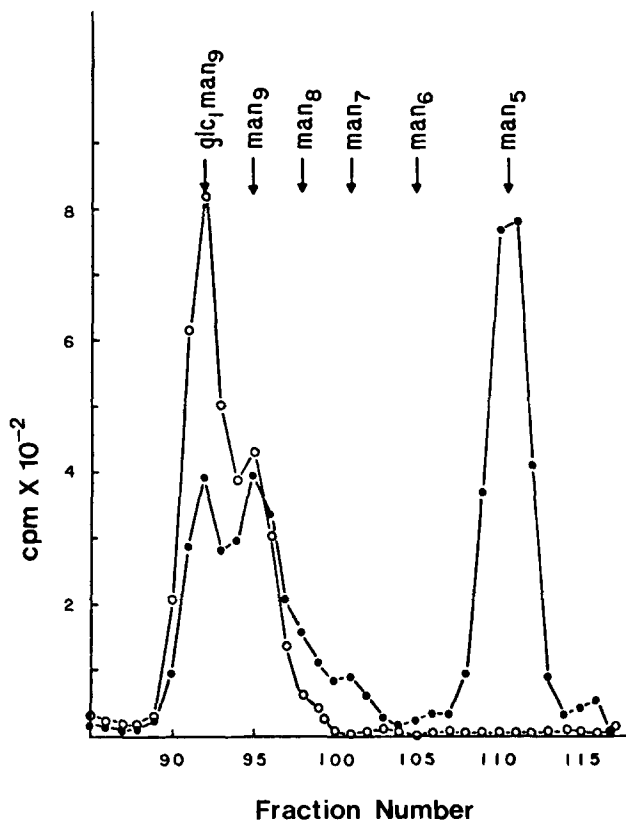


Figure 2. Oligosaccharide size class determination of G protein trimming in vitro. Ts045-infected 15B mitotic cell homogenate labeled with [^3H]mannose (see Materials and Methods) was incubated in vitro for 30 min on ice (*solid circles*), or for 30 min at 32°C (*open circles*). Samples were processed for SDS gel electrophoresis, G protein excised, and the oligosaccharides prepared for chromatography on a Biogel P-4 column (1 \times 120 cm) as described in Materials and Methods. The arrows indicate the elution peaks of the oligosaccharide standards.

cells may be functionally stable to the homogenization conditions necessary to shear the plasma membrane to prepare a crude cell homogenate. If so, such intact mitotic compartments may provide the organelles in a form allowing reconstitution of transport in vitro.

To test the possibility that mitotic organelles may retain the functional capacity for export from the ER in vitro, a post-chromosomal supernatant (PCS) was prepared from mitotic clone 15B cells infected with the ts045 virus. G protein synthesized by ts045-infected mitotic cells during labeling with [^{35}S]methionine at the restrictive temperature (39.5°C) is exclusively localized to the ER (4, 8, 20). When the PCS was incubated at 32°C for 60 min in the presence of ATP, a form of G protein with increased electrophoretic mobility comparable to that observed for the $\text{man}_5\text{GlcNAc}_2$ species formed in vivo was observed upon SDS gel electrophoresis of the incubated fraction (Fig. 1, *B* and *E*). 70% of the total G protein was processed to the form with the lower molecular weight during incubation in vitro, suggesting a highly efficient process (Fig. 1 *B*). In these incubations, intact cells were not detected by light microscopy, eliminating the trivial explanation that the trimming observed in vitro was a G protein transported in living cells present in the incubation. No trimming in vitro was observed when an asynchronous inter-

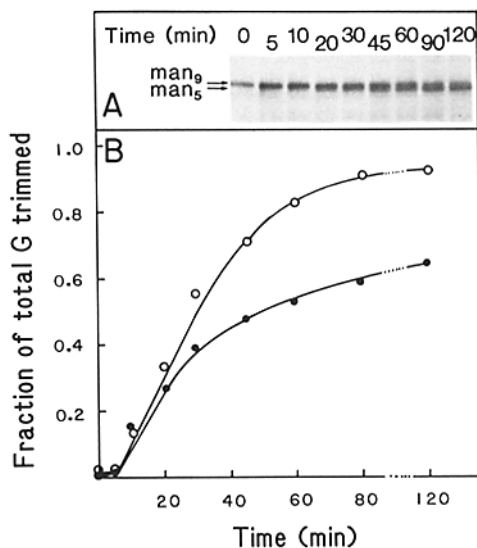


Figure 3. Kinetics of processing of G protein in vitro. A 15B mitotic cell homogenate containing [^{35}S]methionine-labeled ts045 G protein was incubated in vitro for the indicated time at 32°C, processed for SDS gel electrophoresis, and the amount of G protein trimmed to the $\text{man}_5\text{GlcNAc}_2$ form was quantitated as described in Materials and Methods. The autoradiogram (*A*) was used to quantitate the amount of G protein trimmed at each time point in vitro as shown in *B* (*solid circles*). A typical time course for processing of G protein in vivo is shown (*B*, *open circles*) (4).

phase 15B cell population was treated with nocodazole (an inhibitor of microtubule assembly) for 3 h before preparation of cell homogenates (Fig. 1 *C*). These results suggest that preparation of active homogenates is not simply a consequence of the conditions used to accumulate cells at metaphase (see Materials and Methods). However, trimming was observed in vitro when homogenates were prepared from mitotic 15B cells infected with wild-type virus in which the G protein was labeled for 3 min with [^{35}S]methionine (Fig. 1 *D*). Under these conditions, the labeled G protein is localized to the ER before homogenization as a consequence of the short pulse (21, 22), and since all vesicular trafficking between ER and Golgi complex is inhibited in mitotic cells in vivo (20).

