Identification of A Novel, N-ethylmaleimide-sensitive Cytosolic Factor Required for Vesicular Transport from Endosomes to the \textit{trans}-Golgi Network In Vitro

Yukiko Goda and Suzanne R. Pfeffer
Department of Biochemistry, Stanford University School of Medicine, Stanford, California 94305-5307

Abstract. We have recently described a cell-free system that reconstitutes the vesicular transport of 300-kD mannose 6-phosphate receptors from late endosomes to the \textit{trans}-Golgi network (TGN). We report here that the endosome-\textit{to}-TGN transport reaction was significantly inhibited by low concentrations of the alkylating agent, N-ethylmaleimide (NEM). Addition of fresh cytosol to NEM-inactivated reaction mixtures restored transport to at least 80\% of control levels. Restorative activity was only present in cytosol fractions, and was sensitive to trypsin treatment or incubation at 100°C. A variety of criteria demonstrated that the restorative activity was distinct from NSF, an NEM-sensitive protein that facilitates the transport of proteins from the ER to the Golgi complex and between Golgi cisternae. Cytosol fractions immunodepleted of \textasciitilde90\% of NSF protein, or heated to 37°C to inactivate \textasciitilde93\% of NSF activity, were fully able to restore transport to NEM-treated reaction mixtures. The majority of restorative activity sedimented as a uniform species of 50–100 kD upon glycerol gradient centrifugation. We have termed this activity ETF-1, for endosome-\textit{to}-TGN transport factor-1. Kinetic experiments showed that ETF-1 acts at a very early stage in vesicular transport, which may reflect a role for this factor in the formation of nascent transport vesicles. GTP hydrolysis appears to be required throughout the transport reaction. The ability of GTP\textgamma{}S to inhibit endosome-\textit{to}-TGN transport required the presence of donor, endosome membranes, and cytosol, which may reflect a role for guanine nucleotides in vesicle budding. Finally, ETF-1 appears to act before a step that is blocked by GTP\textgamma{}S, during the process by which proteins are transported from endosomes to the TGN in vitro.

In recent years, a number of cell-free systems have been devised that reconstitute the vesicular transport of proteins between membrane-bound organelles of the secretory and endocytic transport pathways. Thus, it is now possible to study the transport of proteins from the ER to the Golgi complex (Beckers et al., 1987; Baker et al., 1988; Ruohola et al., 1988), between Golgi cisternae (Balch et al., 1984; Rothman, 1987), as well as the budding of transport vesicles from the \textit{trans}-Golgi network (TGN) (Bennett et al., 1989; deCurtis and Simons, 1989; Tooze and Hutten, 1990), and their subsequent fusion with the plasma membrane (Woodman and Edwardson, 1986; Howell et al., 1987). In addition, a number of events in the endocytic pathway have also been reconstituted (for review see Gruenberg and Howell, 1989). The availability of these systems has permitted a biochemical analysis of the molecular mechanisms that underlie vesicular transport processes (Balch, 1989; Goda and Pfeffer, 1989; Rothman and Orci, 1990; Wattenberg, 1990).

The transport of proteins between Golgi cisternae has been studied in greatest detail. Rothman and co-workers have purified and characterized an N-ethylmaleimide (NEM)-sensitive protein, termed NSF (NEM-sensitive fusion protein), that is required for intra-Golgi transport in vitro (Glick and Rothman, 1987; Block et al., 1988). NSF is also required for ER-\textit{to}-Golgi transport (Beckers and Balch, 1989), as well as endocytic vesicle fusion (Diaz et al., 1989). The physiological significance of this transport factor is supported by its high degree of homology to the yeast SEC18 gene product (Wilson et al., 1989). Another set of proteins, soluble NSF attachment protein, SNAPs, were also recently purified, and are required for NSF action (Clary and Rothman, 1990). One of the three identified SNAP proteins is likely the product of the yeast SEC17 gene (Clary et al., 1990), consistent with genetic data which indicate an interaction between SEC17 and SEC18 gene products (Kaiser and Schekman, 1990). Finally, GTP hydrolysis is required before the reactions catalyzed by NSF and SNAP (Orci et al., 1989), and may reflect the participation of small ras-like,
GTP-binding proteins (Bacon et al., 1989; Baker et al., 1990; Balch, 1989; Plutner et al., 1990).

