Calcium and GTP: Essential Components in Vesicular Trafficking between the Endoplasmic Reticulum and Golgi Apparatus

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Abstract. Ca²⁺ and GTP hydrolysis are shown to be required for the transport of protein between the ER and the cis-Golgi compartment in semiintact cells, an in vitro system that reconstitutes transport between intact organelles. Transport was inhibited rapidly and irreversibly in the presence of micromolar concentrations of the nonhydrolyzable GTP analogue, GTPγS. The transport block in the presence of GTPγS was found to be distal to a post-ER, pre-Golgi compartment where proteins accumulate during incubation at 15°C. In addition, transport was completely inhibited in the absence of free Ca²⁺. A sharp peak defining optimal transport between the ER and the cis-Golgi was found to occur in the presence of 0.1 μM free Ca²⁺. Inhibition of transport in the absence of free Ca²⁺ was found to be fully reversible allowing the step inhibited by GTPγS to be assigned to a position intermediate between the ER and the Ca²⁺ requiring step. The results suggest that GTP hydrolysis may trigger a switch to insure vectorial transport of protein along the ER/Golgi pathway, and that a free Ca²⁺ level similar to the physiological levels found in interphase cells is essential for a terminal step in vesicle delivery to the cis-Golgi compartment.

The secretory pathway of the eukaryotic cell is populated by an extensive collection of carrier vesicles trafficking protein from the ER through the Golgi compartments to the plasma membrane. The biochemical mechanisms basic to interorganelle transport have only recently become approachable due to the successful reconstitution of specific transport steps using cell-free systems and genetic analysis. ER to Golgi transport (Beckers et al., 1987; Baker et al., 1988) and inter-Golgi transport (Balch et al., 1984; Orci et al., 1987; Melancon et al., 1987) can be readily studied in vitro using organelles prepared from mammalian cell lines. Genetic analysis using the yeast Saccharomyces cerevisiae has resulted in the identification of at least 25 essential genes necessary for transport from the ER to the cell surface (Novick et al., 1980). Of these, at least 11 gene products are essential for ER to Golgi transport (Newman and Ferro-Novick, 1987).

A number of lines evidence are accumulating to implicate a role for GTP hydrolysis in intracellular transport. GTP-binding proteins are essential for protein transport in yeast. The SEC4 gene product is a small (23.5 kD) GTP-binding protein associated with the cytoplasmic face of the plasma membrane and secretory vesicles (Goud et al., 1988; Salmi- nen, 1987). Cells carrying the temperature-sensitive allele of SEC4 (sec4-8) accumulate mature, glycosylated invertase in numerous small vesicles prepared from mammalian cell lines. Genetic analysis using the yeast Saccharomyces cerevisiae has resulted in the identification of at least 25 essential genes necessary for transport from the ER to the cell surface (Novick et al., 1980). Of these, at least 11 gene products are essential for ER to Golgi transport (Newman and Ferro-Novick, 1987).

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from permeabilized rat mast cells. Not all cell lines respond in this fashion. In permeabilized rat insulinoma cells (Vallar et al., 1987), and neutrophils (Barrowman et al., 1986) GTP-mediated stimulation of secretion is independent of Ca++.

In the present study we explore whether GTP hydrolysis and Ca++ are required for transport of protein between the ER and the cis-Golgi compartment using a cell-free system that reconstitutes transport between intact organelles (Beckers et al., 1987). We find that both GTP hydrolysis and Ca++ are essential for this early step in the secretory pathway. Transport is inhibited in the presence of micromolar concentrations of GTPyS at a post-ER, pre-Golgi compartment. Transport in vitro occurs only in the presence of 0.01-0.1 μM free Ca++, physiological concentrations typical of interphase cells. The results support the interpretation that the steps in ER to Golgi transport affected by GTPyS and Ca++ are independent and kinetically distinct.

Materials and Methods

Materials

Semiintact cells were prepared from clone 15B CHO cells infected with the ts045 strain of vesicular stomatitis virus (VSV) using the swelling method as described previously (Beckers et al., 1987). Trans 35S-label ([35S]methionine and [35S]cysteine, >1,000 Ci/mmol) was purchased from ICN Bio- medicals, Inc. (Irvine, CA). Endoglycosidase D (Endo D) was either purchased from Boehringer-Mannheim Biochemicals (Indianapolis, IN), or prepared from the culture supernatant of Diplococcus pneumoniae (Glassgow et al., 1977). Nucleotide analogues were purchased in the form of their phosphorothioates. Semiintact cells were prepared from the culture supernatant of ts045 strain of vesicular stomatitis virus (VSV) using the swelling method described by Howell and Gomperts (1987) to yield the final free Ca++ indicated. In all cases, the final pH of the VSV-CHO/Ca++ buffer was adjusted to pH 7.2 before use. Under these conditions no effect of addition of the VSV-CHO/Ca++ buffer was observed upon the pH of the incubation. Transport efficiency between different preparations of semiintact cells ranges from 30 to 60%, with the routine values of 40-50%. All results reported are highly representative of assays from at least four independent preparations of semiintact cells.