These results tentatively suggest that we have successfully reconstituted in vitro transport between the ER and *cis* Golgi compartment. To provide additional evidence for this interpretation, the following lines of investigation were pursued.

G Protein Is Processed to the $\text{Man}_5\text{GlcNAc}_2$ Species In Vitro

To demonstrate that the observed shift in molecular weight after incubation in vitro is the result of processing of G protein oligosaccharides to the $\text{man}_5\text{GlcNAc}_2$ form, as is observed in vivo, a PCS was prepared from ts045-infected mitotic cells in which the core-oligosaccharides of G protein had been labeled with [^3H]mannose at the restrictive temperature. These homogenates were incubated at 32°C for 30 min in vitro. G protein oligosaccharides were isolated and analyzed using high resolution Biogel P-4 chromatography. As shown in Fig. 2, G protein oligosaccharides were processed from the high mannose $\text{man}_9\text{GlcNAc}_2$ species to the $\text{man}_5\text{GlcNAc}_2$ form as is observed in vivo (4). The

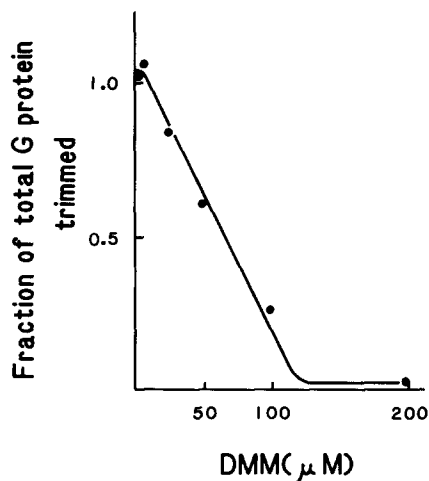


Figure 4. Trimming in vitro is sensitive to DMM. A 15B mitotic cell homogenate containing [³⁵S]methionine-labeled ts045 G protein was incubated in vitro for 60 min at 32°C as described in Materials and Methods in the presence of increasing concentrations of DMM as indicated. DMM was added immediately before initiation of transport. Samples were processed to determine the extent of G protein trimmed to the man₅GlcNAc₂ species as described in Materials and Methods.

intermediate man₇GlcNAc₂ and man₆GlcNAc₂ forms of G protein oligosaccharides were not detected at this time period of incubation in vitro. A similar result is observed in vivo (4). These results suggest that G protein is processed in vitro along the same enzymatic pathway observed in vivo.

Kinetics of G Protein Processing In Vitro

The kinetics of G protein processing by α -mannosidase I to the man₅GlcNAc₂ species is shown in Fig. 3. Processing in vitro occurs with a reproducible 5–10 min lag period, followed by a linear phase of trimming, which approaches a plateau after 60–80 min of incubation (Fig. 3, solid circles). These results closely resemble the kinetics of processing observed in vivo (Fig. 3, open circles) (4).

The extent of processing of ts045 G protein and wild-type G protein in vitro at 32, 37, and 39.5°C were found to be similar after 90 min of incubation (data not shown). Since transport of G protein from the ER to the Golgi complex in vivo is inhibited by incubation of 15B cells at 39.5°C (4), these results suggest that the temperature-sensitive phenotype of G protein has not been reconstituted in vitro using the present homogenization and incubation conditions.

Trimming Is Inhibited by Addition of Deoxymannojirimycin

DMM is a substrate analogue of mannose and a noncompetitive inhibitor of the Golgi α -mannosidase I (10), and ER-associated α -mannosidases that trim resident ER enzymes to the man₇GlcNAc₂ and man₆GlcNAc₂ species (12). In contrast, neither the ER α -mannosidase that cleaves the man₉GlcNAc₂ species to the man₈GlcNAc₂ structure (9), the soluble form of the ER α -mannosidase released during cell homogenization (12), nor lysosomal mannosidases are sensitive to DMM (10, 12). Thus, DMM can be used to determine if the processing observed in vitro is a consequence of delivery of G protein to the Golgi-associated trimming site con-

taining the DMM-sensitive α -mannosidase I, processing high mannose forms to the man₅GlcNAc₂ species. As shown in Fig. 4, addition of increasing concentrations of DMM to the in vitro incubation immediately before initiation of transport resulted in a 50% inhibition of trimming at a concentration of 60 μ M. 50% inhibition of trimming G protein in vivo in clone 15B cells is observed in the presence of 250 μ M DMM (data not shown), similar to concentrations required to inhibit processing in other cell lines (12). No inhibition was observed by the incubation of homogenates in vitro in the presence of up to 1 mM swainsonine, a specific inhibitor of the medial Golgi compartment enzyme α -mannosidase II (71) (data not shown), providing additional proof that the observed trimming is specific to the α -mannosidase I activity present in the 15B *cis* Golgi compartment.

Transport of G Protein Occurs between Sealed Membrane-bound Compartments

To determine if G protein must reside in a sealed membrane-bound compartment for efficient trimming by α -mannosidase I in vitro, crude homogenates were incubated in the presence of 0.1% Triton X-100 to solubilize membranes before incubation. The solubilized form of α -mannosidase I is not inhibited by the addition of Triton X-100 under these conditions (67). As shown in Fig. 5 A, no processing of G protein was observed after incubation for 60 min in the presence of detergent, suggesting that a solubilized form of G protein is not a substrate for trimming by α -mannosidase I under the conditions used to establish transport in vitro. This result further distinguishes the processing observed here from assays developed to measure α -mannosidase I activity in cell fractions (10, 12, 15, 18).