We have recently described a cell-free system that reconstitutes the transport of mannose 6-phosphate (man6P) receptors from late endosomes to the TGN (Goda and Pfeffer, 1988). Man6P receptors carry newly synthesized, soluble lysosomal hydrolases from the TGN to late endosomes, and are then transported back to the TGN to complete a cycle of biosynthetic, lysosomal enzyme transport (Kornfeld and Mellman, 1989). Our endosome→TGN transport assay relies upon the unique localization of sialyltransferase to the trans-Golgi and TGN, and uses a mutant cell line in which glycoproteins are not sialylated (CHO clone 1021; Briles et al., 1977). Radiolabeled man6P receptors, present in late endosomes in a mutant cell extract, acquire sialic acid residues when they are transported to the TGN of wild type Golgi complexes present in reaction mixtures. Sialic acid acquisition by man6P receptors in this system reflects a vesicular transport process, since it is time, temperature, ATP, and cytosol dependent, and also requires GTP hydrolysis (Goda and Pfeffer, 1988). Furthermore, man6P receptors and sialyltransferase remain in sealed membrane compartments throughout the reaction, and nonspecific membrane fusion is ruled out by several criteria (Goda and Pfeffer, 1988).

We have initiated a biochemical analysis of endosome→TGN transport in vitro (Draper et al., 1990). We present here evidence for a novel, NEM-sensitive cytosolic factor that acts at a very early stage in this transport process, before a step involving GTP hydrolysis.

Materials and Methods

Materials

Chemicals were from Sigma Chemical Co. (St. Louis, MO) unless otherwise specified. Pure NSF and mouse monoclonal anti-NSF IgM (4A6) were generous gifts of Dr. James Rothman (Princeton University, Princeton, NJ).

Assay for NEM-Sensitive Restorative Activity

The assay is a modification of the transport assay previously described (Goda and Pfeffer, 1988). Wild type, rat liver Golgi membranes were added to semi-intact cell extracts prepared in reaction buffer containing cytosol, CMP-sialic acid, protease inhibitors, and the ATP-regenerating system. The mixture was treated with ~80 μmol NEM/mg protein for 6 min on ice, and then quenched with a twofold molar excess of DTT. Typically, NEM treatment was carried out in a total volume of 160 μl at a concentration of 0.2-0.3 mM. CHO cytosol or cytosol buffer was then added (40-60 μl), and the mixtures were incubated for 2 h at 37°C.

Ammonium Sulfate Precipitation and Glycerol Gradient Sedimentation

Finely ground ammonium sulfate was added slowly to CHO cytosol, while stirring on ice, to achieve 40% saturation. The precipitate was collected by centrifugation for 5 min at 35,000 g in a centrifuge (Model TL-100; Beckman Instruments, Inc., Palo Alto, CA), and resuspended in one fourth the original volume with 90 mM KCl, 50 mM Hepes-KOH, pH 7.2 (HK buffer). Residual ammonium sulfate was removed by extensive dialysis against 25 mM Tris HCl, pH 8, 50 mM KCl, 1 mM DTT, and the dialysate was clarified by centrifugation for 5 min at 250,000 g. Ammonium sulfate fractionation resulted in a twofold increase in the specific activity since the clarified material retained 77% of the initial restorative activity and total protein yield was ~40%.

Ammonium sulfate-fractionated cytosol (400 μl) was layered onto 4.8 ml linear, 5-25% (wt/vol) glycerol gradients prepared in 25 mM Tris HCl, pH 8, 50 mM KCl. Gradients were centrifuged for 2 h at 50,000 rpm in a rotor (model SW50.1; Beckman Instruments, Inc.): 300 μl fractions were collected from the top. The molecular weight markers used were: apoferritin, 440 kDa; gamma globulin, 158 kDa; bovine albumin, 66 kDa; and chicken ovalbumin, 45 kDa. Generally, 70-90% of restorative activity applied to a glycerol gradient was recovered after centrifugation.

