A Novel Assay Reveals a Role for Soluble N-Ethylmaleimide-sensitive Fusion Attachment Protein in Mannose 6-Phosphate Receptor Transport from Endosomes to the Trans Golgi Network*

(Received for publication, June 11, 1997, and in revised form, September 2, 1997)

Christian Itin‡, Carmen Rancaño§, Yoshiaki Nakajima, and Suzanne R. Pfeffer†

From the Department of Biochemistry, Stanford University School of Medicine, Stanford, California 94305-5307

Soluble N-ethylmaleimide-sensitive fusion protein (NSF) attachment protein (α-SNAP) is a soluble protein that enables the NSF ATPase to associate with membranes and facilitate membrane trafficking events. Although NSF and α-SNAP have been shown to be required for many membrane transport processes, their role in the transport of mannose 6-phosphate receptors from endosomes to the trans Golgi network was not established. We present here a novel in vitro assay that monitors the transport of cation-dependent mannose 6-phosphate receptors between endosomes and the trans Golgi network. The assay relies on the trans Golgi network localization of tyrosine sulfotransferase and sensors transport of mannose 6-phosphate receptors engineered to contain a consensus sequence for modification by this enzyme. Using this new assay we show that α-SNAP strongly stimulates transport in reactions containing limiting amounts of cytosol. Together with α-SNAP, NSF can increase the extent of transport. These data show that α-SNAP, a soluble component of the SNAP receptor machinery, facilitates transport from endosomes to the trans Golgi network.

NSF1 is a trimeric ATPase that is required for the transport of proteins between a variety of membrane-bound compartments in eukaryotic cells (1–3). NSF requires the so-called SNAP proteins to bind to membranes. Three SNAP isoforms have been identified: α, β, and γ (4). SNAPs are soluble proteins that bind saturably to Golgi membranes (5, 6).

When NSF is mixed together with α-SNAP and a detergent extract of salt-washed Golgi membranes, NSF and α-SNAP become incorporated into a 20 S complex (7). Characterization of the proteins contained within this complex led to the identification of the SNAP receptors or SNAREs (8). Pairing of v-SNAREs on transport vesicles and t-SNAREs on their cognate membrane targets is thought to provide specificity for vesicle docking and fusion events (3). ATP hydrolysis by NSF induces a conformational change in the SNARE proteins (9) that triggers SNARE complex disassembly, prior to membrane fusion.

α-SNAP and NSF have been implicated in most heterotypic and homotypic docking and fusion reactions (3, 10–14). However in MDCK cells, transport from the trans Golgi network (TGN) to the apical plasma membrane was independent of NSF and could not be stimulated by α-SNAP (15). In yeast, a late step in the delivery of internalized α-factor to the vacuole requires an NSF homolog, the END13 gene product (16). Furthermore, delivery of carboxypeptidase Y from the TGN to the vacuole was shown to be independent of NSF (17). Carboxypeptidase Y is sorted by a receptor in the TGN for delivery to the endosomal/vacuolar system. The sorting receptor, Vps10p, has to return to the TGN for additional rounds of transport (18). Thus, it was possible that recycling from endosomes to the TGN might also be independent of NSF.

We study the transport of mannose 6-phosphate receptors (MPRs) between endosomes and the TGN, both in cultured cells (cf. Ref. 19) and in an in vitro assay that reconstitutes this transport process (20). In mamalian cells, MPRs carry lysosomal hydrolases from the TGN to endosomes, where they release the hydrolases and then return to the TGN for another round of transport (21). In previous work, we were unable to determine if NSF was required for endosome-to-TGN transport because this step requires a factor that is more sensitive to N-ethylmaleimide than NSF (22). Although NSF-depleted cytosol was active in in vitro transport, it was possible that the abundant, membrane-associated NSF present in those reactions was sufficient for the transport observed. Using a novel in vitro transport assay, we show here that α-SNAP stimulates the recycling of MPRs from endosomes to the TGN. Furthermore, NSF can stimulate transport in the presence of α-SNAP.

EXPERIMENTAL PROCEDURES

GDI and Rab9-GDI complexes were prepared as described (23).

Mouse cation-dependent mannose 6-phosphate receptor (MPR46) cDNA was a gift of Dr. Bernhard Hofack (EMBL, Heidelberg, Germany). The bacterial expression plasmid pMW183 containing His6::NodQ2 was a gift of Dr. Sharon Long (Stanford University). Dr. James Rothman (Sloan-Kettering, New York), Dr. Sidney Whiteheart (Kentucky College of Medicine, Lexington), and Dr. Thomas Mayer (University of Muenster, Germany) kindly provided expression plasmids for His-tagged versions of NSF, α-SNAP, and Rab1b N121I, respectively. The following chemicals (Sigma) were kept as stocks: 100 × protease inhibitor mix: 1 mg/ml leupeptin, 4 mg/ml aprotinin, 100 μM pepstatin A, stored at −80 °C; 20 mg/ml cycloheximide in ethanol and 20 μM primaqmine in H2O, stored at −20 °C.