Endo D Digestion and Quantitation of Transport

After termination of the transport reaction by transfer to ice, the membranes were pelleted by a brief (20 s) centrifugation in an Eppendorf microcentrifuge at top speed. Under these conditions all of the labeled VSV-G protein remains associated with the semiintact cells and is recovered in the pellet. The pellet was subsequently solubilized in an endo D digestion buffer (0.1% Triton X-100, 5 mM EDTA, and 50 mM phosphate, pH 6.5). 2.5 μl of endo D were added, and the mixture incubated at 37°C for at least 5 h. Digestion was terminated by the addition of a 5 x concentrated gel sample buffer (10% SDS, 80 mM DTT, 50% glycerol, 0.001% bromophenol blue in 312.5 mM Tris-HCl, pH 6.8) and boiled for 3 min. The samples were analyzed by SDS-PAGE using 7.5% polyacrylamide gels (Laemmli, 1970). After autoradiography of the dried gels, the fraction of VSV-G protein transported was determined by densitometry (Balch and Beckers, 1987) as calibrated previously using Biogel P4 oligosaccharide analysis (Balch et al., 1986). In brief, all autoradiographs were scanned using a Hoefer Scanning Gel Densitometer connected to an IBM-XT and Hoefer GS350 software. All experiments contain a zero time point to determine background as well as other relevant controls such that each autoradiograph is internally standardized. For each determination, a vertical line is dropped to the baseline at the inflection point separating the manα2-containing band and oligosaccharide-deficient band. The integrated value of the lower band (the transported form of G protein) is divided by the total integrated value of both bands as the measure of the fraction of G protein transported. Only density values in the linear range of the film are used for calculations. Multiple scans of the same gel yields an error range of 5-10% in the calculated fraction of G protein transported. Reproducibility of a given experimental protocol using a preparation of semiintact cells is within 5-10% for all reported values.

Results

GTPyS and AIFc Inhibit Transport between the ER and Golgi

Transport of protein between the ER and the cis-Golgi compartment can be reconstituted in vitro using cells in which the plasma membrane has been physically perforated to allow access to the interior of the cell containing intact ER and Golgi compartments (Beckers et al., 1987). Transport in these "semiintact" cells can be quantitated by following the processing of the high-mannose (manα2) core-oligosaccharide of the VSV-G protein using infected Chinese hamster ovary (CHO) cells. The high-mannose oligosaccharide is rapidly processed to the manα3 oligosaccharide form as a consequence of delivery of VSV-G protein to the first cis-Golgi compartment containing the trimming enzyme α-1,2-mannosidase I (Balch et al., 1986; Tabas and Kornfeld, 1979). In CHO 15B cells this manα3 oligosaccharide form is not further modified during transport to the cell surface. To reconstitute transport in vitro, CHO cells are infected with a temperature-sensitive strain of VSV, strain ts045. Ts045 is defective in the export of G protein from the ER at the restrictive (40°C), but not the permissive (30°C) temperature.
Cells are labeled in vivo with [35S]methionine for 10 min at 40°C to retain the labeled VSV-G protein in the ER. Subsequently, semintact cells are prepared on ice (Beckers et al., 1987). After incubation in vitro, the amount of VSV-G protein transported to the cis-Golgi compartment can be readily quantitated by the appearance of the man5 oligosaccharide. This is accomplished by post incubation in the presence of detergent with endo D which specifically removes the man5 oligosaccharide, leading to an increased electrophoretic mobility of the transported VSV-G protein during SDS-PAGE (Beckers et al., 1987) (Fig. 1, solid circles). Transport from the ER to the cis-Golgi compartment using this cell-free system requires the addition of the soluble cytoplasmic components lost during preparation of semiintact cells, and energy in the form of ATP (Beckers et al., 1987).

A number of recent reports have implicated a role for GTP in the delivery of protein to the cell surface in yeast (Goud et al., 1988; Salminen and Novick, 1987; Segev et al., 1988; Schmitt et al., 1988) and mammalian cells (Burgoyne, 1987; Howell et al., 1987), and in the transport of protein between subcompartments of mammalian Golgi (Melancon et al., 1987). To determine whether transport of protein from the ER to the cis-Golgi compartment requires GTP, we prepared semintact cells that were washed to remove soluble factors. These cells were incubated with ATP and a cytosol fraction that was passed through a G25 gel-filtration column to remove low molecular mass components. No stimulation of transport was observed by the addition of increasing concentrations of GTP to the complete assay, with a slight inhibition of transport (15%) observed at 1 mM GTP (data not shown).

A possible explanation for this lack of stimulation of transport by GTP was that our wash conditions were inadequate to deplete the endogenous guanine-nucleotide pool. Since in vitro transport reactions are performed in the presence of ATP, all guanine nucleotides bound to cytosolic, membrane-associated, or cytoskeletal GTP-binding proteins are recycled and maintained as GTP due to the activity of nucleoside diphosphate kinase which catalyzes transfer of the γ-phosphate group from ATP to GDP yielding ADP and GTP (data not shown). To circumvent the problem imposed by an endogenous GTP pool, analogues of GTP were tested for their effect on transport. GTPγS and other nonhydrolyzable analogues have been previously demonstrated to irreversibly activate cell surface “G” proteins (Gilman, 1987; Casey and Gilman, 1988), inhibit protein synthesis (Bourne, 1988), and augment the assembly of microtubules (Kirschner, 1981). As shown in Fig. 1, addition of 10 μM GTPγS resulted in inhibition (80%) of the VSV-G protein transport to the cis-Golgi compartment. This value was found to vary from 60 to 90% between different semiintact cell preparations. A similar result was observed in the presence of 50 μM Al3+ and 5 mM KF. Al3+ and F⁻ form a complex, AlF4⁻, which in the presence of GDP mimics the γ-phosphate group of GTP (Sternweiss and Gilman, 1982). No inhibition was observed by Al3+ or F⁻ when added separately (data not shown). To test

![Figure 1. GTPγS and AlF4⁻ inhibit in vitro transport of VSV-G protein between the ER and cis-Golgi compartment.](image)
if the inhibition of transport by GTPγS was specific, GTPγS was added to the in vitro assay in the presence of 1 mM GTP. These conditions have been previously demonstrated to block the effects of GTPγS or GTP-binding proteins (Gilman, 1987). In the absence of GTP, maximal inhibition of transport was observed using 1 mM GTPγS (Fig. 2, solid circles) with half-maximal inhibition of transport observed at 0.3 mM GTPγS. In contrast, no inhibition of transport by up to 3 mM GTPγS was observed in the presence of 1 mM GTP (Fig. 2, open circles). AIFc was also found to irreversibly inhibit transport. Half-maximal inhibition was observed at 3 μM AIFc in the presence of 5 mM F− (data not shown). Transport was not inhibited by addition of GDPβS (100 μM) or ATPγS (100 μM) (data not shown). However, the non-hydrolyzable analogue GMP-PCP was found to inhibit transport. Half-maximal inhibition of transport by GMP-PCP was observed at 100 μM (data not shown), indicating that inhibition of ER to Golgi transport by GTPγS is not a consequence of protein kinase-dependent thiolation. Inhibition of transport is highly sensitive, and is specific for the nonhydrolyzable analogues of guanine-nucleotides, suggesting that GTP hydrolysis may play an essential role in trafficking between the ER and Golgi compartment.