G protein is asymmetrically oriented in the ER membrane with a 29 amino acid carboxyl terminus extension on the cytoplasmic face of the ER (30). Before incubation in vitro, 80% of the total G protein in this preparation of cell homogenate was protected from complete tryptic hydrolysis as a consequence of its presence in a sealed membrane-bound compartment (Fig. 5 B, lanes a, b, and e). The protected fragment of G protein, which includes the transmembrane and luminal domains, is found as a truncated form with a higher electrophoretic mobility observed by SDS gel electrophoresis due to the cleavage of the cytoplasmically exposed, trypsin-sensitive carboxyl terminus (Fig. 5 B, lane b) (30). No G protein was detected when detergent was added before trypsin digestion (Fig. 5 B, lane e). When the cell homogenate was incubated for 60 min at 32°C, 60% of total G protein was processed to the trimmed form in vitro (Fig. 5 B, lane c). After incubation, 65% of the total G protein was found to be trypsin-resistant (Fig. 5 B, lane d). Of this total trypsin-resistant G protein found after incubation in vitro (Fig. 5 B, lane d), nearly 80% was found to be in the trimmed form (Fig. 5 B, lane d). These results suggest that for this homogenate preparation, the observed 60% efficiency of transport of total G protein present in the incubation (Fig. 5 B, lane c) is an underestimate of the actual efficiency of transport of G protein present in sealed ER structures before incubation. Trypsin latency of G protein present in different preparations ranged from 50 to 85%, suggesting that the ER is a fragile structure.

These results indicate that the form of G protein found in sealed vesicles before incubation is trimmed to the man₅-

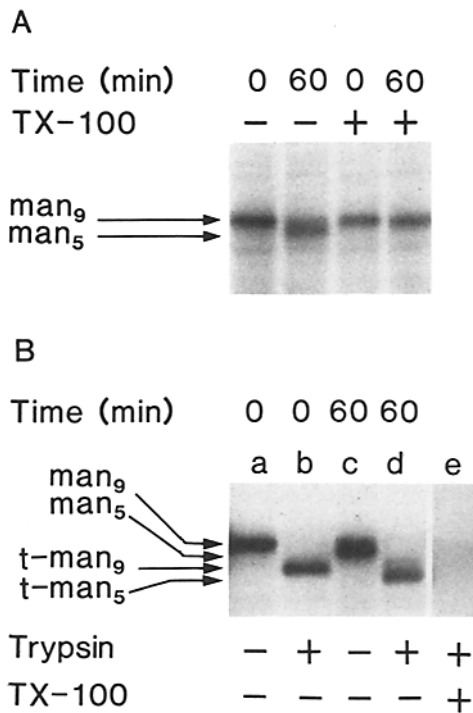


Figure 5. G protein is found in a sealed, membrane-bound compartment before and after incubation in vitro. (A) A 15B mitotic cell homogenate containing [³⁵S]methionine-labeled ts045 G protein was incubated in vitro for 60 min at 32°C as described in Materials and Methods in the presence or absence of TX-100 at a final concentration of 0.1%. (B) 15B cell homogenates containing [³⁵S]methionine-labeled ts045 G protein were incubated in duplicate in vitro either on ice, or at 32°C for 60 min as described in Materials and Methods. The samples shown in lanes a and c were incubated for an additional 5 min at 32°C in the presence of a cocktail containing a mixture of trypsin and soybean trypsin inhibitor at a final concentration of 100 and 200 µg/ml, respectively. The samples shown in lanes b and d were incubated for an additional 5 min at 32°C in the presence of 100 µg/ml trypsin before the addition of 200 µg/ml trypsin inhibitor. The sample shown in lane e was incubated with trypsin as described for lanes b and d in the presence of 0.1% TX-100 to solubilize the membranes. The migration of the man₉GlcNAc₂ and man₅GlcNAc₂ oligosaccharide forms of the truncated form of G protein missing the carboxyl terminus is indicated by the prefixes t-man₉ and t-man₅, respectively.

GlcNAc₂ form with high efficiency, and is found in a sealed membrane-bound compartment with the proper asymmetric membrane orientation after incubation in vitro.

Role of Cytosolic and Membrane-bound Components on G Protein Transport

When the mitotic cell homogenate containing G protein was pelleted and washed to remove soluble cytoplasmic factors, trimming was not observed during incubation in vitro (Fig. 6). When increasing concentrations of cytosol were added to the incubation containing washed membranes, both the extent and efficiency of transport observed in the control (unwashed membranes) was recovered (Fig. 6). Mitotic cytosol can be quantitatively replaced with cytosol prepared from a nonsynchronized (interphase) cell population, indicating that the requirement for preparation of homogenates from mitotic cells is specific to the membrane fraction prepared