Recombinant DNA and Cell Lines

MPR46 cDNA (24) was inserted as an EcoRI fragment in Bluescript SK (Stratagene). First a tyrosine sulfation site (25) was introduced at

This paper is available online at http://www.jbc.org
cDNA position 241 of mouse MPR46 by polymerase chain reaction and cloned via a primer-based 5’-NcoI site to the 3’-end of an influenza virus hemagglutinin leader sequence fused to a FLAG epitope tag that was derived from pB6G5SF32 (26). Next, the sequence encoding the FLAG epitope tag was replaced by polymerase chain reaction with a cassette coding for an [His]6::NodQ2 tag (His6::NodQ2) (22). The modified cDNA was cloned back to the MPR46 DNA via a ScaI site at position 346, replacing its 5’-region. This construct was designated MPR46HMY and confirmed by DNA sequencing. MPR46HMY was cloned as an XhoI/XbaI fragment into pME18S (R. Maruyama, University of Tokyo). This construct and pSV2neo (ratio 10:1) were cotransfected into CHO cells. Stable transformants were selected with geneticin (Sigma), 100 units/ml inorganic pyrophosphatase (Sigma), and 10 μCi/ml [35S]sulfate (ICN) in a-MEM supplemented with 7.5% fetal calf serum. Of 23 isolated clones, 11 were positive by Western blot and indirect immunofluorescence (27) using monoclonal antibody 9E10 against the c-myc epitope (28). Clones were maintained in the above medium. All studies were carried out with clone 3.

**Synthesis of 35S-Labeled 3’-Phosphoadenosine 5’-Phosphosulfate (PAPS)**

The synthesis of [35S]labeled 3’-phosphoadenosine 5’-phosphosulfate (PAPS) was modified from Ebhrhardt et al. (29). The reaction is outlined in Fig. 1C. A typical synthesis reaction, total volume of 200 μl, consisted of 1 μCi of sodium [35S]sulfate (ICN) in reaction buffer (10 mM Tris/HCl, pH 8, 30 mM KCl, 5 mM MgCl2, 1 mM EDTA, 1 mM dithiothreitol, 10% glycerol, 10% DMSO, 100 units/ml AP phosphatase (Sigma), 100 units/ml inorganic pyrophosphatase (Sigma), and 1 mM recombinant His-tagged NodQ2 gene product from Rhizobium meliloti (NodQ2 has both ATP-sulfurylase and APS kinase activity (49)). The reaction was incubated for 2 h at 30 °C, and enzymes were inactivated by boiling for 1 min and aggregates pelleted for 2 min in a microcentrifuge. The reaction mixture was diluted to 2 μl with distilled H2O yielding a specific activity of 0.5 Ci/μmol (corresponding to 0.3 μmol PAPS). The extent of conversion of [35S]PAP to [35S]PAPS was monitored by spotting 2 μl of reaction product onto a polyethyleneimine-cellulose F (Merck) TLC plate, previously washed for 5 min with H2O and dried. The reaction products were resolved by 0.9 M LiCl.

**Metabolic Labeling and Immunoprecipitation**

Cells were grown to subconfluence, washed with TD (25 mM Tris/HCl, pH 7.4, 5.4 mM KCl, 137 mM NaCl, 0.3 mM Na2HPO4), and preincubated in labeling medium for 15 min (α-MEM lacking methionine and cysteine but containing 7.5% dialyzed fetal calf serum). Cells were pulsed for 15 min with 1 μCi/ml [35S]methionine and -cysteine (Tran35S-label, ICN), transferred to ice, washed twice with chase medium (α-MEM, 7.5% fetal calf serum, 10 mM methionine, 10 mM cysteine), and chased for the indicated times. Cells were transferred to ice, washed with TD, harvested in RIPA (50 mM Tris-HCl, pH 7.8, 150 mM NaCl, 1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% sodium dodecyl sulfate (SDS), 1.5% Triton X-100) in the presence of 1 mM phenylmethylsulfonyl fluoride and, solubilized for 1 h. After centrifugation at 100,000 × g for 10 min at 4 °C, the supernatant was transferred to a new tube; 5 μl of the supernatant was removed, and snap-frozen on a metal plate cooled on dry ice. After thawing the cells for 4 min at room temperature, they were transferred on ice and washed 2 × with KH to remove cytosol. The plates were drained extensively; 250 μl of SEAT buffer (10 mM triethanolamine, 10 mM acetic acid, pH 7.4, 1 mM EDTA, 0.25 M sucrose) was added, and the cells were scraped with a rubber policeman and pooled.

**Preincubation with Non-labeled PAPS, Collection, and Freezing Membranes**

Broken cells were preincubated with unlabeled PAPS in the absence of added cytosol to reduce Golgi-derived background. 250 μl of broken cells in SEAT were mixed with 80 μl of RB buffer (25 mM HEPES-KOH, pH 7.2, 15 mM magnesium acetate, 25 mM KCl), 120 μl of ATP mix (10 mM ATP, pH 7, 150 mM creatine phosphate, 20 mM MgCl2, 210 units/ml creatine phosphokinase, 0.1 mg/ml leupeptin, 0.4 mM phenylmethylsulfonyl fluoride, 10 μg of pepstatin A), 260 μl of 0.75 M sucrose, 5 μl of 5 mM PAPS (Sigma, −25 μm final), and H2O to a final volume of 1 ml. After 15 min at 37 °C the mix was overlayed on a snap-frozen sucrose cushion (20 μl of 1 M, 40 μl of 0.5 M sucrose). The sucrose was thawed for 5 min on ice, and the membranes were pelleted for 5 min at 4 °C in a microcentrifuge at top speed. 850 μl of the supernatant was removed, and the membranes were resuspended in the remaining volume. Suspended membranes were pooled, aliquoted, and snap-frozen in liquid nitrogen and stored at −80 °C.