Preparation of 35S-Cys/Met-Labeled Cytosol

CHO clone 1021 cells were grown and labeled as previously described (Goda and Pfeffer, 1988) except 0.14 nCi Tran35S label (ICN Radiochemicals, Irvine, CA) was used per plate and cells were chased in complete medium for only 10 min. Labeled cells were washed three times in ice-cold hypotonic buffer (10 mM Hepes KOH, pH 7.2, 15 mM KCl) and left to swell on ice for 10 min. The buffer was aspirated and cells were then scrapped into HK buffer containing 10 mM MgCl2, 5 mM ATP, 5 mM DTT, and protease inhibitors. The semi-intact cells were further disrupted by five passes through a 27-gauge needle, and the extract was cleared of membranes by centrifugation for 5 min at 250,000 g in a centrifuge (model TL-100; Beckman Instruments, Inc.). The supernate was concentrated threefold in an ultrafiltration membrane cone (Centricon CF25; Amicon Corp., Danvers, MA), and desalted by centrifugation through a 2 ml column of PD6 (Bio-Rad Laboratories, Richmond, CA) equilibrated in HK containing 2 mM MgCl2, 2 mM DTT, and 0.5 mM ATP. The resulting cytosol was 6 mg/ml. Unlabeled CHO wild-type cytosol was prepared as described (Balch et al., 1984), and was also desalted in parallel.

Immunodepletion of NSF from CHO Cytosol

Anti–NSF IgM and a control IgM (specific for danylabeled proteins; Sigma Chemical Co.) were each bound to anti-mouse, IgM, μ-chain specific-agarose (Sigma Chemical Co.) according to the manufacturer, at 4°C overnight, to yield 2 mg IgM per ml of resin. Cytosol was incubated for 2 h at 4°C with agarose coupled to either anti-NSF or control IgM at a ratio of 50 μl agarose beads per mg of cytosolic protein. Agarose beads were pelleted by low speed centrifugation, and the supernate was recovered for further analysis. When immunodepletion was carried out with 35S-cys/met-labeled cytosol, the agarose beads, after pelleting, were washed with 5 ml, 10 mM K-phosphate, pH 7.2, 0.5 M KCl. The antigen was then stripped from the beads with 1 ml of 0.1 M glycine, pH 2.0, 0.15 M KCl, and analyzed by TCA precipitation followed by 7.5% SDS-PAGE and autoradiography as described (Goda and Pfeffer, 1988).

Other Procedures

Cis-mediated Golgi transport was carried out in semi-intact cell extracts as described (Goda and Pfeffer, 1988). Protein was determined according to the method of Bradford (1976) using reagent (Bio-pad Laboratories) and BSA as standard. Autoradiograms were quantified using a densitometric scanner (model 300A; Molecular Dynamics, Sunnyvale, CA).

Results

Transport of man6P receptors from late endosomes to the TGN, carried out in semi-intact cell extracts, is stimulated two- to threefold by the addition of a crude cytosol fraction (Goda and Pfeffer, 1988; and see below). Nevertheless, appreciable transport is observed in the absence of exogenously added cytosol, presumably because of cytosolic proteins present in the extracts. To identify cytosolic proteins that facilitate endosome→TGN transport, it was necessary to define conditions in which transport was strictly dependent upon the addition of exogenous cytosol. For this purpose, we used the broadly reactive, sulfhydryl group-alkylating reagent, NEM, to inhibit the in vitro endosome→TGN transport reaction. After brief incubation with this inhibitor, unreacted NEM was quenched with excess DTT. We then tested if cytosolic components could restore activity to a NEM-inactivated reaction mixture. This experimental approach has expedited the analysis of cytosolic factors that mediate
Because of proteins present in the semi-intact cell extract, transport to restore transport to an NEM-treated reaction mix (data not shown). These data show that endosome~TGN transport appeared to be entirely cytosolic. We therefore compared the physical properties of our restorative activity with those of NSF, an NEM-sensitive transport factor (NSF) that facilitates a number of in vitro transport reactions (Rothman and Orci, 1990). Preliminary experiments suggested that our NEM-sensitive, restorative activity was distinct from NSF. NSF activity is present on Golgi membranes and also in the cytosol (Glick and Rothman, 1987). In contrast, the restorative activity required for endosome~TGN transport appeared to be entirely cytosolic.

**The NEM-Sensitive Restorative Activity Is Distinct from NSF**

Rothman and co-workers have recently identified, and purified to homogeneity, an NEM-sensitive transport factor (NSF) that facilitates a number of in vitro transport reactions (Rothman and Orci, 1990). Preliminary experiments suggested that our NEM-sensitive, restorative activity was distinct from NSF. NSF activity is present on Golgi membranes and also in the cytosol (Glick and Rothman, 1987). In contrast, the restorative activity required for endosome~TGN transport appeared to be entirely cytosolic.

