Identification of A Novel, N-ethylmaleimide-sensitive Cytosolic Factor Required for Vesicular Transport from Endosomes to the 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 trans-Golgi network (TGN). We report here that the endosome→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 immuno-depleted of ≥90% of NSF protein, or heated to 37°C to inactivate ≥93% 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→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 GTPγS to inhibit endosome→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 GTPγS, 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 trans-Golgi network (TGN) (Bennett et al., 1989; deCurtis and Simons, 1989; Tooze and Huttner, 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→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). 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,

1. Abbreviations used in this paper: GlcNAc, N-acetylglucosamine; Man6P, mannose 6-phosphate; NEM, N-ethylmaleimide; SNAP, soluble NSF attachment protein; TGN, trans-Golgi network; VSV, vesicular stomatitis virus.
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 constitutes the transport of mannos-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 kD; gamma globulin, 158 kD; bovine albumin, 66 kD; and chicken ovalbumin, 45 kD. 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 mCi Tras35S 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 resuspended 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 passages 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 (Centriflo CF25; Amicon Corp., Danvers, MA), and desalted by centrifugation through a 1 ml column of PD6DG (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 dansylated 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→medial 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-Rad 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, sulphydryl 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 an 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.

A variety of other cellular processes (cf. Hortsch et al., 1986; Glick and Rothman, 1987; Block et al., 1988; Nicchitta and Blobel, 1989; Newmeyer and Forbes, 1990).

When 35S-labeled semi-intact cells and wild-type Golgi membranes were pretreated with 0.2 mM NEM on ice, the transport of man6P receptors from endosomes to the TGN was significantly inhibited (Fig. 1, compare 1 and 2). However, addition of fresh cytosol to an NEM-treated reaction mix restored transport to 80% of control levels (Fig. 1, 3). The majority of the restorative activity present in cytosol was itself, sensitive to NEM inactivation (Fig. 1, 4), and was at least partly proteinaceous, since both trypsin treatment, or incubation at 100°C, abolished its activity (Fig. 1, 5 and 6).

When reaction components were pretreated with NEM, transport was diminished at least 60% in the absence of cytosol, and could be restored in a concentration-dependent manner by addition of fresh cytosol (Fig. 2, ▲). In addition, when cytosol was added back to a final concentration of 1 mg/ml, at least 72% of the restorative activity was NEM sensitive (Fig. 1, 2–4). Together, these experiments demonstrate that the transport of proteins from endosomes to the TGN is strictly dependent upon cytosolic proteins. At least some of the cytosolic factors required are sensitive to NEM inactivation. Moreover, essential cytosolic proteins, including NEM-sensitive factors, are present in limiting quantities in semi-intact cell extracts.

If reactions were treated for longer times or with higher concentrations of NEM, the background transport observed in the absence of exogenous cytosol was decreased. However, under these conditions, cytosol addition no longer restored transport, probably because of concurrent inactivation of essential membrane components. It was thus essential to define precise conditions of NEM treatment that would ensure efficient recovery of transport activity upon addition of fresh cytosol.

**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. We therefore compared the physical properties of our restorative activity.
monitors the transport-coupled addition of N-acetylgalactosamine (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 poly peptide 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

<table>
<thead>
<tr>
<th>Reaction treated with: 0.2 mM NEM</th>
<th>Extent transport in semi-intact cells</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>endosome–TGN</td>
</tr>
<tr>
<td>+ 0.16 μg NSF*</td>
<td>&lt;0.1</td>
</tr>
<tr>
<td>+ NSF activity-free cytosol†</td>
<td>&lt;0.1</td>
</tr>
<tr>
<td>+ NSF activity-free cytosol and NSF</td>
<td>0.8</td>
</tr>
</tbody>
</table>

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 man6P receptors; for cis→medial Golgi transport, the mock-treated sample represented 3,524 cpm 3H-GlcNAc incorporated into VSVG 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.
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 secretion pathway.

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

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 immuno-depletions are shown (lanes 1–3). (b) Cytosols, immunodepleted by a single passage over either a control (C) 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%).