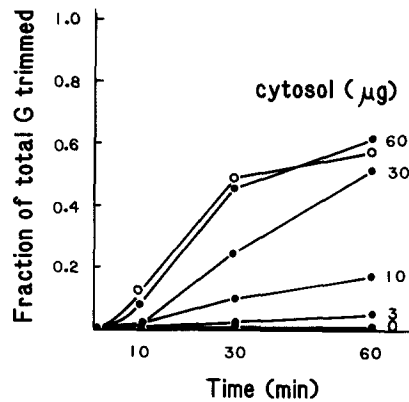


Figure 6. Trimming in vitro requires a soluble protein fraction (cytosol) prepared from cell homogenates. A 15B mitotic cell homogenate containing [³⁵S]methionine-labeled ts045 G protein was washed to remove soluble cytosolic proteins as described in Materials and Methods, and 25 µg of the membrane fraction was incubated in the presence of increasing concentrations of a high speed supernatant (cytosol) of a mitotic cell homogenate prepared as described in Materials and Methods. The time course of trimming in vitro for 60 µg of the unwashed cell homogenate is shown by the open circles. At each time point, transport was terminated by transfer to ice, and samples processed to determine the extent of G processed to the man₅GlcNAc₂ form as described in Materials and Methods.

from 15B cells (data not shown). Cytosol prepared from the mouse mutant L cell line clone 6, defective in processing of G protein oligosaccharides beyond the man₃GlcNAc₂ form (25, 67), can quantitatively replace the cytosol requirement provided by the mitotic 15B cell homogenate (data not shown). In addition, mitotic cytosol was not found to promote transport of G protein between the ER and Golgi compartments prepared from interphase cells (data not shown).

No transport was observed when ts045 G protein-containing mitotic cell homogenate was treated for 10 min on ice with trypsin, followed by incubation in the presence of uninfected, unlabeled 15B mitotic cell homogenate and trypsin inhibitor (Table I). Inhibition was also observed when the ts045 G protein-containing homogenate was treated for 5 min on ice with 1 mM *N*-ethylmaleimide (NEM) before quenching of the unreacted NEM by addition of DTT and incubation in the presence of uninfected, unlabeled 15B mitotic cell homogenate (Table I). These results suggest that, in addition to the acceptor Golgi compartment containing α -mannosidase I, component(s) found on the donor compartment membrane and in the cytosol are required for transport in vitro.

Appearance of the Trimmed Form of G Protein Is ATP-dependent

We have recently established that export of G protein from the ER in vivo requires ATP (4). If the events observed here reflect those occurring in vivo, transport in vitro should be ATP-dependent. When extracts were incubated in the absence of an ATP regenerating system, the extent of G protein processed was reduced by only 15% (Table I). Since cell homogenates contain a substantial pool (at least 1 mM) of ATP, extracts were depleted of endogenous ATP by the addition of hexokinase and glucose. Under these conditions, pro-

Table I. Requirement for ATP in vitro

Fraction	Addition or omission	Fraction of total G trimmed at 60 min of incubation at 32°C
(A) 15B Mitotic homogenate	None (complete)	0.65
	+Trypsin	0.05
	+NEM	0.05
(B) 15B Mitotic homogenate	-ATP/CP/CPK	0.55
	-ATP/CP/CPK, +HK, +Glc	0.05
	+HK	0.55
	+Glc	0.60
	+EDTA	0.05
(C) Washed 15B mitotic homogenate	-Cytosol	0.05
	+Cytosol (+ADP)	0.15
	+Cytosol, +ATP/CP/CPK	0.55

(A) A 15B mitotic cell homogenate containing [³⁵S]methionine-labeled ts045 G protein was washed as described in Materials and Methods. Resuspended membranes (50 µg) were treated with 5 µg of trypsin for 10 min on ice before the addition of trypsin inhibitor (20 µg), or were treated with NEM at a final concentration of 1 mM for 5 min on ice before the addition of DTT at a final concentration of 2 mM. 25 µg treated membranes were incubated in vitro in the presence of 100 µg untreated, uninfected mitotic 15B homogenate as described in Materials and Methods. Control incubations that contained equivalent concentrations of a premixture of trypsin and trypsin inhibitor, or NEM and DTT, showed no inhibition of processing (data not shown). (B) 15B mitotic cell homogenate containing [³⁵S]methionine-labeled ts045 G protein was incubated in the presence, or absence of an ATP-regenerating system (2.5 mM ATP, 5 mM CP, 0.2 IU of CPK) per 50 µl incubation cocktail. To remove endogenous ATP, hexokinase (HK; 1 IU/50 µl incubation cocktail), and glucose (1 mM final) were added to the incubation cocktail where indicated. (C) Washed mitotic extracts were prepared and incubated with a cytosol fraction prepared by filtration over Sephadex G-25 to remove low molecular compounds as described in Materials and Methods. ADP was added to the incubation where indicated at a final concentration of 2.5 mM. EDTA (Na form; pH 7.0) was added to the incubation where indicated at a final concentration of 3 mM.

cessing of G protein to the man₅GlcNAc₂ form was completely inhibited (Table I). A similar inhibition of transport was observed in the absence of ATP when membranes were washed and incubated in the presence of a cytosol fraction depleted of ATP by Sephadex G-25 gel filtration (Table I). Processing of G protein in the presence of ATP was inhibited by the addition of EDTA to the assay (Table I).