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**Figure 1.** An NEM-sensitive cytosolic protein is required for endosome~TGN transport. Lane 1, complete reaction. Lanes 2–6; 35S-labeled, semi-intact CHO clone 1021 cells and rat liver Golgi membranes were preincubated in reaction buffer with 0.2 mM NEM for 6 min at 0°C. Transport reactions (2 h) were then initiated by addition of 40 μl of either HK buffer (lane 2) or CHO cytosol (5 mg/ml; lane 3). Alternatively, cytosol was pretreated with 0.2 mM NEM for 6 min on ice and then quenched with 0.4 mM DTT (lane 4), or boiled for 3 min and clarified by centrifugation for 10 min at 200,000 g (lane 5), or incubated with 0.2 mg/ml trypsin for 20 min at 37°C followed by addition of egg white trypsin inhibitor (2.0 mg/ml; lane 6). Lane 1 represents a reaction in which 20.4% of CHO clone 1021 man6P receptors acquired sialic acid.

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**Figure 2.** Titration of cytosolic restorative activity. 35S-labeled, semi-intact CHO clone 1021 cells, crude cytosol, and rat liver Golgi membranes were preincubated in reaction buffer with 0.3 mM NEM for 6 min at 0°C, and excess NEM was quenched with 0.6 mM DTT (△). Alternatively, the reaction mix was mock-treated by incubating with 0.6 mM DTT before addition of 0.3 mM NEM (□). Reactions were then carried out for 2 h in the presence of increasing cytosol concentrations. An extent of transport of 1.0 represents the acquisition of sialic acid by 22.0% of man6P receptors.
monitors the transport-coupled addition of N-acetylglycosamine (GlcNAc) residues to vesicular stomatitis viral (VSV) glycoprotein oligosaccharides. We used semi-intact, CHO clone 1021 cells as the GlcNAc transferase-containing acceptor fraction, and purified Golgi complexes from VSV-infected, CHO clone 15B cells as the donor fraction.

As shown in Table I, gentle NEM treatment significantly inhibited endosome→TGN transport, yet only slightly inhibited the transport of proteins between Golgi cisternae. Since NSF is absolutely essential for intra-Golgi transport, the relatively mild conditions of NEM-treatment employed were not sufficient to inactivate the majority of the NSF activity required for the intra-Golgi transport reaction. This experiment demonstrated that endosome→TGN transport was more sensitive than intra-Golgi transport, to NEM treatment. It was possible that endosome→TGN transport simply required more NSF than intra-Golgi transport. This was ruled out, however, by the observation that addition of pure NSF did not restore man6P receptor transport (Table I). Furthermore, cytosol which was depleted of NSF activity by incubation at 37°C for 20 min (in the absence of ATP; Block et al., 1988) fully restored endosome→TGN transport. Moreover, when pure NSF was added in conjunction with cytosol devoid of NSF activity, no additional restorative activity was detected.

When cis→medial Golgi transport was assayed in NEM-treated, semi-intact cell extracts, a small stimulation was observed upon addition of NSF activity-free cytosol (Table I). We do not believe that this reflects residual NSF activity in the cytosol, because independent assays showed that the cytosol had lost ≥93% residual NSF activity (see legend of Table I). It is very possible that cis→medial Golgi transport, assayed in semi-intact cells, is stimulated by other NEM-sensitive cytosolic factors (Balch and Rothman, 1985), such as protease inhibitors, that may not be as essential in reactions that use purified membrane components.

To rule out, unequivocally, any possible overlap between NSF and the endosome→TGN restorative activity, we immunodepleted NSF protein from cytosol fractions. Cytosol was applied to agarose columns to which anti-NSF IgM had been attached. The effectiveness of antigen depletion was then determined using cytosol prepared from metabolically labeled CHO cells. Fig. 4a shows the amount of NSF polypeptide that was depleted from cytosol after three successive rounds of immunodepletion. Densitometric analysis of the

### Table I. Comparison of NSF and the Endosome→TGN Restorative Activity

| Reaction treated with: 0.2 mM NEM | endosome→TGN | cis→medial Golgi |
|-----------------------------------|-------------|-----------------|
| + 0.16 μg NSF*                    | <0.1        | 0.65            |
| + NSF activity-free cytosol†      | <0.1        | ND              |
| + NSF activity-free cytosol and NSF | 0.8        | 1.0             |
|                                   | 0.8         | 1.0             |

The extent of transport was calculated relative to mock-treated reactions: for endosome→TGN transport, this represented the acquisition of sialic acid by 6.8% of mann6P receptors; for cis→medial Golgi transport, the mock-treated sample represented 3,524 cpm [3H-GlcNAc] incorporated into VSV G glycoprotein.

