The Small GTP-binding Protein rab6 Functions
in intra-Golgi Transport

Olivier Martinez,* Anne Schmidt,‡ Jean Salaméro,§ Bernard Hoflack,‡ Michèle Roa,* and Bruno Goud*

*Unité de Génétique Somatique (Centre National de la Recherche Scientifique URA 361), Institut Pasteur, 75724 Paris Cedex 15, France; ‡European Molecular Biology Laboratory, Postfach 102209, D-6900 Heidelberg, Germany; and §Institut National de la Santé et de la Recherche Medicale U 255, Institut Curie, 75231 Paris Cedex 05, France

Abstract. Rab6 is a ubiquitous ras-like GTP-binding protein associated with the membranes of the Golgi complex (Goud, B., A. Zahraoui, A. Tavitian, and J. Saraste. 1990. *Nature (Lond.).* 345:553–556; Antony, C., C. Cibert, G. Géraud, A. Santa Maria, B. Maro, V. Mayau, and B. Goud. 1992. *J. Cell Sci.* 103: 785–796). We have transiently overexpressed in mouse L cells and human HeLa cells wild-type rab6, GTP (rab6 Q72L), and GDP (rab6 T27N) -bound mutants of rab6 and analyzed the intracellular transport of a soluble secreted form of alkaline phosphatase (SEAP) and of a plasma membrane protein, the hemagglutinin protein (HA) of influenza virus. Overexpression of wild-type rab6 and rab6 Q72L greatly reduced transport of both markers between *cis*/medial (α-mannosidase II positive) and late (sialyl-transferase positive) Golgi compartments, without affecting transport from the endoplasmic reticulum (ER) to *cis*/medial-Golgi or from the *trans*-Golgi network (TGN) to the plasma membrane. Whereas overexpression of rab6 T27N did not affect the individual steps of transport between ER and the plasma membrane, it caused an apparent delay in secretion, most likely due to the accumulation of the transport markers in late Golgi compartments. Overexpression of both rab6 Q72L and rab6 T27N altered the morphology of the Golgi apparatus as well as that of the TGN, as assessed at the immunofluorescence level with several markers. We interpret these results as indicating that rab6 controls intra-Golgi transport, either acting as an inhibitor in anterograde transport or as a positive regulator of retrograde transport.

THREE classes of GTPases play an important role in the molecular mechanisms underlying vesicular transport and membrane trafficking in eukaryotic cells. Members of the ADP-ribosylating factors family participate in the assembly and disassembly of coatomers associated with COP-coated vesicles and in the formation of the clathrin coat (Ostermann et al., 1993; Stamnes and Rothman, 1993). Recent studies have pointed out the role of several heterotrimeric G proteins in the formation of transport vesicles (Leyte et al., 1992). The third class of GTPases is the Sec4/Ypt/rab family, proteins closely related to the p21 ras proto-oncogenes (Valencia et al., 1991).

Genetic studies in yeast have revealed that the small GTPases of the Sec4/Ypt/rab family are potent regulators of membrane traffic. Thus Sec4 and Ypt1, two yeast *Saccharomyces cerevisiae* proteins, are involved in the Golgi to plasma membrane and in the ER to Golgi transport, respectively (Salminen and Novick, 1987; Goud et al., 1988; Bacon et al., 1989; Segev et al., 1988; Segev, 1991). Within

the past few years, about 30 proteins sharing strong homology with Sec4 and Ypt1, the rab proteins, have been characterized in mammalian cells (Touchot et al., 1987; Zahraoui et al., 1989; Chavrier et al., 1990b; Elferink et al., 1992; Zahraoui et al., 1994).

Rab proteins are believed to regulate specific transport steps of membrane traffic. Consistent with this notion, localization studies have now well documented that they can be associated with distinct organelles of the endocytic or the biosynthetic/secretory pathways (Zerial and Stenmark, 1993). A number of in vivo and in vitro studies have also shown that rab proteins are involved in precise steps of vesicular transport: rab1a, rab1b, and rab2 regulate ER to Golgi transport (Plutner et al., 1991; Tisdale et al., 1992; Nuoffer et al., 1994), rab 4 and rab5 early steps in endocytosis (Gorvel et al., 1991; Van der Sluijs et al., 1992; Bucci et al., 1992), rab9 late endosomes to TGN transport (Lombardi et al., 1993; Riederer et al., 1994), rab3a and b, two proteins expressed in neuronal and neuro-endocrine cells, regulated exocytosis (Lledo et al., 1993; Johannes et al., 1994; Holz et al., 1994). Recently, rab8 has been shown to function in vesicular traffic between the TGN and the basolateral plasma membrane in polarized cells (Huber et al., 1993a,b). Since nonfunctional rab proteins lead to the accumulation of trans-