**GTPγS Inhibition Is Rapid and Irreversible**

To establish whether inhibition by GTPγS requires incubation conditions that support ER to Golgi transport, the complete reaction mixture containing semintact cells, ATP, and cytosol was first preincubated in the presence of 3 mM GTPγS for 60 min on ice, conditions which do not promote VSV-G transport, before the addition of 1 mM GTP (to quench free GTPγS), and reincubation at the permissive temperature. No inhibition of transport was observed (data not shown), suggesting that GTPγS must be actively metabolized into the GTPγS sensitive site. In contrast, when cells were preincubated for increasing time at 30°C in the presence of GTPγS before the addition of GTP (Fig. 3, solid circles), inhibition of transport was found to be rapid (tn ~ 3 min). After 20 min preincubation in the presence of GTPγS at 30°C, transport to the cis-Golgi compartment could not be rescued by the addition of 1 mM GTP to the assay, and is therefore irreversibly inactivated at this time point. Since delivery to the cis-Golgi compartment occurs with a tn of ~30 min, it is clear that GTPγS inhibition precedes processing by α-1,2-mannosidase I.

**Only the Membrane Fraction Is Sensitive to GTPγS**

Transport in vitro between the ER and Golgi compartments in semintact cells requires the addition of a soluble cytoplasmic fraction. To determine if this soluble fraction was sensitive to GTPγS, cytosol and ATP were preincubated in the absence (Fig. 4, lanes a and b) or presence (lanes c and d) of 3 μM GTPγS for 20 min at 30°C. Subsequently, semintact cells and GTP (to quench GTPγS) were added to the assay to determine if the cytosol still retained the capacity to support transport. As shown in lanes c and d (Fig. 4), preincubation of cytosol in the presence of GTPγS did not inhibit the effect on transport.

While no inhibition was observed by preincubation of the cytosol and ATP in the presence of GTPγS, a second possibility was that a factor(s) sensitive to GTPγS in the cytosol required coincubation with the ER or Golgi compartments for expression of inhibitory activity. We first tested whether GTPγS inhibition was sensitive to the concentration of cytosol, or ratio of the cytosol and membrane fractions present in the assay. As shown in Fig. 5, equivalent inhibition of transport by GTPγS was observed across the entire range of cytosol concentrations tested. We next tested whether a factor present in the cytosol could be selectively inactivated or removed by preincubation of membranes, cytosol and GTPγS. To address this question, a large excess (sixfold) of uninfected, unlabeled semiintact cells were preincubated in the presence of cytosol and GTPγS for 20 min at the permissive temperature. Under these conditions, inhibition of transport by GTPγS is complete. The membranes were dis-
Preincubation of cytosol without (Fig. 4, lanes b and d) or with (Fig. 4, lanes f and h) membranes in the presence or absence of GTPγS did not result in the inactivation of any factor required for inhibition of transport by GTPγS. A similar experimental protocol was followed using up to 10 μg of purified ER or Golgi membranes (Balch et al., 1984) with identical results (data not shown). These results show that none of the cytosolic factors required for transport are irreversibly inhibited by GTPγS and that a factor required for inhibition cannot be removed from the cytosol.

From the above results, it was likely that the GTPγS sensitive component in the assay was membrane associated. To provide additional evidence in support of this conclusion, the effect of GTPγS on semiintact cells was examined. Infected, labeled semiintact cells were preincubated in the presence of cytosol, ATP, and GTPγS for 20 min at 30°C. Membranes were pelleted and resuspended in the presence of fresh cytosol, ATP, and GTP. As shown in Fig. 6 (compare lanes b and e), no activity was observed, suggesting that GTPγS inactivates the membrane compartments provided by the semiintact cells. Incubation of inactivated membranes in the presence of fresh cytosol and excess unlabeled semiintact cells was similarly ineffective in recovering transport (data not shown). We conclude from these results that GTPγS irreversibly inhibits a membrane-associated reaction that cannot be reactivated by addition of a factor present in untreated cytosol or supplied in trans by untreated membranes. Since preincubation of semiintact cells in the absence of cytosol or ATP results in an irreversible inactivation of transport (Beckers, C. J. M., and W. E. Balch, unpublished data) it is presently not possible to test whether preincubation of mem-

Figure 4. The cytosol fraction is not affected by preincubation in the presence of GTPγS. Cytosol was preincubated at 30°C with an ATP regenerating system in the absence (a, b, e, and f) or presence (c, d, g, and h) of 3 μM GTPγS. Unlabeled semiintact cells (SIC) (150 μg of protein) were included in reactions e, f, g, and h. After 20 min the reaction mixtures were centrifuged for 10 min in an air-fuge (100,000 g; Beckman Instruments Inc.) to remove the membranes. The supernatant fraction was supplemented with GTP (a, c, e, and g) or GTPγS (b, d, f, and h) at final concentrations of 1 mM and 3 μM, respectively, and reincubated in the presence of fresh labeled semiintact cells for 60 min.

carded by pelleting, and the cytosol fraction was used in a second incubation that contained infected, labeled semiintact cells and 1 mM GTP. As shown in Fig. 4 (lanes e and g), full activity was observed using cytosol preincubated with membranes in either the presence or absence of GTPγS.