**Transport Reaction**

Routinely, transport reactions were carried out in a final volume of 150 μl. A standard transport reaction consisted of 1 μl of RB, 12 μl of ATP mix, 1.4 μl of 2 mM sodium chloride, H2O, 10 μl of 0.5 μCi/ml [35S]PAPS (~20 μCi final), 6 μl of membranes, rapidly thawed on ice, and 14 μl of 50 mM sodium carbonate (pH 9.5), 1 μCi/ml [35S]PAPS (Sigma, −25 μCi final), and H2O to a final volume of 1 ml. After 15 min at 37 °C the mix was overlayed on a snap-frozen sucrose cushion (20 μl of 1 M, 40 μl of 0.5 M sucrose). The sucrose was thawed for 5 min on ice, and the membranes were pelleted for 5 min at 4 °C in a microcentrifuge at top speed. 850 μl of the supernatant was removed, and the membranes were resuspended in the remaining volume. Suspended membranes were pooled, aliquoted, and snap-frozen in liquid nitrogen and stored at −80 °C.

**Purification and Quantification of Tyrosine-sulfated MPR46HMY**

The membranes were solubilized with 500 μl of RIPA, 25 mM imidazole for 2 h on ice; insoluble material was pelleted in a microcentrifuge for 5 min, and the supernatant was transferred onto 10 μl of prewashed Ni-NTA agarose beads. MPR46HMY were allowed to bind for 1 h at 4 °C, followed by 4 washes with RIPA, 25 mM imidazole and eluted with 100 μl of 25 mM EDTA in RIPA, 25 mM imidazole.

**Preparation of Recombinant α-SNAP, NSF, and His6:·NodQ2**

Histidine-tagged bacterially expressed α-SNAP and NSF were purified essentially as described by Whiteheart et al. (32). For NSF the only change is that the final step is an S-300 column instead of a Superose 6 Fast Flow (Pharmacia Biotech Inc.). ATPase assays were carried out as described in Tagaya et al. (35, data not shown). His6:·NodQ2, the ATP-sulfurylase/APS kinase from R. meliloti, was expressed in XL-1 blue cells grown to an OD660 of 0.8 at 37 °C, induced for 4 h at 30 °C with 20 μg isopropyl-β-D-galactopyranoside. The cells were harvested by centrifugation and resuspended in 50 mM sodium phosphate, pH 8, 300 mM NaCl, 20 mM imidazole, 10% glycerol, and disrupted by French press. Henegens were cleared by centrifugation, and the supernatant was incubated with 1 ml of Ni-NTA agarose for 30 min at 4 °C. The column was packed, washed with 100 volumes of buffer, and eluted with 500 mM imidazole in buffer. Essentially pure fractions were pooled and dialyzed against 20 mM Tris/HCl, pH 8, 30
The in vitro (His) acid sequence is shown in one-letter code. The abbreviations used are: sulfation site.

will be referred to as MPR46HMY.

myc-tag for detection (Ref. 28; Fig. 1)

N-terminal histidine tag for purification (38), followed by a peptide can serve as a substrate for tyrosine sulfation when sequence derived from the C terminus of the cholecystokinin engineered to contain a tyrosine sulfotransferase recognition minus of the cation-dependent, murine MPR (MPR46, 24) was specific enzyme, tyrosine-sulfotransferase.

zyme, tyrosine-sulfotransferase. A, MPR46HMY, the reporter used in the in vitro assay, is a modified MPR46 that contains a triple tag histidine tag; c-myc epitope tag; Y-sulfation, tyrosine sulfation site.

mA KCl, 5 mM MgCl2, 1 mM EDTA, 1 mM dithiothreitol, 10% glycerol, aliquoted, and stored at −80 °C. Activity of His6-NodQ2 was verified measuring the conversion of radiolabeled sulfate to radiolabeled PAPS and separation of the reaction products by TLC as described above (see Fig. 3B).

RESULTS

In previous work, we were unable to determine if NSF was required for endosome-to-TGN transport because this step requires a factor that is more sensitive to N-ethylmaleimide than NSF (22). In these experiments, it was possible that the abundant, membrane-associated NSF was sufficient for the transport observed. Thus, we sought to establish a facile assay that reconstituted the same transport process but might show greater dependence upon added cytosolic factors.

Our previously established in vitro transport reaction makes use of the TGN localization of sialyltransferase and a CHO mutant cell line in which N-linked oligosaccharide sialylation does not occur (20). Transport requires the presence of purified, “wild type” rat liver Golgi membranes, as well as ATP and cytosolic proteins. Although this assay has enabled us to show a role for the Rab9 GTPase and Rab9-GDI complexes in MPR trafficking (23, 34), it requires time-consuming metabolic labeling, tedious chromatography steps, SDS-PAGE, and fluorography.