port intermediates, the current hypothesis is that rab proteins could function in specifying accurate targeting/docking of transport vesicles with their acceptor membranes (Goud and McCaffrey, 1991; Zerial and Stenmark, 1993). However, these targeting processes may also require other important components, the so-called v-SNARES and t-SNARES, recently shown to be involved in specific interactions between vesicles and target membranes (Söllner et al., 1993; Rothman and Warren, 1994).

We have previously shown that rab6, a ubiquitous protein expressed in mammalian cells (Goud et al., 1990) as well as in yeast and plants (Hengst et al., 1990; Bednarek et al., 1994) is associated with medial and *trans*-Golgi cisternae (Goud et al., 1990), and membranes of the TGN (Antony et al., 1992). In addition to the membranes of the Golgi complex, rab6 has also been detected on post-Golgi organelles in some specialized cell types (Jasmin et al., 1992; Tixier-Vidal et al., 1993; Deretic and Papermaster, 1993). This localization suggests that rab6 could be involved in intra-Golgi and/or post-Golgi transport events. To pinpoint the site of action of rab6, we have expressed dominant mutants of rab6 taking advantage of the fact that mutations introduced in the conserved guanine nucleotide binding domains profoundly alter their GDP/GTP binding and GTP hydrolysis activities (Valencia et al., 1991; Bourne et al., 1991). These mutations, which are found in oncogenic or dominant inhibitor forms of ras proteins, can lock the proteins into their GDP or GTP-bound conformations, either by altering affinities for nucleotides or by affecting the interactions with regulatory proteins such as exchange factors or GTPase-activating proteins (GAP)[1] (Boguski and McCormick, 1993). Overexpression of such rab mutants in cells have been used to dramatically affect intracellular transport, thereby showing that an intact GDP/GTP cycle is critical for rab function (Walworth et al., 1992; Tisdale et al., 1992; Van der Sluijs et al., 1992; Bucci et al., 1992; Li and Stahl, 1993; Stenmark et al., 1994; Nuoffer et al., 1994; Riederer et al., 1994).

Using a T7 recombinant vaccinia virus, we have expressed wild-type (WT) rab6, GTP and GDP-bound mutants of rab6 and analyzed their effects on the intracellular transport of a soluble secreted form of alkaline phosphatase (SEAP) and of the hemagglutinin protein (HA) of influenza virus, a transmembrane protein. Using pulse/chase experiments, we show that overexpression of wild-type rab6 or a mutant impaired in GTP hydrolysis (rab6 Q72L), greatly reduced transport of both markers between *cis*/medial (α-mannosidase II positive) and late (sialyl-transferase positive) Golgi compartments. While the overexpression of a GDP-bound mutant (rab6 T27N) did not affect the kinetics of transport between these two compartments, we found that it caused an apparent accumulation of the markers in late Golgi compartments. In addition, both mutants drastically alter the morphology of the Golgi complex. These results indicate that rab6 regulates intra-Golgi transport.

Materials and Methods

Cell Culture

HeLa cells were grown in α-MEM (TechGen International, France) sup-

---

1. *Abbreviations used in this paper*: endo H, endoglycosidase H; GAP, GTPase-activating protein; HA, influenza hemagglutinin; SEAP, secreted alkaline phosphatase; STI, soybean trypsin inhibitor; WT, wild-type.

plemented with 10% FCS (Biological Industries, Israel) and penicillin-streptomycin in a 6% humidified CO_2 incubator. Growth medium for mouse L cells consisted of DMEM containing 4.5 g/l glucose and 0.11 g/l sodium pyruvate (Gibco, Scotland) supplemented with 10% FCS, 2 $\times$ 10^{-8} M β-mercaptoethanol and antibiotics. BHK-21 cells were grown in G-MEM (Gibco) supplemented with 10% tryptose phosphate broth (Gibco), 10% FCS, and antibiotics.