Figure 5. Inhibition by GTPγS is not affected by the concentration of the cytosol used to support transport. Standard transport reactions were performed with the indicated amount of cytosol in the absence (●) or presence (○) of 3 μM GTPγS.

Figure 6. The membrane fraction is irreversibly inactivated by GTPγS. Labeled semiintact cells (SIC) were incubated for 20 min using standard conditions at 30°C in the absence (a, b, and c) or presence (d, e, and f) of 3 μM GTPγS. After 20 min the membranes were pelleted by a brief (5 s) centrifugation in an Eppendorf microcentrifuge. Cells were resuspended in buffer and either transferred to ice (a and d) or reincubated with fresh cytosol and ATP in the presence of 1 mM GTP (b and e) or 3 μM GTPγS (c and f).
branes with GTPγS in the absence of cytosol or ATP would similarly inactivate the donor ER or acceptor Golgi function.

**Transport Rapidly Proceeds Past the GTPγS-sensitive Step**

The transport of VSV-G protein from the ER to the cis-Golgi compartment proceeds through biochemically distinguishable kinetic steps. Processing of G protein by α-1,2-mannosidase I occurs immediately upon delivery of G protein to the cis-Golgi compartment (Beckers, C. J. M., and W. E. Balch, unpublished data). Both in vivo (Balch et al., 1986) and in vitro (Fig. 1), a lag of 10-15 min is followed by a period in which the delivery of VSV-G protein to the cis-Golgi compartment is linear with time. The lag period probably represents steps in transport related to vesicle formation and targeting to the cis-Golgi compartment. To define the temporal location of the GTPγS-sensitive step with respect to possible transport intermediates, a complete cocktail containing semintact cells, cytosol, and ATP was preincubated for increasing time at 30°C before the addition of GTPγS (Fig. 7). After addition of GTPγS the incubation was continued until a total incubation time of 90 min had elapsed. This protocol allows any VSV-G protein that has been transported past the GTPγS-sensitive step at the time of addition of inhibitor to complete its journey to the cis-Golgi compartment. If the GTPγS-sensitive step occurs at a late step in transport, then the fraction of VSV-G protein between the GTPγS step and the cis-Golgi, which continues transport to the cis-Golgi, will be small. On the other hand, if the GTPγS-sensitive step is an early event in transport (during the lag period), then the fraction of VSV-G protein between the GTPγS-sensitive step and the cis-Golgi, which continues on to cis-Golgi, will be significantly larger. The amount of G protein processed by α-1,2-mannosidase I will depend on the quantity of VSV-G transported beyond the GTPγS sensitive step at the time of addition of inhibitor. As shown in Fig. 7 (open circles), processing is observed even at the earliest time points, suggesting that some VSV-G protein has progressed through the GTPγS-sensitive step. 30% of the VSV-G protein is transported beyond the GTPγS sensitive step by 10 to 15 min. Since transport to the cis-Golgi compartment occurs with a t1/2 of 30 min (Fig. 7, closed circles) and inhibition of transport by GTPγS occurs with a t1/2 of 3 min (Fig. 3), we conclude that GTP hydrolysis regulates an early event preceding Golgi fusion and processing by α-1,2 mannosidase I.

**Transport from the 15°C Intermediate to the cis-Golgi Is GTPγS Sensitive**

Incubation of cells at reduced temperatures has proven to be an exceptionally useful approach for the accumulation of secretory protein in unique subcellular compartments of the secretory pathway (Griffiths and Simons, 1986; Saraste and Kuismanen, 1984; Saraste et al., 1986; Tartakoff, 1986). We have previously established that VSV-G protein can be selectively accumulated in a post-ER, pre-Golgi compartment by preincubation of cells in vivo at 15°C (Balch et al., 1986). The compartment(s) occupied by VSV-G and other proteins after preincubation of cells for 60-90 min at 15°C (the "15°C intermediate") are morphologically heterogeneous, appearing to consist of vesicles and a collection of unique tubulovesicular smooth membrane compartments between the ER and the Golgi complex (Saraste and Kuismanen, 1984; Bergmann and Singer, 1983; Tooze et al., 1988). To test if transport of VSV-G protein present in this post-ER, pre-Golgi intermediate is sensitive to inhibition by GTPγS, VSV-G protein was labeled with [35S]methionine at the restrictive temperature and chased for 60 min at 15°C before in vivo preparation of the semintact cells. Transport of G protein from the 15°C intermediate to the cis-Golgi in vitro requires ATP and cytosol, but shows no lag period and is insensitive to homogenization conditions which inactivate export from the ER (Beckers, C. J. M., and W. E. Balch, unpublished data). As shown in Fig. 8, when these semintact cells were incubated in the presence of GTPγS, transport was efficiently blocked. Since the transport of the bulk of G protein from the ER to a site that is insensitive to GTPγS in vitro occurs with a t1/2 of 10-15 min, we conclude that export of G pro-

**Figure 7.** Transport proceeds rapidly past the GTPγS sensitive step. Incubations of semintact cells (SIC) were performed as described in Materials and Methods. At the indicated time (Δt) samples were either transferred to ice (○), or supplemented with GTPγS to a final concentration of 3 μM and incubation continued for a total reaction time of 90 min (●).