Our new assay (Fig. 2) takes advantage of another TGN-specific enzyme, tyrosine sulfotransferase (35, 36). The N terminus of the cation-dependent, murine MPR (MPR46, 24) was engineered to contain a tyrosine sulfotransferase recognition sequence derived from the C terminus of the cholecystokinin precursor (37). Leitinger et al. (25) have shown that this nonapeptide can serve as a substrate for tyrosine sulfation when transplanted to the C termini of the asialoglycoprotein receptor H1 subunit or α1-proteinase inhibitor. In addition, we added an N-terminal histidine tag for purification (38), followed by a myc-tag for detection (Ref. 28; Fig. 1B). The modified receptor will be referred to as MPR46HMY.

MPR46HMY Is Properly Targeted to the MPR Recycling Pathway in Vivo—At steady state in cultured cells, endogenous MPRs are found predominantly in late endosomes; a smaller number are present in the TGN and early endosomes, and less than 5–10% are located at the cell surface (21). We first analyzed the biochemical properties of MPR46HMY in vivo. CHO cells stably expressing MPR46HMY were pulse-labeled for 15 min and chased for up to 29 h. MPR46HMY was immunoprecipitated with anti-myc tag mAb 9E10, separated by SDS-PAGE, and visualized by fluorography. Fig. 2A shows that MPR46HMY is stable for more than 29 h. The protein was first detected as a 46-kDa glycoprotein which then increased to ~50 kDa due to oligosaccharide maturation; it was converted to its mature form, as judged by endo H cleavage, after ~1 h (Fig. 2B). As shown previously, mature MPR46 contains both endo H-resistant and endo H-sensitive oligosaccharide chains (cf. Ref. 50). These experiments indicate that transport of tagged MPRs through the endoplasmic reticulum and up to the medial Golgi is not delayed due to inefficient folding.

To test the functionality of the tyrosine sulfation tag, untransfected CHO cells (Fig. 2C; lane 1) or cells expressing MPR46HMY (Fig. 2C; lane 2) were labeled with [35S]sulfate for 2 h, and MPR46HMY was purified by detergent solubilization and nickel agarose binding. Only the transfected cells expressed a sulfate-labeled polypeptide that bound to the nickel resin; only a single prominent polypeptide was detected that had the mobility expected for MPR46HMY. This confirmed that the tyrosine sulfation tag was utilized by tyrosine sulfotransferase in vivo and also that [35S]sulfate-labeled MPR46HMY can be purified in one step by virtue of its histidine tag.

The presence of the TGN-specific, tyrosine sulfate modification showed that MPR46HMY was localized at or beyond the TGN. Moreover, the stability of MPR46HMY is a strong indication that it was not mis-sorted to lysosomes, as trafficking mutants of MPR46 are delivered to lysosomes and rapidly degraded there (39). Correct localization was confirmed by indirect immunofluorescence microscopy (Fig. 3). Upon transient transfection in COS cells, MPR46HMY showed excellent co-localization with the 300-kDa, cation-independent MPR.
FIG. 3. Localization of MPR46HMY by immunofluorescence microscopy. A and B, transient expression in COS cells. Cells were double-labeled (19) with polyclonal anti-300-kDa MPR (1:50), Texas Red goat anti-rabbit (1:500, A), mouse anti-myc antibody (1:2000) followed by fluorescein-conjugated goat anti-mouse (1:500, B). C, CHO cells stably expressing MPR46HMY (clone 3) visualized with anti-myc antibody as in B. In all panels, cells were pretreated for 4 h with cycloheximide (20 μg/ml) to drain the secretory pathway. Bar at bottom represents 10 μm.

(Fig. 3, A and B). Unfortunately, the anti-300-kDa MPR antibodies do not recognize the CHO cell form of this protein. Nevertheless, in stably expressing CHO cells, MPR46HMY showed predominantly endosomal staining consistent with its normal localization (Fig. 3C).

Resialylation experiments were carried out to test recycling of MPR46HMY from the plasma membrane to the TGN in cultured cells. Cells were pulse-labeled with [35S]sulfate, and glycoproteins reaching the plasma membrane were desialylated with neuraminidase. The cells were then recultured for several hours in the absence of neuraminidase to permit glycoproteins recycling to the TGN to re-acquire sialic acid. The rate of resialylation, determined by slug binding (20), was identical to that reported for the cation-independent MPR in CHO wild type cells (data not shown; Ref. 19). We therefore conclude that MPR46HMY is functional in terms of its ability to recycle to the TGN in living cells.

In Vitro Sulfation as a Measure of Transport—The new in vitro reaction utilizes [35S]3'-phosphoadenosine 5'-phosphosulfate (PAPS) as the precursor for tyrosine sulfation. PAPS is normally synthesized in the cytosol from sulfate and ATP in a two-step process (Fig. 4A). Sulfate and ATP are first converted to phosphoadenosine 5'-phosphosulfate (APS) by ATP-sulfurylase; APS is then converted to PAPS by APS kinase. ATP-sulfurylase is reversibly inhibited by chloride at millimolar concentrations, both in vivo and in vitro (40).