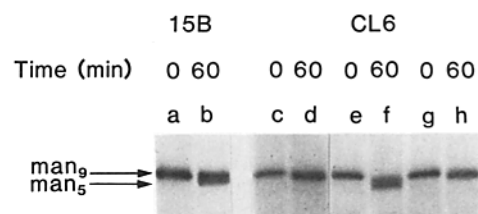


Figure 7. Interphase donor ER is active in vitro in the presence of mitotic homogenates. 15B (lanes a and b) or clone 6 (lanes c-h) interphase cell homogenates (15 µg) containing [³⁵S]methionine-labeled ts045 G protein were incubated alone (lanes c and d), in the presence of 55 µg protein of 15B mitotic cell homogenate (lanes a, b, e, and f), or in the presence of 55 µg 15B interphase cell homogenate (lanes g and h), as described in Materials and Methods, on ice (0 min), or at 32°C for 60 min. Samples were processed for SDS gel electrophoresis to separate the untrimmed and trimmed forms of G protein as described in Materials and Methods.

Interphase Donor Homogenates are Active In Vitro in the Presence of Mitotic Homogenates

Several interpretations could account for the efficient activity of mitotic cell homogenates for export of G protein from the ER. The first possibility is that the mitotic homogenates provide either an active donor ER, an active acceptor Golgi compartment, or are essential for preparation of both compartments. A second possibility is that during incubation of the mitotic cell homogenate in vitro, physiologically disassembled organelles are reassembled to form functional compartments. This interpretation is based on the recent evidence that the nuclear envelope (and possibly other organelles) present in homogenates prepared from CHO cells are reassembled in vitro using similar incubation conditions (14).

To test for these various possibilities, a donor ER membrane containing homogenate was prepared from an asynchronous interphase 15B cell population that contained [³⁵S]methionine-labeled G protein in the ER. As shown previously, G protein is not processed when interphase cell homogenates are incubated in vitro (see Fig. 1 A). If the donor ER homogenate containing G protein prepared from interphase cells is incubated with a mitotic 15B cell homogenate containing the acceptor Golgi compartment and trimming of G protein is not observed, then it must be concluded that the interphase donor ER is a labile compartment that has to be provided by the mitotic cell homogenate. On the other hand, if trimming is observed, then mitotic homogenates provide either an active Golgi acceptor compartment, or activate transport of G from the interphase donor ER compartment in some other fashion. As shown in Fig. 7 (lane b), incubation of an interphase donor ER homogenate containing G protein with the mitotic cell homogenate resulted in 50% of the total G protein being processed to the trimmed form.

To provide additional evidence that export of G protein can occur from an ER compartment prepared from interphase cells, a donor fraction was prepared from the mouse L cell line clone 6 (25). In the infected clone 6 cell line, G protein is transported to the cell surface with oligosaccharides in the high mannose (man₉GlcNAc₂) form (42), indicating that processing of G protein, not transport, is the only apparent defect in this mutant (25, 42). For trimming to occur in vitro, G protein must be transferred from the ER membranes found in the homogenate of the clone 6 cell line to the acceptor Golgi compartment containing α-mannosidase I provided by the separate addition of the 15B cell homogenate to the incubation (see Fig. 10). When homogenates were prepared from interphase clone 6 cells in which ts045 G protein was labeled at the restrictive temperature with [³⁵S]methionine, no trimming was observed during incubation in vitro in the presence of uninfected interphase 15B cell homogenates (Fig. 7, lane h). In contrast, 60% of the G protein oligosaccharides were trimmed in vitro by incubation in the presence of a homogenate prepared from the uninfected mitotic 15B cell line (Fig. 7, lane f). The extent of G protein oligosaccharide processing was found to be proportional to the concentration of the mitotic 15B membranes present in the assay (Fig. 8). These results emphasize the importance of interorganelle transfer of G protein in vitro, and support the interpretation that the efficiency previously observed during transport of G protein present in the ER of mitotic cell homogenates (Fig. 1 B) is not simply a consequence of a labile donor ER compartment.

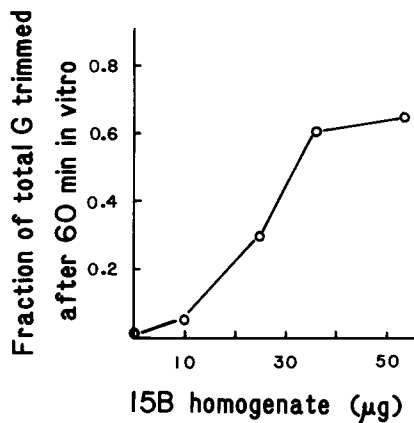


Figure 8. Dependence of processing in vitro on the acceptor Golgi compartment containing α -mannosidase I. Clone 6 interphase cell homogenates (20 μ g protein) containing [35 S]methionine-labeled ts045 G protein were incubated in vitro in the presence of increasing concentrations of a washed 15B mitotic homogenate, and 60 μ g of 15B mitotic cytosol (see Materials and Methods). Samples were incubated for 60 min at 32°C, processed for SDS gel electrophoresis, and the fraction of the total G protein in the trimmed form was determined as described in Materials and Methods.