**Figure 8.** Transport from the 15°C intermediate to the cis-Golgi is inhibited by GTPγS. Semintact cells (SIC) were prepared from cells immediately after termination of the labeling period (a, b, and c), or after the labeled cells had been incubated for 60 min at 15°C (d, e, and f) (see Materials and Methods). The semintact cells were either kept on ice (a and d) or incubated for 90 min at 30°C under standard conditions in the absence (b and e) or presence (c and f) of 3 μM GTPγS.
Transport to the cis-Golgi Compartment Requires Ca^{2+}

YPTlp is a yeast GTP-binding protein (Schmitt et al., 1986) for which a number of mammalian homologs have been identified (Haubruck et al., 1987; Touchot et al., 1987). It is an essential component for orderly progression through the cell cycle and organization of the cytoskeleton (Schmitt et al., 1986), and for the transport of protein through the Golgi (Segev et al., 1988; Schmitt et al., 1988). Recently, second site mutations that suppress, in a temperature-dependent fashion, the dominant lethal phenotype of the yptlp^{+} mutant allele, were found to be rescued by raising the Ca^{2+} concentration of the growth medium at the nonpermissive temperature. In light of the wide range of calcium-sensitive activities disrupted by a YPTlp defect, it was proposed that YPTlp function may be essential in regulation of intracellular Ca^{2+} (Schmitt et al., 1988).

To test for a possible role of Ca^{2+} in the transport of protein between the ER and Golgi in mammalian cells, a complete cocktail containing semiintact cells, ATP, and cytosol was supplemented with 5 mM EGTA. Under these conditions the free Ca^{2+} is calculated to be less than 10^{-10} M. As shown in Fig. 9 (A, lane c) no processing of G protein to the man_{5} oligosaccharide form was observed. To determine if the transport of VSV-G protein between the ER and the cis-Golgi requires a free Ca^{2+} concentration relevant to physiological levels of Ca^{2+} found in vivo, semiintact cells were incubated in the presence of cytosol, ATP, and a EGTA/Ca^{2+} buffer to provide free Ca^{2+} over the concentration range of 10^{-11} to 10^{-10} (pCa range of 3 to 10). As shown in Fig. 10, optimal transport was only observed in the limited range of free Ca^{2+} of 0.01 to 0.1 μM. This level of free Ca^{2+} is similar to levels observed in interphase cells (0.1 to 0.15 μM) (Poenie et al., 1986). The transport efficiency was reduced at least fourfold when the free Ca^{2+} was raised from 0.1 (pCa = 7) to 0.3 μM (pCa = 6.5) or higher.

A trivial but unlikely explanation for these results was that processing by the α-1,2-mannosidase I in the lumen of the cis-Golgi compartment was inhibited by the membrane impermeant EGTA. Solubilized α-1,2-mannosidase IA is sensitive to EDTA (Kornfeld and Tabas, 1979). However, its activity is not stimulated by either Mg^{2+} or Ca^{2+} (Tulsiani and Touster, 1988). To provide additional evidence against the possibility that EGTA was inhibiting α-mannosidase I processing as opposed to transport, the kinetics of release of G protein from the EGTA block was examined. Semiintact cells were preincubated in the presence of cytosol, ATP, and EGTA for 60 min. Subsequently, the cells were pelleted and reincubated in the presence or absence of a Ca^{2+}/EGTA buffer (free Ca^{2+}, 0.1 μM), cytosol and ATP. As shown in Fig. 9 (B, lane b), reincubation in the presence of Ca^{2+}, ATP, and cytosol results in the reversal of transport inhibition and recovery of the equivalent fraction of transport observed in the control (Fig. 9 A, lane b [no preincubation in the pres-
of EGTA). Reversal of transport inhibition requires both ATP and cytosol (Fig. 9, lanes c and d), making it likely that additional transport steps must be completed before delivery of G protein to the cis-Golgi compartment for processing by \( \alpha \)-1,2 mannosidase I.

The GTP-\( \gamma \)S-sensitive Step Precedes the Ca\( ^{2+} \) Requiring Step

The observation that Ca\( ^{2+} \) will relieve the temperature-sensitive block by YPTlp in vivo (Schmitt et al., 1988) suggested that GTP hydrolysis and Ca\( ^{2+} \) dependency of ER to Golgi transport may be temporarily or functionally related. Since Ca\( ^{2+} \) depletion in vitro is fully reversible, semiintact cells provide a model system to test this hypothesis. To address this question in vitro, semiintact cells were first preincubated in the presence of cytosol, ATP, EGTA, and GTP\( \gamma \)S for 60 min at 30\(^\circ\)C (Fig. 11). Subsequently, the cells were pelleted and resuspended in the presence of Ca\( ^{2+} \) and GTP.

No activity was observed when cells were preincubated in the presence of both EGTA and GTP\( \gamma \)S (A, lane b), suggesting that free Ca\( ^{2+} \) is not required for transport of VSV-G protein to the GTP\( \gamma \)S-sensitive step. In contrast, when semiintact cells preincubated in the presence of cytosol, ATP, and EGTA (but not GTP\( \gamma \)S) were pelleted and reincubated in the complete reaction mixture in the absence (Fig. 11 B, lane b) or presence (lane c) of GTP\( \gamma \)S, no inhibition of transport by GTP\( \gamma \)S was observed. These results show that the GTP\( \gamma \)S-sensitive step precedes a Ca\( ^{2+} \), ATP, and cytosol-dependent step required for delivery of VSV-G to the cis-Golgi compartment.

To determine whether GTP\( \gamma \)S inhibition of transport could be reversed by the addition of free Ca\( ^{2+} \) and GTP. A complete cocktail was preincubated in the presence of GTP\( \gamma \)S for 20 min to inactivate transport. Subsequently, free Ca\( ^{2+} \) was adjusted in the assay in the concentration range of 0.01 to 100 \( \mu \)M (as a Ca\( ^{2+} \)/EGTA buffer) in the presence of 1 mM GTP. No reversal of transport inhibition was observed over the concentration range tested (data not shown). Similarly, when GTP\( \gamma \)S was coincubated with a wide range of free Ca\( ^{2+} \) concentrations (0.01-100 \( \mu \)M), no differences were detected in extent of inhibition observed (data not shown). From these results we conclude that no direct relationship exists between the GTP\( \gamma \)S sensitive step and the Ca\( ^{2+} \) requirement for the transport of VSV-G to the cis-Golgi compartment.