[35S]PAPS can be synthesized in vitro from [35S]sulfate and ATP with an efficiency of more than 95% (Fig. 4B), using commercially available ATP-sulfurylase and inorganic pyrophosphatase together with recombinant APS kinase from R. meliloti (29). [35S]PAPS synthesis was monitored by thin layer chromatography (Fig. 4B) and could be used in in vitro transport reactions without further purification. [35S]PAPS was unstable at 37 °C in the presence of cytosolic proteins. However, inclusion of 10 mM chlorate in reaction mixtures provided significant stabilization of the PAPS substrate (Fig. 4B).

To use tyrosine sulfation as a measure of endosome-to-TGN transport, the rate of tyrosine sulfation must be significantly faster than the rate of the overall transport process. We therefore examined the kinetics of [35S]PAPS translocation and its subsequent incorporation into trichloroacetic acid-precipitable material using purified rat liver Golgi membranes. In the presence of 20 μM [35S]PAPS, the half-time for the incorporation of the label into trichloroacetic acid-precipitable counts was reached after 5 min (not shown), which is equivalent to the transport reaction lag phase time period (Ref. 20; this study, Fig. 5C). This concentration of PAPS exceeds that reported for saturation of the PAPS translocation machinery (5 μM, Ref. 41). Together, these data suggest that at a concentration of 20 μM [35S]PAPS and in the presence of chloride (to stabilize PAPS), the kinetics of sulfation should be fast enough to use as an indicator of TGN arrival in vitro.

Tyrosine Sulfation-based Endosome-to-TGN Transport Assay—The new assay utilizes donor membranes containing unsulfated MPR46HMY receptors. To accumulate unsulfated receptors, cultured cells are grown for 3 days in sulfate-free medium containing 10 mM chloride. Four hours prior to cell harvest, cycloheximide is added to chase newly synthesized MPR46HMY to their steady state localization. After as little as
In independent experiments. When energy dependence was measured, the
MPR46HMY band did not increase with time, despite the
table. Reactions were incubated for up to 2 h at 37 °C, stopped on ice, and
detergent-solubilized. MPR46HMY receptors are collected by
binding to nickel agarose, which is washed and then eluted
with EDTA. The reaction product is essentially pure, as shown
by SDS-PAGE and fluorography (Fig. 2C, lane 3). The purity of
the signal permitted direct quantification of the extent of
MPR46HMY sulfation by liquid scintillation counting.

Fig. 5A shows the general features of the in vitro transport
process, monitored by the sulfation of MPR46HMY. Reactions
were carried out for 2 h at 37 °C. Under these conditions,
sulfation was stimulated significantly by cytosol; we routinely
observed 5–7-fold stimulation by cytosol addition (Fig. 5, A and
B). No transport was observed at 4 °C or in the presence of an
ATP depletion system. Moreover, Rab GTPases were required
because addition of Rab GDP dissociation inhibitor-α (GDI)
inhibited transport significantly (42). Thus, Rab proteins are
required for efficient transport-coupled sulfation. These control
experiments confirmed that the sulfate incorporation observed
was not due to MPR46HMY that might have been localized to
the TGN at steady state.

To rule out the possibility that we were monitoring intra-
Golgi transport, we added primaquine, a reagent shown to
completely block intra-Golgi transport without affecting endo-
some-to-TGN transport (20, 43). As shown in Fig. 5A, prima-
quione had no effect on the extent of MPR46HMY sulfation. In
addition, a recombinant dominant negative form of Rab1b (mu-
tation N121I) added at final concentrations of up to 2.7 µg/ml
had no effect (not shown), confirming that endoplasmic reticu-
Ium-to-Golgi and intra-Golgi transport processes were not
being scored (44–46). Together, these data show that we are not
monitoring intra-Golgi transport.

In vitro transport was maximal at 0.5 mg/ml cytosol (Fig.
5B). In addition, the reaction was complete after 60 min (Fig.
5C). The slight lag seen between 0 and 5 min may represent the
time required for the formation of transport intermediates;
alternatively, it may simply be a consequence of the presence of
cold PAPS in the lumen of the TGN at the start of the reaction.
The reaction process is somewhat faster than that reported
previously (20). This difference is likely due to the fact that
receptor sialylation is less efficiently detected than receptor
tyrosine sulfation.

In summary, these data suggest that MPR46HMY sulfation
can be used to measure the extent of transport of this receptor
from endosomes to the TGN in vitro. Confirmation of this
conclusion comes from our observation that Rab9 participates
in this process (see below).

α-SNAP, Rab9, and NSF Stimulate Endosome-to-TGN
Transport in Vitro—When recombinant α-SNAP was added to
reactions containing limiting amounts of cytosol (Fig. 6A),
transport could be stimulated to the same level observed in the
presence of saturating cytosol concentrations (Fig. 6B). To
verify that this stimulation was not due to a potential enhance-
ment of intra-Golgi transport, we showed that stimulation of
transport by α-SNAP was unaffected by primaquine (Fig. 6C).
These data suggest that α-SNAP is a very limiting component
under the conditions of this assay and can stimulate endosome-
to-TGN transport.