When wild-type G protein was labeled with [35 S]methionine in infected interphase clone 6 cells for 3 min and chased in vivo at 37°C before preparation of cell homogenates, trimming in vitro was lost with a half-time of \sim 12 min (Fig. 9). Under these conditions, G protein oligosaccharides are found in the $\text{man}_5\text{GlcNAc}_2$ form during transport to the cell surface in vivo and are thus a possible substrate for α -mannosidase I at each stage of the secretory pathway (see Fig. 10). We can conclude from these results that G protein only transiently occupies an early donor ER compartment in vivo, which is active in vitro for transport to the *cis* Golgi compartment containing α -mannosidase I. These results rule out the possibility that nonspecific fusion between membrane struc-

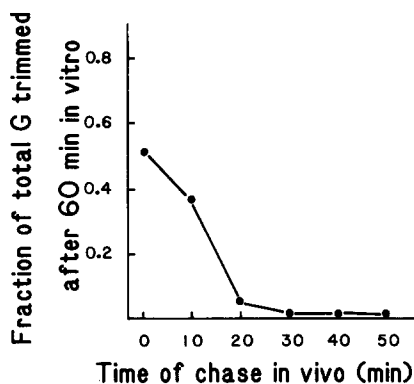


Figure 9. G protein only transiently occupies a compartment during transport to the cell surface in vivo that is active in vitro. Wild-type virus-infected clone 6 interphase cells were labeled with [35 S]methionine for 3 min at 37°C before chase in the presence of excess unlabeled methionine. At each time point a cell homogenate was prepared and 25 μ g was incubated in vitro in the presence of a cell homogenate prepared from uninfected mitotic 15B cells (75 μ g) for 60 min at 37°C as described in Materials and Methods. Samples were processed, and the fraction of the total G protein trimmed to the $\text{man}_5\text{GlcNAc}_2$ form in vitro for each time point of chase in vivo was determined as described in Materials and Methods.

tures containing G protein and the *cis* Golgi compartment is responsible for the observed trimming, and clearly illustrates the high specificity of the reconstituted transport step.

Discussion

Trimming Measures Transport of G Protein between the ER and a *cis* Golgi Compartment

The reconstitution of transport of protein through the secretory pathway in vitro poses a major challenge in developing novel assays that faithfully measure the steps occurring in vivo. A number of criteria are important to establish the validity of the events observed in vitro to those occurring in vivo. It is necessary to define the cellular localization of the transported protein before incubation (donor compartment), and the processing activity marking the acceptor compartment. These data identify the segment of the total cellular pathway being studied in vitro. A second consideration is to demonstrate that the events occurring in vitro are a consequence of transfer between sealed membrane-bound compartments. In addition, authentic transport in vitro should occur using physiological conditions with the efficiency, specificity, and kinetics approaching those observed in vivo, and measure a vectorial process that is dependent upon both donor and acceptor membranes. Using these criteria, we have provided evidence that a new segment of the secretory pathway has been reconstituted in vitro, transport of protein between the ER and the *cis* Golgi compartment.

To ensure that the events measured here were not a measure of inter-Golgi transport (3, 21, 22), the mutant virus ts045 was used to prepare extracts in which G protein was localized to the ER in the high mannose ($\text{man}_{8-9}\text{GlcNAc}_2$) form before preparation of cell homogenates (4). The acceptor compartment was chosen to reflect an early Golgi-associated processing step conferred by α -mannosidase I. α -Mannosidase I has been purified from Golgi fractions (67). Trimming of G protein to the $\text{man}_5\text{GlcNAc}_2$ species is the activity characteristic of the Golgi-associated α -mannosidase I and not the ER-associated mannosidases that trim resident ER proteins to, but not further than, the $\text{man}_{8-6}\text{GlcNAc}_2$ species (11, 28, 29, 43, 58). This result is supported by kinetic studies in which trimming in vivo to the $\text{man}_5\text{GlcNAc}_2$ form (4) is coincident with the morphological data showing transfer of G protein to the Golgi complex in vivo (7, 8). The Golgi stack is a polarized organelle consisting of *cis*, *medial*, and *trans* compartments (17). While the morphological localization of α -mannosidase I in the Golgi stack remains to be established with immunoelectron microscopy, we recently provided functional evidence that α -mannosidase I occupies a *cis* Golgi compartment (1) preceding the *medial* Golgi compartment containing *N*-acetylglucosaminyl transferase I (16). Thus, transport in this assay measures a stage preceding inter-Golgi transfer in vitro (22). Since both ts045 G protein and wild-type G protein are transported from the ER with similar efficiency in vitro, the transport events observed here are not simply a measure of transfer of ts045 G protein from a unique temperature-sensitive site in the ER.

The following lines of evidence suggest that transport of G protein in vitro occurs along the same compartmentalized pathway observed in vivo. (a) Addition of detergent to solu-

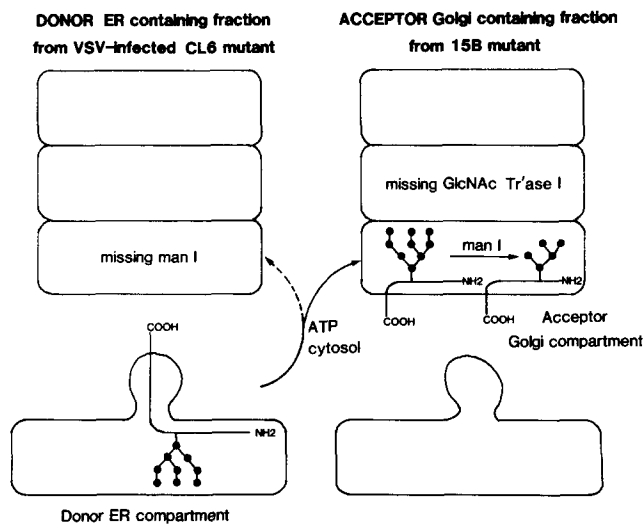


Figure 10. Working model for transport of G protein between the ER and the Golgi complex in vitro. The donor ER compartment is prepared from the clone 6 mutant cell line defective in the processing of G protein past the $\text{man}_8\text{GlcNAc}_2$ form in vivo. The donor ER is incubated in vitro in the presence of an acceptor Golgi compartment containing α -mannosidase I prepared from the 15B cell line. Trimming of G protein in vitro to the $\text{man}_5\text{GlcNAc}_2$ species occurs only when G protein is transported between the two distinct compartments in the presence of cytosol and ATP.