Discussion

Inhibition by GTP-\( \gamma \)S

Semiintact cells provide a versatile model system to explore mechanisms of interorganelle transport in vitro. While the exocytic organelles remain functionally intact, the loss of soluble cytoplasmic components as a consequence of plasma membrane perforation has allowed us to begin to dissect for the first time the contributions of the intact membrane compartments and soluble components to transport of protein at this early stage of the secretory pathway.

We have presented evidence to support the conclusion that GTP hydrolysis is an essential step in the transport of protein from the ER to the cis-Golgi compartment. Inhibition of transport by GTP\( \gamma \)S was sensitive, rapid, and irreversible; but could be blocked in the presence of GTP when added before incubation. A similar level of inhibition was observed in the presence of AlF\(_4^-\). These results are consistent with the effect of GTP\( \gamma \)S and AlF\(_4^-\) on a typical GTP-binding protein (Gilman, 1987). GTP\( \gamma \)S has also been reported to inhibit transport of protein from the ER through the Golgi apparatus in yeast (Baker et al., 1988). Under their incubation conditions, 50\% inhibition of transport was observed at 5 \( \mu \)M GTP\( \gamma \)S. Whether GTP\( \gamma \)S inhibition of transport in yeast is a consequence of a block in ER to Golgi transport, inter-Golgi transport (Melancon et al., 1987), or both remains to be determined due to the unknown distribution of processing enzymes in the yeast Golgi apparatus.

Our results suggest that the GTP\( \gamma \)S-sensitive factor(s) is a constitutive component of the transport pathway between the ER and the cis-Golgi compartment. Inhibition of transport was only observed when semiintact cells containing ER and Golgi compartments, cytosol, and ATP were incubated at 30\(^\circ\)C, suggesting that the GTP\( \gamma \)S-sensitive step could be effectively blocked only under conditions in which the components required for transport could actively exchange GTP\( \gamma \)S for GTP/GDP. The evidence further suggested that the GTP\( \gamma \)S-sensitive component(s) is a nondiffusible com-

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ponent associated with the membrane fraction, at least in the inhibited state. The latter point stems from the observation that VSV-G protein transport inactivated by the addition of GTPγS cannot be reactivated in trans by the incubation in the presence of fresh cytosol and untreated semintact cells in the presence of GTP. Under these conditions transport of VSV-G is unable to resume despite the fact that addition of fresh membranes and cytosol initiates a new round of vesicular transport between the ER and cis-Golgi compartment. GTP hydrolysis may be essential in triggering a conformational switch to insure vectorial flow of protein along the ER/Golgi pathway.

Transport between subcompartments of the Golgi has previously been reported to be sensitive to GTPγS (Melancon et al., 1987). In contrast to our results, inhibition of transport between the cis and medial subcompartments of the Golgi apparatus by GTPγS requires a soluble factor that can be selectively removed from the cytoplasmic fraction (Melancon et al., 1987). Removal of this factor renders the inter-Golgi assay insensitive to GTPγS inhibition, but does not impede the ability of cytosol to support further rounds of transport. The acceptor (medial) Golgi function was found to be irreversibly inhibited by GTPγS (Melancon et al., 1987). One of several suggestions to account for their results was that the GTPγS-sensitive factor is not a constitutive component of the transport system, but may play a regulatory role in transport of protein between Golgi compartments. For example, it may serve to inhibit membrane traffic as is observed during mitosis (Featherstone et al., 1985; Warren, 1985).

In ER to Golgi transport, we currently cannot distinguish whether the post-ER 15°C intermediate, or the acceptor (cis) Golgi compartment in semintact cells is the target for GTPγS inhibition. However, it is clear that preincubation of cytosol with GTPγS in the presence of a large excess of cells (or purified Golgi and ER membranes) does not selectively remove (inactivate?) a factor from the soluble cytoplasmic pool required for inhibition by GTPγS, nor does it reduce the capacity of the cytosol to support transport. We therefore favor the interpretation that the reaction in ER to Golgi transport inhibited by GTPγS is a constitutive part of the transport machinery.

The explanation for the observed differences between ER to Golgi transport and inter-Golgi transport may reflect major differences in the in vitro systems being used to reconstitute transport. Semintact cells provide a reservoir of membrane and cytoskeleton-associated proteins normally found in the living cell. In contrast, purified Golgi-compartments may have a more limited pool of membrane-associated factors after purification by sucrose density gradient centrifugation (Balch et al., 1984). Loss of sensitivity of inter-Golgi transport to GTPγS (by preincubation of cytosol and Golgi membranes with GTPγS) may reflect differences in this membrane-associated pool. Alternatively, the observed differences may reflect the existence of unique subsets of GTPγS-sensitive components with differing roles in transport along the secretory pathway. The latter interpretation is supported by the observation that SEC4p and YPT1p, both highly related low molecular mass GTP-binding proteins, are found to function at different stages of the secretory pathway (Salminen and Novick, 1987; Segev et al., 1988; Schmitt et al., 1988).