We have previously shown that prenylated Rab9 (in complex
with GDI) is required for endosome-to-TGN transport, both in vivo
and in vitro (19, 23, 34). Here, we tested whether
Rab9-GDI complexes could stimulate transport in the presence of purified recombinant α-SNAP. Fig. 7A shows that in the presence of limiting cytosol, Rab9-GDI complexes were only slightly stimulatory at the concentration employed. However, in the presence of 50 or 150 ng/ml α-SNAP, the same amount of Rab9-GDI was significantly more effective at enhancing transport, suggesting that α-SNAP is a limiting component required for the detection of Rab9 function.

The ability of Rab9 to stimulate in vitro transport provides the most stringent confirmation that the transport event we have reconstituted here reflects the physiological process by which MPRs travel between endosomes and the TGN (19). Moreover, both the original and new transport assays show parallel requirements for each of the requisite protein factors identified to date. These findings strongly support the notion that we have reconstituted, endosome-to-TGN transport. α-SNAP is thought to attach NSF to membrane-bound SNAP receptors (reviewed in Ref. 3). We therefore tested whether α-SNAP and NSF showed synergy in their abilities to stimulate the transport reaction. Limiting cytosol yielded 35% maximal transport. NSF or α-SNAP added alone to limiting cytosol raised transport to 52 or 65%, respectively, of that measured with full cytosol. However, when both of these proteins were added in concert, transport was stimulated to ~110% of that seen with saturating amounts of cytosol (Fig. 7B).

In summary, these experiments demonstrate that α-SNAP can stimulate endosome-to-TGN transport. The stimulation by α-SNAP was specific, because it occurred in the presence of primaquine and also increased the stimulation observed in reactions containing Rab9-GDI complexes. Furthermore, α-SNAP increased stimulation of transport by NSF, supporting a role for NSF in endosome-to-TGN transport.2

DISCUSSION

We have shown here that α-SNAP stimulates endosome-to-TGN transport in vitro. This is a strong indication that the SNARE machinery is also utilized for transport of MPRs from endosomes to the TGN. To reveal this requirement, we used a new in vitro assay that shows significantly greater cytosol dependence than our previous transport assay (20).

The new assay was designed to make use of the TGN localization of tyrosine sulfotransferase and required a cell line expressing MP46HMY, a cation-dependent MPR modified at its N terminus to contain His- and myc-tags, as well as a site for

2 Y. Nakajima, C. Itin, and S. R. Pfeffer, submitted for publication.
tyrosine sulfation. We showed that MPR46-HMY rapidly acquires endo consistent with its failure to be mislocalized to lysosomes for degradation. Moreover, the protein traversed from the cell surface to the TGN with the same kinetics as the cation-independent MPR in CHO cells. These features strongly support the conclusion that we have not altered the trafficking signals within MPR46. In addition, the fact that MPR46-HMY was a substrate for tyrosine sulfotransferase showed that the consensus polypeptide derived from the cholecystokinin precursor can function when introduced near the N terminus of MPR46.

Several lines of evidence confirm that we have reconstituted the transport of MPR46 from endosomes to the TGN. Most importantly, the reaction is stimulated by the Rab9 GTPase and is completely unaffected by primaquine. These characteristics are hallmarks of MPR recycling (20, 23, 34) and strongly rule out the possibility that we are measuring intra-Golgi transport or endoplasmic reticulum-to-Golgi transport. In further support of a vesicular transfer, the reaction is stimulated by α-SNAP and NSF and is inhibited by GDI, as would be expected of a Rab-dependent process.

At steady state in NRK cells and CHO cells, greater than 90% of 300-kDa MPRs reside in late endosomes (51). Colocalization of MPR46 and the 300-kDa MPR has failed to reveal significant differences in the steady state distribution of these proteins between cellular compartments (cf. Ref. 52). Thus, it seems likely that the protein we follow begins transport in the late endosomal compartment. In addition, any protein that might have been present in the TGN at the beginning of the reaction will be masked by the cold pre-labeling protocol employed here.

Can we be certain that tyrosine sulfotransferase marks arrival in the TGN and not another cellular compartment? This enzyme is localized to the TGN in PC12 cells (36), MDCK cells (25), and IgM-secreting B cells (40). In one study by Spiess and co-workers (25), an overexpressed substrate appeared to acquire tyrosine sulfate before galactose and sialic acid when it was slowed in its export from the TGN by 20 °C incubation of COS-7 cells but not MDCK cells. Although we have not directly immunolocalized tyrosine sulfotransferase in CHO cells, it seems most likely that the enzyme resides in the TGN because MPRs recycle to the TGN but not compartments containing galactosyltransferase in CHO cells (53). Moreover, Rab9 facilitates transport of cation-independent MPRs from late endosomes to the TGN in living cells and in vivo (19, 34), the process reconstituted here.