* The activities of NSF and NSF-free cytosol were measured as described by Block et al. (1988) using NEM-treated, donor and acceptor Golgi fractions. Values obtained were: no cytosol added, 167 cpm; + NSF-free cytosol, 367 cpm; + NSF-free cytosol, + NSF (0.08 μg of this preparation), 3,001 cpm [3H-GlcNAc] incorporated.

† This cytosol was prepared by incubation for 20 min at 37°C in the absence of ATP (Block et al., 1988) and added to a final concentration of 1 mg/ml. ND, not determined.
Figure 4. The restorative factor required for endosome→TGN transport is present in cytosol depleted of NSF protein. (a) 35S-labeled proteins depleted from cytosol by passage over either control (C) or anti-NSF (N) antibody columns. Proteins were eluted from antibody columns as described in Materials and Methods; the results of three successive immunodepletions are shown (lanes 1–3). (b) Cytosols, immunodepleted by a single passage over either a control (A) or an anti-NSF (n) antibody column, were assayed for restorative activity. The background transport observed in the absence of added cytosol (4.4%) was subtracted to obtain cytosol-restored transport values; a level of 1.0 represents sialic acid acquisition by 12.1% of man6P receptors (16.5%–4.4%).

Figure 5. GTP prevents GTPγS inhibition when added at early times. At the times indicated, GTP (1.5 mM, final concentration) was added to standard, 200 µl transport reactions, containing 50 µM GTPγS and 1.8 mg/ml cytosol, that were in progress at 37°C. Incubations were carried out for a total length of 2 h. The extent of transport was calculated relative to a standard transport assay containing 1.5 mM GTP, in which 16.5% of man6P receptors acquired sialic acid. In the absence of 1.5 mM GTP, 24.1% of man6P receptors acquired sialic acid.

Figure 6. Kinetics of acquisition of resistance to NEM or GTPγS. Reactions were either stopped on ice (—), treated with 0.3 mM NEM for 6 min on ice (○), or GTPγS was added to final concentration of 0.1 mM (▲). Reactions containing NEM or GTPγS were allowed to continue such that the total incubation time at 37°C was 2 h. Arrows indicate the respective half times for acquisition of resistance to each inhibitor. An extent transport of 1.0 represents acquisition of sialic acid by 20.7 and 11.8% of man6P receptors for the experiments involving GTPγS or NEM addition, respectively; background levels of transport observed when inhibitors were added at the beginning of the reactions have been subtracted: 13.6% for GTPγS and 6.9% for NEM.

ETF-1 Acts at An Early Stage in Transport, before GTP Hydrolysis

GTP hydrolysis is required for a variety of vesicular transport steps. In mammalian cells, GTP hydrolysis is absolutely required for both ER→Golgi and cis→medial Golgi transport (Beckers and Balch, 1989; Melançon et al., 1987). In yeast, the SEC4 gene product is 30% homologous to mammalian, GTP-binding ras proteins, and is required for the delivery of secretory vesicles to the plasma membrane (Salminen and Novick, 1987). Another yeast gene, YPT1, also encodes a GTP-binding protein, and its gene product appears to be involved in ER→Golgi and intra-Golgi transport in yeast (Segev et al., 1988; Bacon et al., 1989; Baker et al., 1990). Recently, Zerial and colleagues have demonstrated the differential localization of mammalian YPT1/SEC4 protein homologues to specific compartments along the secre-
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by 60 min of incubation, endosome--TGN transport was no longer sensitive to inactivation by low concentrations of ETF-1 or GTP hydrolysis were used to form a specific reac-

marion of that intermediate, when the addition of NEM or

experiment did not distinguish the point in transport at which

reflection after a few minutes of incubation cannot be assumed to

import of man6P receptors from endosomes to the TGN in vitro

is likely to reflect a requirement for GTP hydrolysis to rescue the NEM block and complete transport.

Therefore, GTP hydrolysis is likely to be required at the same point or at least one point beyond the action of ETF-1. GTP hydrolysis may also be required before the formation of the NEM-resistant intermediate.