To localize the site of GTPγS inhibition, we took advantage of both kinetic studies and the observation that VSV-G protein can be selectively accumulated in an intermediate compartment between the ER and the Golgi by preincubation of cells in vivo at 15°C before preparation of semintact cells (Balch et al., 1986). Several lines of evidence indicate that transport in vitro from this site can be distinguished from ER export. In particular, incubation of semintact cells in which VSV-G protein was accumulated in the 15°C intermediate in vivo shows no lag after initiation of transport, and transport from this intermediate compartment is insensitive to cell homogenization (Beckers, C. J. M., and W. E. Balch, unpublished data), a procedure that destroys ER export activity (Beckers and Balch, 1987). While the 15°C compartment is biochemically uncharacterized, it has been proposed to contain a population of vesicles trafficking protein to the Golgi compartment (Palade, 1976; Saraste and Kuismannen, 1984; Saraste et al., 1987; Balch et al., 1986). Since transport of VSV-G from this population of vesicles in vitro is inhibited by GTPγS, the GTPγS-sensitive step may block subsequent delivery and fusion to the cis-Golgi compartment. Incubation of Golgi in vitro in the presence of GTPγS results in the striking accumulation of nonclathrin coated vesicles (Melancon et al., 1987). These vesicles are the likely precursors for uncoated vesicles responsible for subsequent delivery of protein to the medial Golgi compartment (Orci et al., 1986; Malhotra et al., 1988). During inter-Golgi transport the uncoating and delivery of carrier vesicles, but not their formation, is inhibited in the absence of GTP hydrolysis. If GTP hydrolysis plays a similar role in ER to Golgi transport, then it is likely that the 15°C intermediate preceding the GTPγS-sensitive step may be similar to or a precursor of such coated vesicles. Since the experimental protocol used only detects the latest step blocked by GTPγS, the possible sensitivity of earlier steps in export remains to be tested.

**Inhibition of Transport by EGTA**

Transport from the ER to the cis-Golgi compartment requires Ca²⁺. It is unlikely that EGTA simply inhibits the processing enzyme α-1,2 mannosidase I. First, α-1,2 mannosidase I is in a sealed compartment inaccessible to the membrane impermeant EGTA. In support of this, the Mn²⁺ requiring medial-Golgi enzyme N-acetylglucosamine transferase I is insensitive to EGTA when present in a sealed Golgi compartment (Balch, W. E., unpublished data). Second, while the solubilized form of α-1,2 mannosidase is sensitive to EDTA, suggesting a divalent metal ion requirement, its activity is not stimulated by the addition of either Ca²⁺ or Mg²⁺ (Tuliani and Touster, 1988). The concentration of free Mg²⁺ is 1.25 mM. The concentration of free Mn²⁺ is <10⁻¹⁰ M. It is unlikely that either divalent ion is responsible for the observed inhibition of processing. Third, the EGTA block is fully reversible. Recovery of activity requires in addition to 0.1 mM free Ca²⁺, both cytosol and ATP indicating that further transport must occur for G protein to reach the site of processing by α-1,2 mannosidase I. Were only processing to be inhibited, G protein would be expected to be transported to the cis-Golgi, or possibly past the cis-Golgi compartment to later Golgi compartments. In the former case, processing would be expected to be ATP and cytosol independent; in the
latter, no processing would be observed. In addition, transport from the Ca\textsuperscript{2+}-sensitive step to the Golgi is also sensitive to N-ethylmaleimide, a sulfhydryl alkylating reagent. N-ethylmaleimide inhibits a step in ER to Golgi transport preceding α-1,2 mannosidase I processing (Beckers, C. J. M., and W. E. Balch, unpublished data). Finally, the levels of Ca\textsuperscript{2+} required for transport of VSV-G protein (0.01-0.1 μM) fall within the narrow range of physiologically free Ca\textsuperscript{2+} values measured for mammalian interphase cells of 0.1-0.15 μM (Poenie et al., 1986). It is unlikely that if the inhibition observed by addition of EGTA was solely due to Ca\textsuperscript{2+}-dependent processing, that excess Ca\textsuperscript{2+} would also result in inhibition. Indeed, these results provide a strong piece of evidence that the semintact cell model system used here to study ER to Golgi transport accurately reflects events occurring in vivo.

In a more general sense, the importance of a calcium buffer in other in vitro systems currently being studied should not be ignored considering the potential general role of Ca\textsuperscript{2+} in vesicle delivery and fusion. A role for Ca\textsuperscript{2+} has been well-documented in the delivery and fusion of secretory granules to the cell surface in a number of cell types which regulate their secretion (Howell et al., 1987; Burgoyne, 1987; Crabb and Jackson, 1985). In addition, Bennett et al. (1988) have provided evidence that suggests that the addition of EGTA to an in vitro assay using perforated MDCK cells which reconstitutes export from the trans-Golgi network results in the release of exocytotic transport vesicles into the assay buffer before their delivery to the plasma membrane. One implication of these results is that the absence of Ca\textsuperscript{2+} promotes release of transport vesicles through inhibition of potential cytoskeletal or membrane associations essential for trafficking to the cell surface. The relationship, if any, between the function of Ca\textsuperscript{2+} for targeting to the cell surface and early steps in the intracellular transport pathway remains to be established.

The requirement for Ca\textsuperscript{2+} in ER to Golgi transport may have important implications for vesicular trafficking during mitosis. As indicated in Results, transport is inhibited by both Ca\textsuperscript{2+} depletion and excess Ca\textsuperscript{2+}. Whether inhibition by excess Ca\textsuperscript{2+} affects the same step as Ca\textsuperscript{2+} depletion remains to be determined. However, it has been previously recognized that during mitosis, free Ca\textsuperscript{2+} levels rise transiently to 0.5-0.8 μM in mammalian cells (Poenie et al., 1986) and to higher concentrations in plant cells (1-3 μM) (Hepler and Callaham, 1986). During mitosis intracellular transport of protein is blocked at a stage before the Golgi complex (Featherstone et al., 1985; Warren, 1985). While in vitro transport of VSV-G between the ER and the cis-Golgi compartment is most efficient at a free Ca\textsuperscript{2+} concentration of 0.1 μM (pCa = 7), transport was reduced fourfold when the free Ca\textsuperscript{2+} was adjusted to 0.3 μM or higher, a value characteristic of the transient raise during mitosis. The cessation of intracellular protein transport in mitotic cells might be explained by this transient increase concentration of cytosolic free Ca\textsuperscript{2+} during mitosis.