Multiple features of the new in vitro transport assay can account for its increased cytosol dependence. In the new assay, MPR46-HMY-expressing CHO wild type cells provide both donor and acceptor compartments. In contrast, the previous complementation scheme required addition of rat liver Golgi membranes (20) which do contain significant concentrations of peripheral membrane proteins (1, 4). Furthermore, in the new assay, membranes are washed by centrifugation through a sucrose cushion, which removes cytosolic and, perhaps also, some peripherally associated membrane proteins. These differences readily explain the increased level of stimulation seen with added cytosolic proteins.

The new assay seemed especially limiting for α-SNAP. Wickner and colleagues (47) have shown that α-SNAP dissociates from yeast vacuolar membranes in the presence of ATP with a half-time of less than 10 min. This condition is very similar to the non-radioactive PAFS preincubation step used in our new assay, which involves a 15-min incubation with an ATP-regenerating system. It is likely that the preincubation step has revealed a role for α-SNAP in endosome-to-TGN transport by depleting the protein from the membranes.

We have previously described an N-ethylmaleimide-sensitive protein required for MPR transport that differed from NSF (22). In contrast to NSF, this factor acted early in transport, presumably in the formation of transport vesicles. The fact that this protein was more sensitive to N-ethylmaleimide than NSF made it difficult for us to determine whether NSF was also required. Very recently, we have used mutant NSF proteins to demonstrate a role for NSF in endosome-to-TGN transport using our previous assay. The enhanced cytosol dependence of the new assay also permitted us to confirm a role for NSF in endosome-to-TGN transport, without needing to inactivate endogenous NSF by treatment of membranes with N-ethylmaleimide.

The discovery of a role for α-SNAP in endosome-to-TGN transport will now allow us to investigate SNARE complex formation for this transport process. Our working hypothesis is that the Rab9 GTPase, present on transport vesicles in its active, GTP-bound conformation, recruits docking factors onto vesicles to direct their association with the TGN. We have proposed that this docking process leads to depletion of the SNARE proteins, permitting SNARE pairing and subsequent fusion (48). The new in vitro transport assay will greatly facilitate the identification of novel docking factors, helping to expand our understanding of the link between Rab GTPases and the docking and fusion machinery.

Acknowledgments—We thank Drs. James Rothman, Sidney Whiteheart, and Thomas Mayer for expression plasmids encoding NSF and α-SNAP, respectively, and Drs. Sharon Long and David Ehrhardt for the expression plasmid encoding R. meliloti AP5 kinase and their initial help with the in vitro synthesis of [35S]PAPS. Our special thanks go to Drs. Dana Auit-Riché, Nirit Uilitzur, and Adam D. Linstedt for helpful discussions and insights during the course of this study.

REFERENCES
1. Block, M. R., Glick, B. S., Wilcox, C. A., Wieland, F. T., and Rothman, J. E. (1988) Proc. Natl. Acad. Sci. U. S. A. 85, 7852–7856
2. Whiteheart, S. W., Rossnagel, K., Buhrow, S. A., Brunner, M., Janicke, R., and Rothman, J. E. (1994) J. Cell Biol. 126, 945–954
3. Rothman, J. E. (1994) Nature 372, 55–63
4. Clary, D. O., Griff, I. C., and Rothman, J. E. (1990) Cell 61, 709–721
5. Weidman, P. J., Melano, B., Block, M. R., and Rothman, J. E. (1989) J. Cell Biol. 108, 1589–1596
6. Whiteheart, S. W., Brunner, M., Wilson, D. W., Wiedmann, M., and Rothman, J. E. (1992) J. Biol. Chem. 267, 12239–12243
7. Wilson, D. W., Whiteheart, S. W., Wiedmann, M., Brunner, M., and Rothman, J. E. (1992) Cell 117, 531–538
8. Söllner, T., Whiteheart, S. W., Brunner, M., Erdjument-Bromage, H., Geronemus, S., Tempst, P., and Rothman, J. E. (1993) Nature 362, 318–323
9. Hanson, P. I., Otto, H., Barton, N., and Jahn, R. (1995) J. Biol. Chem. 270, 16955–16961
10. Rabouille, C., Levine, T. P., Peters, J.-M., and Warren G. (1995) Cell 82, 905–914
11. Arkhauy, U., Jacobs, R., Peters, J.-M., Watson, N., Farquhar, M. N., and Malhotra, V. (1995) J. Cell Biol. 129, 577–589
12. Haas, A., and Wickner, W. (1996) EMBO J. 15, 3396–3405
13. Rodrigues, L., Stirling, C. J., and Woodman, P. G. (1994) Mol. Cell. Biol. 5, 775–783
14. Morgan, A., and Burgoyne, R. D. (1995) EMBO J. 14, 232–239
15. Ikonen, E., Tagaya, M., Ullrich, O. Monteuccio, C., and Simons, K. (1995) Cell 81, 571–580
16. Munn, A. L., and Riezman, H. (1994) J. Cell Biol. 127, 373–386
17. Graham, T. R., and Emr, S. D. (1991) J. Cell Biol. 114, 207–218
18. Cooper, A. A., and Stevens, T. H. (1990) J. Cell Biol. 125, 529–541
19. Riederer, M. A., Soldati, T., Shapiro, A. D., Lin, J., and Pfeffer, S. R. (1994) J. Cell Biol. 125, 573–582
20. Guda, Y., and Pfeffer, S. R. (1988) Cell 55, 309–320
21. Kornfeld, S., and Mellman, I. (1989) Annu. Rev. Cell Biol. 5, 483–525
22. Guda, Y., and Pfeffer, S. R. (1991) J. Cell Biol. 112, 823–831
23. Dirac-Sveistrup, A. B., Soldati, T., Shapiro, A. D., and Pfeffer, S. R. (1994) J. Biol. Chem. 269, 15327–15340
24. Ludwig, T., Ruther, U., Metzger, R., Copeland, N. G., Jenkins, N. A., Lebel, P., and Hofflack, B. (1992) J. Biol. Chem. 267, 12121–12129
25. Leitinger, B., Brown, J. L., and Spiess M. (1994) J. Biol. Chem. 269, 8116–8121
26. Guan, X.-M., Kohlbka, T.-S., and Kohlbka, B. (1992) J. Biol. Chem. 267, 21995–21998
27. Warren, G., Davoust, J., and Cockcroft, A. (1984) EMBO J. 3, 2217–2225
28. G. Griffiths, personal communication.
α-SNAP in Endosome-to-TGN Transport