bilize membranes before incubation inhibited trimming of G protein to the $\text{man}_5\text{GlcNAc}_2$ species. (b) Incubation of donor membranes with trypsin before and after incubation indicated that G protein is predominately found in a sealed vesicle with the proper asymmetric membrane orientation. (c) Transport in vitro reflected the efficiency and kinetics observed in vivo. In homogenates that were devoid of intact cells, up to 80% of the G protein that was found in a sealed membrane-bound compartment before incubation was processed to the $\text{man}_5\text{GlcNAc}_2$ species after 60 min of incubation. This result is in contrast to a recent report in which an in vitro assay for transport of invertase from the ER to the Golgi complex using interphase yeast cell homogenates was proposed (27). Efficiency of transport to the Golgi complex was found to be 2–5% of the total invertase present in the ER before incubation in vitro. This low level of transport could not be reliably detected during incubation of 15B interphase homogenates with the present assay. (d) Transport in vitro requires energy in the form of ATP, as is required in vivo (4). (e) Transport requires factors present in the soluble fraction (cytosol) of crude cell homogenates. Soluble cytosolic components are also essential for inter-Golgi transport in vitro (3, 21).

We have not been able to reproducibly reconstitute in vitro the temperature-sensitive phenotype of ts045 export from the ER observed in vivo. Whether this reflects a problem related to homogenization of cells at the permissive temperature (on ice), or is indicative that at least some aspects of the initial temperature-sensitive step observed during export in vivo are not faithfully reconstituted in vitro, remains to be determined. With the recent report of the differences in the oligomeric structure of ts045 G protein at the permissive and restrictive temperatures in vivo (36), we can test these possibilities directly.

Specificity of trimming was assessed by several different criteria. The requirement for G protein in a sealed membrane compartment before and after incubation argues against the interpretation that a soluble form of α -mannosidase I is responsible for the trimming observed in vitro. This result is consistent with the observation that cytosol prepared from homogenates of the clone 6 cell line defective in processing of G protein past the $\text{man}_8\text{GlcNAc}_2$ oligosaccharide form in vivo supports trimming in vitro (25, 67). G protein oligosaccharides were shown to be directly processed in vitro to the α -mannosidase I product, the $\text{man}_5\text{GlcNAc}_2$ oligosaccharide form. The intermediate $\text{man}_6\text{GlcNAc}_2$ and $\text{man}_7\text{GlcNAc}_2$ structures, products characteristic of trimming by ER-associated α -mannosidases in vivo (12, 43, 58), were not observed. Ts045 G protein is slowly processed to these intermediate structures in vivo by prolonged incubation (60 min) of cells at the restrictive temperature (4, 29), or during incubation of mitotic cells at the permissive temperature in the presence of nocodazole (20). These results suggest that oligosaccharides present on G protein are potential substrates for these ER-associated α -mannosidases that trim resident ER proteins to the $\text{man}_6\text{GlcNAc}_2$ form, but that G is likely to be exported rapidly to the Golgi stack where it is principally processed by α -mannosidase I. Incubation in the presence of the inhibitors DMM and swainsonine provided direct evidence that processing to the $\text{man}_5\text{GlcNAc}_2$ form in vitro by the acceptor Golgi compartment is a result of the DMM-sensitive α -mannosidase I.

A second line of evidence for specificity was provided by preparation of the donor ER compartment from the clone 6 cell line. As shown in the model presented in Fig. 10, G protein present in the donor clone 6 ER was trimmed in vitro only when provided with a 15B acceptor Golgi compartment containing α -mannosidase I. This result eliminates the trivial explanation that the processing observed here reflects an anomalous mixing (fusion) of ER and Golgi membranes during homogenization of cells, and provides direct evidence for transfer between distinct donor ER and acceptor Golgi compartments in vitro. A rapid loss of G protein from a donor compartment that is active in vitro was observed during transport to the cell surface in vivo in the clone 6 cell line. While it is not possible to determine biochemically the rate of G protein transport from the ER to the Golgi complex in the clone 6 cell line (since the first Golgi-associated processing step is defective), we have found that the transport time of G protein to the cell surface in clone 6 is similar to that observed in the clone 15B cell line (Balch, W. E., unpublished results). Since loss of donor activity in vitro was similar to the half-time observed for export of G protein from the ER in the 15B cell line (4), these data support the conclusion that the principal donor in vitro is the ER. A transient donor population is also observed for inter-Golgi transfer in vitro (22, 59, 60). However, in this assay, G protein must be transported to the *cis* Golgi compartment before activity is observed in vitro. Both the transient nature of the ER donor compartment containing G protein, and the high efficiency of trimming observed in vitro argue against a model in which nonspecific fusion between compartments is responsible for the events observed here.