A GTPγs-sensitive Transport Component Requires Late Endosomes for its Activity

Since ETF-1 appeared to be required before a step involving GTP hydrolysis, we sought to further characterize the inhibitory effects of GTPγS during endosome--TGN transport. As shown in Fig. 7 A, the ability of GTPγS to inhibit transport was maximal at high concentrations of CHO cytosol. GTPγS inhibited the ability of cytosol to stimulate transport, analogous to other vesicular transport steps that require GTP hydrolysis (Melanoñon et al., 1987; Beckers and Balch, 1989; Mayorga et al., 1989; Wessling-Resnick and Braell, 1990). These data indicate that a cytosolic factor(s), that may or may not itself by the target of GTPγS, is (are) used to generate a GTP hydrolysis-requiring intermediate.

It has been postulated that GTP-binding proteins are required for accurate targeting of transport vesicles to the acceptor membrane. Accordingly, results from ER--Golgi, intra-Golgi, and endocytic vesicle fusion assays indicate that GTP-binding proteins are required at a late stage in transport, most likely preceding vesicle fusion (Melanoñon et al., 1987; Orri et al., 1989; Wessling-Resnick and Braell, 1990; Beckers and Balch, 1989; Baker et al., 1990; Beckers et al., 1990). In addition, the ability of GTPγS to inhibit vesicle transport has been shown to require membrane components (Melanoñon et al., 1987; Beckers and Balch, 1989; Mayorga et al., 1989; Wessling-Resnick and Braell, 1990).

We carried out preincubation experiments with individual reaction components to determine the site (or sites) at which GTPγS can inhibit transport. Semi-intact cell extracts and/or rat liver Golgi membranes were incubated separately in the presence of GTPγS, ATP, and cytosol at 37°C. Transport assays were then carried out in the presence of excess GTP to prevent any subsequent transport inhibition by GTPγS. The results of this experiment are presented in Fig. 7 B. Pre-

incubation of the 35S-labeled semi-intact cell extract, which provides the donor, late endosome compartment, was sufficient to significantly inhibit the transport reaction. In contrast, preincubation of the acceptor, rat liver Golgi membranes did not reduce their ability to support transport (Fig. 7 B).

Since the donor, semi-intact cells are more complex than rat

Table II. ETF-1 Acts before or Coincident with a Step Requiring GTP Hydrolysis

| Pretreatment         | Stage 2 | Extent transport |
|----------------------|---------|------------------|
| None                 | 2h, 37°C| 1.00             |
| (a) without NEM      |         | 0.49             |
| (b) + GTPγS          |         | 0.49             |
| +NEM, 60', 37°C      |         | 1.00†            |
| (c) + cytosol        |         | 0.50             |
| (d) + buffer         |         | 0.50             |
| (e) + cytosol and GTPγS|       | 0.50             |

In reaction (a), an extent of transport of 1.0 represents the acquisition of acidic acid by 36.5% of man6P receptors.

* GTPγS inhibition was less than that observed in Fig. 5 because these reactions were not carried out at high cytosol concentrations optimal for inhibition. A similar cytosol dependence for GTPγS inhibition has been observed by others (cf. Melanoñon et al., 1987).

† For NEM-pretreated samples, the extent of transport was calculated relative to the amount of transport observed in reaction (c), in which 38.8% of man6P receptors acquired acidic acid. Background levels of transport observed in the absence of cytosol (12.4%) were subtracted from values shown in (c-e). The average of duplicate reactions is shown.

dory pathway (Chavrier et al., 1990). It has been proposed that GTP hydrolysis is coupled to the targeting of transport vesicles to ensure the proper delivery of vesicle contents (Bourne, 1988). However, the observation that GTP hydroly-
sis is involved in the budding of secretory storage granules (Tooze and Huttner, 1990) indicates that GTP may play other roles as well.

We have previously shown that GTPγS inhibits the trans-
port of man6P receptors from endosomes to the TGN in vitro (Goda and Pfeffer, 1988). As shown in Fig. 5, this inhibition is likely to reflect a requirement for GTP hydrolysis transport, since excess GTP or GDP, but not excess ATP or CTP (not shown), abrogated the inhibitory effect of GTPγS. Ex-
cess GTP blocked the ability of GTPγS to inhibit transport only if it was added within the first few minutes of incubation to transport reactions containing GTPγS (Fig. 5). Similar kinetics were observed in ER--Golgi and intra-Golgi trans-
port (Beckers and Balch, 1989; Melanoñon et al., 1987). Since GTPγS might bind tightly (and rapidly) to a later act-
ing component, the inability of GTP to block GTPγS inhibi-
tion after a few minutes of incubation cannot be assumed to reflect an early requirement for GTP hydrolysis. Thus, this experiment did not distinguish the point in transport at which GTP hydrolysis was required.