28. Evan, G. I., Lewis, G. K., Ramsay, G., and Bishop J. M. (1985) *Mol. Cell Biol.* **5**, 3610–3616
29. Ehrhardt, D. W., Atkinson, E. M., Faull, K. F., Freedberg, D. J., Sutherland, D. P., Armstrong, R., and Long, S. R. (1995) *J. Bacteriol.* **177**, 6237–6245
30. Laemmli, U. K. (1970) *Nature* **227**, 680–685
31. Acharya, U., McCaffery, J. M., Jacobs, R., and Malhotra, V. (1995) *J. Cell Biol.* **129**, 577–589
32. Whiteheart, S. W., Griffin, I. C., Brunner, M., Clary, D. O., Mayer, T., Bahrow, S. A., and Rothman, J. E. (1993) *Nature* **362**, 353–355
33. Tagaya, M., Wilson, D. W., Brunner, M., Arango, N., and Rothman, J. E. (1993) *J. Biol. Chem.* **268**, 2662–2666
34. Lombardi, D., Soldati, T., Riederer, M. A., Goda, Y., Zerial, M., and Pfeffer, S. R. (1993) *EMBO J.* **12**, 677–682
35. Baeuerle, P. A., and Huttner, W. B. (1987) *Biochem. Biophys. Res. Commun.* **141**, 870–877
36. Ross, F., Mantovani, S., Rosboch, R., and Huttner, W. B. (1992) *J. Biol. Chem.* **267**, 12227–12232
37. Rosenquist, G. L., and Nicholas, H. B. (1993) *Protein Sci.* **2**, 215–222
38. Hochuli, E., Bannwarth, W., Doebeli, H., Gentz, R., and Stueber, D. (1988) *Biotechnology* **6**, 1321–1325
39. Rohrer, J., Schweitzer, A., Johnson, K. F., and Kornfeld, S. (1995) *J. Cell Biol.* **130**, 1297–1306
40. Baeuerle, P. A., and Huttner, W. B. (1986) *J. Cell Biol.* **129**, 577–589
41. Schwarz, J. K., Capasso, J. M., and Hirschberg, C. B. (1984) *J. Biol. Chem.* **259**, 3554–3559
42. Pfeffer, S. R., Dirac-Svejstrup, A. B., and Soldati, T. (1995) *J. Biol. Chem.* **270**, 17057–17059
43. Hiebsch, R. R., Rauh, T. J., and Wattenberg, B. W. (1991) *J. Biol. Chem.* **266**, 20323–20328
44. Plutner, H., Cox, A. D., Pind, S., Keshravi-Far, R., Bourne, J. R., Schwanger, R., Der, C. J., and Balch, W. E. (1991) *J. Cell Biol.* **115**, 51–63
45. Pind, S. N., Noller, C., McCaffery, J. M., Plutner, H., Davidson, H. W., Farquhar, M. G., and Balch, W. E. (1994) *J. Cell Biol.* **122**, 239–252
46. Davidson, H. W., and Balch, W. E. (1993) *J. Biol. Chem.* **268**, 4216–4226
47. Mayer, A., Wickner, W., and Haas, A. (1996) *Cell* **85**, 83–94
48. Pfeffer, S. R. (1996) *Annu. Rev. Cell Dev. Biol.* **12**, 441–461
49. Schwedoch, J. S., Liu, C., Leyh, T. S., and Long, S. R. (1994) *J. Bacteriol.* **176**, 7055–7064
50. Hille, A., Waheed, A., and von Figura K. (1990) *J. Cell Biol.* **110**, 963–972
51. Griffiths, G., Matteoni, R., Back, R., and Hoflack, B. (1990) *J. Cell Sci.* **95**, 441–461
52. Klumperman, J., Hille, A., Veermendaal, T., Oorschot, V., Stoovogel, W., von Figura K, and Geuze, H. J. (1993) *J. Cell Biol.* **121**, 997–1010
53. Duncan, J., and Kornfeld, S. (1988) *J. Cell Biol.* **106**, 617–628