Cytoplasmically oriented components present on the donor compartment are required for transport in vitro. This was demonstrated by loss of activity after trypsin digestion,

or by treatment of membranes with *N*-ethylmaleimide (NEM). Compartments active in inter-Golgi transport in vitro are similarly trypsin- and NEM-sensitive (2, 5). Trypsin lability of the donor compartment may reflect a requirement for additional ER membrane components. Alternatively, since the proteolysis conditions used here cleave the carboxyl terminus of G protein, these results may reflect a requirement for the cytoplasmic domain of G protein as is observed in vivo (30). G protein expressed from the cloned cDNA of the membrane-anchored construction missing the cytoplasmic carboxyl terminus domain is blocked in export from the ER (57). Characterization of the transport in vitro of the various truncated forms of G protein present in the ER of homogenates prepared from cells transfected with deletion mutants of the cloned cDNA of G protein (24, 56, 57) should provide evidence to address these possible requirements for export.

In the present analysis we cannot unequivocally rule out the interpretation that the α -mannosidase I present in the 15B Golgi cisternae is delivered to the ER in vitro in a fashion counter to the normal vectorial flow of protein in vivo. In this interpretation, the Golgi compartments containing α -mannosidase I could putatively fuse with the ER compartment containing G protein, resulting in trimming of the ER-associated G protein molecules. A prediction from this interpretation is that incubation of the clone 6 donor ER with mitotic acceptor Golgi prepared from wild-type CHO cells that contain additional Golgi-associated processing enzymes would result in processing of G protein to the complex oligosaccharides forms containing additional *N*-acetylglucosamine, galactose, and sialic acid. This result is not observed. G protein is trimmed to the $\text{man}_5\text{GlcNAc}_2$ form, as is observed in the presence of an acceptor Golgi prepared from the 15B cell line (Beckers, C., and W. E. Balch, unpublished results), emphasizing the point that the present assay conditions promote efficient transfer only between the ER and the *cis* Golgi compartment. While the kinetics, efficiency, and high specificity of the processing events observed here support a model in which transport between ER and Golgi stack has been reconstituted, direct evidence for transfer of G protein to the Golgi cisternae will require application of immunoelectron microscopy, a line of investigation we are currently pursuing.

The Role of Mitotic Homogenates for Transport In Vitro

Based on the observation that the Golgi apparatus and nuclear envelope are disassembled during mitosis, we have used electron microscopy and serial thin sections of mitotic cells to determine that the nonnuclear RER is similarly disassembled (Hermanowsky, A., and W. E. Balch, manuscript in preparation). It was proposed that such physiological disassembly would provide organelles more amenable to cell breakage protocols than the intact structures found in interphase cell populations. The appearance of the trimmed form of G protein during co-incubation of an interphase donor ER and a mitotic acceptor Golgi compartment ruled out the simple interpretation that mitotic homogenates are only important for preparation of a functional donor ER compartment. The data presently support two other models regarding the role of mitotic membranes in transport in vitro.

In the first interpretation of the role of mitotic membranes in the assay, the acceptor Golgi compartment is the homog-

enization-sensitive component. This is because G protein present in an interphase clone 6 ER compartment was efficiently processed in vitro when incubated with a mitotic 15B cell homogenate. The converse experiment, incubation of G protein present in mitotic clone 6 donor ER with an acceptor Golgi compartment provided by an asynchronous interphase 15B homogenate, has not been possible due to our inability to prepare infected, mitotic clone 6 cell populations. The reason(s) behind the apparent ability of the interphase *cis* Golgi compartment to function efficiently as a donor during inter-Golgi transfer in vitro (3, 21, 22), but not as an acceptor for export from the ER in vitro may reflect either inactivation of the compartmentalized trimming activity of α -mannosidase I per se, or differential sensitivity to homogenization of Golgi cisternae function in these two stages of the secretory pathway.

A second interpretation consistent with our results is that the mitotic homogenate promotes reassembly or repair of organelles in vitro. It has recently been established that in addition to organelle disassembly, all vesicular trafficking is arrested in mitotic cells (20). G protein accumulates in the ER in the $\text{man}_{8-9}\text{GlcNAc}_2$ oligosaccharide forms in mitotic cells arrested at metaphase with nocodazole (20). Three lines of evidence support the interpretation that factors required for maintenance of the mitotic state are inactivated during cell homogenization. In addition to appearance of the trimmed form of G protein in vitro reflecting reinitiation of transport, interphase cytosol was found to substitute for mitotic cytosol, and we have observed that both the nuclear envelope and the RER are reassembled during incubation of the donor and acceptor crude homogenates in vitro (Hermanowsky, A., and W. E. Balch, unpublished observations). The appearance of reassembled nuclei in vitro from CHO homogenates using similar incubation conditions has been well documented (14). In addition, nuclei reassembled in vitro have been shown to be a useful model system for studying import of protein into the nucleus in a cell-free system (47), attesting to the remarkable facility of reassembled organelles to authentically reconstitute functions observed in vivo. If the efficiency of G protein export observed here is a consequence of reassembly of donor ER and Golgi acceptor membranes into functional organelles, then the apparent donor activity of interphase ER membranes containing G protein may be a result of their incorporation into the reassembling mitotic ER.

Both interpretations of the role of mitotic homogenates in the transport of G protein in vitro support a model in which we are measuring a specific transport event between the ER and a *cis* Golgi compartment. Through selective enrichment of intermediate steps in transfer in vitro, and fractionation of the required components, a number of new approaches can now be used to understand the biochemical machinery required for both the export and delivery steps. In addition, the choice of G protein as a model system for ER export provides the potential for contrasting the requirements for this early stage to the requirements for inter-Golgi transfer (5, 49, 73, 74).

Providing an enzymological definition to the mechanisms that regulate transport from the ER should provide insight into the biochemical rules for organization of the secretory pathway in the living cell.

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