During intra-Golgi transport, GTP hydrolysis is required at a stage before transport vesicle fusion (Melanoñon et al., 1987), before the action of NSF (Orri et al., 1989). We car-
rried out kinetic experiments to map the point along the vesic-
ular transport reaction pathway at which the endosome--TGN reaction becomes resistant to inhibition by GTPγS and NEM.

If ETF-1 or GTP hydrolysis are used to form a specific reac-
tion intermediate, there should be a point in time, after for-
mation of that intermediate, when the addition of NEM or GTPγS no longer blocks the completion of transport.

We have previously shown that the in vitro transport of man6P receptors from endosomes to the TGN displays an initial lag of ~18 min, and then proceeds linearly for the next 150 min (Goda and Pfeffer, 1988). As shown in Fig. 6 (c) by 60 min of incubation, endosome--TGN transport was no longer sensitive to inactivation by low concentrations of NEM. Resistance to NEM was acquired with a half-time of ~25 min. In other words, ETF-1 action was half completed at a point in the reaction when negligible transport had taken place. Thus, ETF-1 acts at an early stage in the transport process. In contrast, GTPγS inhibited transport throughout the course of incubation (Fig. 6, a); resistance to GTPγS addition was achieved with a half-time of ~45 min.

The kinetic experiments presented in Fig. 6 suggested that ETF-1 acted before a step requiring GTP hydrolysis. To confirm this, we preincubated an NEM-treated reaction mix for 1 h at 37°C to block transport at the point at which ETF-1 is first required. Cytosol was then added to allow transport to continue. If GTP hydrolysis is required for a subsequent step, inclusion of GTPγS at this stage should inhibit transport. As summarized in Table II, GTPγS inhibited the ability of cytosol to rescue the NEM block and complete transport.

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incubation of the 35S-labeled semi-intact cell extract, which provides the donor, late endosome compartment, was sufficient to significantly inhibit the transport reaction. In contrast, preincubation of the acceptor, rat liver Golgi membranes did not reduce their ability to support transport (Fig. 7 B). Since the donor, semi-intact cells are more complex than rat

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The experiments that led to this conclusion were carried out in such a way that they detected the latest possible point in transport that a factor was required. If ETF-1 represents more than one component, they must each act within the same early time frame. Second, we have used very low concentrations of NEM. While these conditions abolish endosome-\(\rightarrow\)TGN transport, they had little effect on ETF-1 activity. Moreover, kinetic experiments showed that ETF-1 acts at a very early stage in vesicular transport, before a step involving GTP hydrolysis. This is in direct contrast with NSF, which has been shown by Rothman and co-workers to act subsequent to GTP hydrolysis, at a late stage along the transport reaction pathway.

It is not yet known whether ETF-1 represents a single transport factor or a mixture of transport factors. However, several lines of evidence suggest that ETF-1 represents a limited number of components, at most. First, the NEM-sensitive, restorative activity was required at a well defined, early point in transport. The experiments that led to this conclusion were carried out in such a way that they detected the latest possible point in transport that a factor was required. If ETF-1 represents more than one component, they must each act within the same early time frame. Second, we have used very low concentrations of NEM. While these conditions abolish endosome-\(\rightarrow\)TGN transport, they had little effect on intra-Golgi transport, carried out in parallel. It is unlikely that a general alkylating agent, used at limiting concentrations, inhibits a large number of cytosolic factors in one

\textbf{Discussion}

The innovation of semi-intact (or perforated) cells (Simons and Virta, 1987; Beckers et al., 1987) has been key to the reconstitution of a number of vesicular transport processes in vitro. However, identification of cytosolic factors required in such systems is complicated for the following reason. While procedures used to generate semi-intact cell extracts advantageously maintain cytoplasmic organization, they fail to remove a significant residue of cytosolic proteins. Several groups have circumvented this problem by washing their semi-intact cell preparations (cf. Beckers et al., 1987), however this results in a substantial loss of endosome-\(\rightarrow\)TGN transport activity (Goda and Pfeffer, 1988). To overcome this difficulty, we have established conditions that selectively inhibit essential cytosolic factors, using a general alkylating agent, NEM. We have described here the identification of an NEM-sensitive, cytosolic factor that is required for the transport of man6P receptors from late endosomes to the TGN in vitro. This factor, which we have termed ETF-1, was identified by virtue of its ability to restore transport to NEM-inhibited reaction mixtures, and has an apparent mass of 50-100 kD, as determined by glycerol gradient sedimentation.