**Role of GTP and Calcium in Transport**

GTP-binding proteins comprise a growing superfamily. One group encompasses the ubiquitous heterotrimeric "G" proteins that transduce hormonal and sensory signals across the plasma membrane (Gilman, 1987). A second group includes the tubulins and the initiation and elongation factors of protein synthesis which use GTP hydrolysis to control their assembly and disassembly from oligomeric complexes (Kirschner, 1981; Bourne, 1988). A third class of proteins include the ras related homologs (Barbacid, 1987). Some members of this group have been shown to be required for transport at different stages of the secretory pathway. SEC4p is a 23.5-kD GTP-binding protein required for targeting of secretory vesicles to the cell surface in yeast (Goud et al., 1988; Salmi- nen and Novick, 1987). YPTlp, a 23-kD GTP-binding protein is essential for orderly progress of the cell cycle in yeast (Schmitt et al., 1987), and more recently, has been demonstrated to be essential for transport of protein through the Golgi complex (Segev et al., 1988; Schmitt et al., 1988). A temperature-sensitive YPT1 mutation results in incompletely glycosylated invertase and proliferation of membranes and vesicles (possibly ER-like structures) at the restrictive temperature (Segev et al., 1988). YPTlp homologs exhibiting sequence identities of >70% with respect to the yeast protein have been identified in mouse and other eukaryotic species (Haubruck et al., 1987; Touchot et al., 1987). While SEC4p has been demonstrated to be tightly associated with secretory vesicles and the plasma membrane (Goud et al., 1988), the YPTlp gene product is probably located on yeast Golgi structures (Segev et al., 1988). A protein that cross-reacts with antibodies to YPTlp has also been found to be associated with the Golgi complex in mammalian cells (Segev et al., 1988). From these studies, it is becoming increasingly apparent that a novel class of 20-25-kD GPT-binding proteins may play an important role in transport between compartments of the secretory pathway. It is interesting to speculate that the GTP-binding protein being studied here may be a YPTlp homolog, a point we are currently investigating.

The newly identified requirement for GTP hydrolysis in vesicular transport has provided ground for speculation as to its possible function in transport (for a recent discussion see Bourne, 1988). While both the SEC4p and YPTlp are associated with components of the secretory apparatus, it remains to be established whether their role in transport is direct or indirect. The only known defect of conditional SEC4 mutations is the accumulation of post-Golgi secretory vesicles (Novick and Schekman, 1983). On the other hand, mutations in YPTlp lead to a broad spectrum of defects in cell cycle regulation, cytoskeleton, and transport (Schmitt et al., 1986; Schmitt et al., 1988). In the latter case, the presence of elevated calcium in the external medium will relieve the inhibitory phenotype of at least one temperature-sensitive mutation at the restrictive temperature (Schmitt et al., 1988). It has been suggested from these studies that Ca\textsuperscript{2+} directly influences transport, and that YPTlp indirectly inhibits transport through reduction of intracellular Ca\textsuperscript{2+} (Schmitt et al., 1988). While we have provided evidence for an essential role for Ca\textsuperscript{2+} in transport of the VSV-G protein from the ER to the Golgi, we were unable to establish a direct relationship between GTPyS inhibition of transport and the Ca\textsuperscript{2+} requirement. If the GTP-sensitive component was indirectly regulating transport through control of intracellular Ca\textsuperscript{2+}, a prediction would be that GTPyS inhibition in vitro should be rescued by controlling the free Ca\textsuperscript{2+} concentration at physiological (or nonphysiological) levels with a Ca\textsuperscript{2+}/EGTA buffer. Both approaches were unsuccessful in relieving the
GTPγS-induced inhibition. We conclude that GTPγS sensitivity and the Ca2+ requirement for transport appear to be independent events.

What is the role of the transport GTPase in vesicular trafficking? We have explored the possibility that the ER to Golgi transport GTPase is similar in activity to the G proteins (Gα, Gβ, or Gγ) involved in signal transduction at the cell surface. The following pharmacological agents related to the activity of these surface G proteins have been found to have no effect on inhibition by GTPγS or transport per se (data not shown): cAMP (1 μM-1 mM), cGMP or 8 Br-cGMP (1 μM-1 mM), IP3 (1 μM-1 mM), diacylglycerol (0.1-10 μM), phorbol-myristic acid (0.1-10 μM), cholera or pertussis toxin (50 μg/ml in the presence of 1 mM NAD) (data not shown). Pretreatment of cells with cholera or pertussis toxin for 3 h before labeling and preparation of seminat cells had no effect on transport in vivo or in vitro (data not shown). An alternative to surface G proteins could be the GTP-binding protein tubulin which is provided by the cytosol. In this case transport may be inhibited as a consequence of the arrest of microtubule growth by GTPγS (Kirschner and Yarbrough, 1981). We find this to be an unlikely explanation since neither nocadazole nor taxol (100 μM) inhibited transport or prevented inhibition of transport by GTPγS when added to the assay (data not shown).

In the absence of evidence for a role of known cell surface or cytoskeletal GTP-binding proteins in transport, the data are interpreted to support the model presented in Fig. 12. Here, we suggest that the role of GTP hydrolysis (by a constitutive component of the transport machinery) is to trigger a switch essential for vectorial flow between the ER and the cis-Golgi compartment. GTP hydrolysis may not only drive progress through the pathway, but may also provide a simple mechanism for the recycling of protein components in the system for use in multiple rounds of transport. This model is consistent with the observation that all GTP-binding proteins examined to date also use GTP hydrolysis to control a wide range of pathways. The Ca2+ requiring step subsequent to the GTPγS-sensitive step may be involved in delivery or fusion. In this case Ca2+ is likely to serve as a cofactor in late steps in transport, distinct in function from the chemical levels of Ca2+ (1 mM) used to induce nonspecific fusion of liposomes (Papahadjopoulos and Bangham, 1966). Through the type of in vitro biochemical analysis presented here we hope to generate a detailed picture of the transport intermediates involved in ER to Golgi trafficking to elucidate the role of GTP and Ca2+ in transport.

Figure 12. Model for the role of GTP and Ca2+ in ER to Golgi transport.

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