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

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. At the times indicated, standard, 200 μl transport 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 100 μM (▲). 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.
by 60 min of incubation, endosome→TGN transport was initially lag of ~18 min, and then proceeds linearly for the next man6P receptors from endosomes to the TGN displays an no longer sensitive to inactivation by low concentrations of

If ETF-1 or GTP hydrolysis are used to form a specific reaction intermediate, there should be a point in time, after for-experiment did not distinguish the point in transport at which the endosome→TGN reaction becomes resistant to inhibition by GTPγS and NEM.

Since GTPγS might bind tightly (and rapidly) to a later acting component, the inability of GTP to block GTPγS inhibition 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 (Mélançon et al., 1987), before the action of NSF (Orci et al., 1989). We carried out kinetic experiments to map the point along the vesicular 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 reaction intermediate, there should be a point in time, after formation 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 mixture 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. 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 (Mélançon 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 be 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 (Mélançon et al., 1987; Orci 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 (Mélançon 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, pretreatment 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...
branes. 3S-labeled semi-intact cells (E) or rat liver Golgi mem-
dicated concentrations of cytosolic proteins. Extent transport rep-
either in the presence or absence of 50 μM GTP3,S with the in-
rentations. Standard transport reactions (200 μl) were carried out
branes (G) were preincubated separately in the presence (+) or
absence (-) of 50 μM GTP3,S for 20 min at 37°C; all preincubation
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→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

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→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→Golgi, intra-Golgi, and endo-
some fusion reactions. Most convincing, in this regard, was the observation that cytosol fractions immunodepleted of ≥90% of NSF protein, or heated to 37°C to inactivate ≥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.

liver Golgi membranes, it was important to rule out the possi-
ability that nonendosomal components in the semi-intact cell extract were responsible for the GTPγS inhibition observed. In control experiments, inclusion of nonradiolabeled, semi-intact cells during the GTPγS preincubation of rat liver Golgi complexes did not alter their insensitivity to the non-
hydrolyzable GTP analogue (data not shown).

The kinetic experiments presented in Fig. 5 suggest that GTPγS binds rapidly and tightly to transport components, by a process that is facilitated by cytosolic factors (Fig. 7 A). The experiment presented in Fig. 7 B indicates that the ability of GTPγS to inhibit transport is also dependent upon the presence of the donor membranes containing radiolabeled man6P receptors. Although we have no information regarding the precise point in transport at which bound GTP is hydrolyzed, our data strongly suggest that a guanine nucleo-
tide is stably recruited onto nascent, budding transport vesi-
cles at the beginning of transport. GTP hydrolysis could then accompany the budding process (Tooze et al., 1990) and/or fusion of transport vesicles with their target membrane.

Figure 7. (A) GTPγS shows maximal inhibition at high cytosol concentrations. Standard transport reactions (200 μl) were carried out either in the presence or absence of 50 μM GTPγS with the indicated concentrations of cytosolic proteins. Extent transport rep-
resents the fraction of transport observed in the presence of GTPγS relative to the control. (B) GTPγS preincubation inhibits the activ-
ity of the donor, endosome fraction but not of acceptor Golgi mem-
branes. 35S-labeled semi-intact cells (E) or rat liver Golgi mem-
branes (G) were preincubated separately in the presence (+) or
absence (−) of 50 μM GTPγS for 20 min at 37°C; all preincubation
experiments contained ATP, an ATP-regenerating system (Goda and
Pfeffer, 1988) and 2.5 mg/ml crude cytosol. Fractions were subse-
quently combined as indicated and incubated for 1 or 2 h at 37°C
in the presence of 1.5 mM GTP.
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 man6P 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, assayed 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, GTP7S inhibited transport throughout the course of the reaction. Nevertheless, GTP7S 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 GTP7S only inhibits the activity of the acceptor membranes (Melançon 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 ETF-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.

We are grateful to Dr. James Rothman for providing anti-NSF IgM and reagents for the intra-Golgi transport experiments, and thank Dr. Morey Slodki for providing Haserella holotis phosphomannan. We also acknowledge the contributions of Dr. Rockford Draper who tested the effect of nucleotides in preventing GTP7S inhibition, and Dr. Sophie Gomez, who initiated gel filtration analyses of ETF-1 activity.

This work was supported by National Institutes of Health grant DK73732. Y. Goda was a predoctoral fellow of the Markey Foundation.

Received for publication 28 August 1990 and in revised form 8 November 1990.

References