A variety of criteria demonstrate that ETF-1 is distinct from NSF, an NEM-sensitive, oligomer of 76-kD subunits that plays a key role in ER-\(\rightarrow\)Golgi, intra-Golgi, and endosome fusion reactions. Most convincing, in this regard, was the observation that cytosol fractions immunodepleted of \(\geq 90\%\) of NSF protein, or heated to 37°C to inactivate \(\geq 93\%\) of NSF activity, were fully able to restore transport to NEM-treated reaction mixtures. In addition, pure NSF protein was unable to substitute for ETF-1 activity. Moreover, kinetic experiments showed that ETF-1 acts at a very early stage in vesicular transport, before a step involving GTP hydrolysis.

This is in direct contrast with NSF, which has been shown by Rothman and co-workers to act subsequent to GTP hydrolysis, at a late stage along the transport reaction pathway.
vesicular transport reaction, without significantly affecting another transport process. Purification of ETF-1 will fully resolve any possible complexity of this transport factor.

Vesicular transport involves the collection of proteins into transport vesicles which then bud from their donor organelle, identify their respective targets, and fuse with a specific, recipient compartment. Many of these processes are likely to involve "general" transport factors that play a similar role in multiple steps. NSF is an excellent candidate for a general transport factor because of its broad role in membrane traffic (Wilson et al., 1989). In addition, every vesicular transfer involves a distinct target; target specification will require unique proteins. It has been proposed that GTP-binding proteins serve this function (Bourne, 1988; Chavrier et al., 1990). Furthermore, since endosome→TGN transport involves the selective retrieval of mann6P receptors from late endosomes, an additional class of proteins that confers cargo selectivity is also likely to function exclusively in this transport process.

The experiments presented here provide a preliminary indication regarding the potential diversity of ETF-1 action, and suggest that ETF-1 may not be required for intra-Golgi transport. This tentative conclusion is based upon the observation that intra-Golgi transport is more resistant to NEM treatment than endosome→TGN transport (summarized above). In intra-Golgi transport, assays with isolated Golgi fractions, addition of pure NSF is sufficient to restore transport to NEM-inactivated reactions (Orci et al., 1989). Since ETF-1 should have been inactivated under these conditions, it appears that intra-Golgi transport does not require this factor. If this proves to be correct, it would open the possibility that ETF-1 represents a factor unique to endosome→TGN transport.

ETF-1 acts at a very early stage in transport. We favor a role for ETF-1 in the formation of transport vesicles. At this point in the reaction, ETF-1 could facilitate cargo selection, or participate in the physical deformation of the late endosome membrane to form, and/or pinch off, a nascent transport vesicle. Unlike ETF-1, GTPyS inhibited transport throughout the course of the reaction. Nevertheless, GTPyS bound rapidly and tightly to the transport machinery, and its inhibitory effect was dependent on the presence of donor membranes and cytosol. Thus, it appears that GTP-binding proteins are recruited onto budding vesicles during the initial phase of transport. This is in direct contrast with intra-Golgi transport, in which GTPyS only inhibits the activity of the acceptor membranes (Melençon et al., 1987). It is possible that GTP is recruited onto a budding transport vesicle and then is hydrolyzed upon accurate docking with the acceptor membrane. Alternatively, GTP hydrolysis may accompany both budding and fusion events. Since multiple steps may require GTP hydrolysis, it remains to be determined whether the GTP that is bound during vesicle budding represents the same molecule of GTP that is hydrolyzed subsequent to (or coincident with) ETF-1 action. Elucidation of the potential role of specific rab proteins (Chavrier et al., 1990) should help to clarify the precise site of action of ETF-1 during the vesicular transport process.

It is not yet known whether NSF (Block et al., 1988) or SNAPs (Clary et al., 1990) are required for the fusion of endosome-derived transport vesicles with the TGN. Unfortunately, attempts to inhibit endosome→TGN transport using anti-NSF antibodies have yielded inconclusive results, to date. The elucidation of the mechanism by which EGF-1 facilitates the transport of proteins from late endosomes to the TGN, and interacts with other transport components to accomplish vesicular transport, will be greatly facilitated by the purification of this early-acting, cytosolic factor.

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