Plasmid Construction and Mutagenesis

cDNAs for human rab6 (Zahraoui et al., 1989), human SEAP (Millàn, 1986; Berger et al., 1988), and HA (virus strain A/Japan/305/57, Gething and Sambrook, 1981) were subcloned into the pGEM-1 plasmid (Promega, Madison, WI). The rab6 mutants T27N and ΔC were generated by oligonucleotide-directed mutagenesis on M13 mp10 vector of rab6 cDNA using the following primers: 5' GTG ATC AAA GAA TTC TTT CCA ACG 3' (T27N) and 5' TTA GCA GGA TCA GCC TCC T 3'(ΔC). After sequencing of the mutated sequences, restriction fragments were inserted into pGEM-rab6. Rab6 Q72L was constructed by directed mutagenesis using a PCR-based protocol (Landt et al., 1990) with the mutant primer 5' GAA CCG CTC TAG ACC TGC T 3' and two other convenient primers. A suitable restriction fragment from the PCR-amplified DNA was reinserted into pGEM-rab6 after complete sequencing. pGEM-rab6 T27N and rab6 ΔC were constructed by Drs. Gress Kadaré and Jean de Gunzburg (INSERM U 248, Paris, France). pGEM-rab5, rab5 N133I, rab7, and rab8 were a generous gift of Dr. Marino Zerial (EMBL, Heidelberg, Germany).

Infection with Vaccinia Virus and Transfection Procedure

L cells and HeLa cells (2 $\times$ 10^5 and 8 $\times$ 10^4, respectively) were plated into 24 well tissue culture dishes 18–24 h before the experiments (Costar Corp., Cambridge, MA). After washing in serum-free medium, cells were infected with the vT7 recombinant vaccinia virus (Fuerst et al., 1986; Bucci et al., 1992). Infection was carried out for 30 min at 37°C in serum free medium containing 25 μg/ml soybean trypsin inhibitor (STI) (Sigma Chem. Co., St. Louis, MO) and 10 mM Hepes, pH 7.2. More than 90% of cells were usually infected as indicated by the expression of the 14-kD late phase vaccinia marker (Rodriguez et al., 1985). After removal of the inoculum, HeLa cells were cotransfected using DOTAP (Boehringer, Mannheim, Germany) with pGEM-HA (1 μg) and either pGEM-1 (control cells) or plasmids encoding for various rab constructs (2 μg). L cells were transfected using Transfectam (Sepracor, Strasbourg, France) with pGEM-SEAP (0.4 μg) and either pGEM-1 or plasmids encoding for rab constructs (0.8 μg). For some experiments (sulphatation and steady-state labeling), L cells (7.5 $\times$ 10^5) and HeLa cells (4 $\times$ 10^5) were seeded in 6-well Falcon plates and 4.5 times more DNA and transfection reagents were used. Transfection was carried out in serum free medium in the presence of 10 mM hydroxyurea (Sigma Chem. Co.) to inhibit maturation of vaccinia particles. Routinely, 50–100% of cells overexpressed rab6 or mutants and among them, more than 95% also expressed the transport markers (SEAP or HA), as determined in double immunofluorescence experiments.

Transport of SEAP

Transport of SEAP was monitored in mouse L cells in which no endogenous form of this marker was detected by immunofluorescence or immunoprecipitation experiments. 4 h 15 min after transfection, cells were incubated for 15 min in medium without methionine and cysteine (ICN Biomedicals, Costa Mesa, CA) and metabolically labeled for 10 min with 100 μCi Express ^{35}S-labeling mix (New England Nuclear, Boston, MA). After washing in serum free medium, cells were chased for various lengths of time in complete medium supplemented with 2.5 mM methionine and cysteine. All the above steps were performed in the presence of hydroxyurea. Media were collected and adjusted to 1% Triton X-100. Cells were lysed in buffer I (20 mM Tris-HCl, pH 8, 150 mM NaCl, 5 mM EDTA, 1% Triton X-100, 0.2% BSA, 1 mM PMSF and a mixture of protease inhibitors consisting of 0.1 μg/ml leupeptin, chymostatin, pepstatin, antipain, and aprotinin). After removal of nuclei and cell debris by centrifugation, SEAP present in cell lysates or in the medium was immunoprecipitated overnight using a polyclonal antibody directed against human placental alkaline phosphatase (Dako, Denmark) and protein A-Sepharose (Sigma Chem. Co.). Immune precipitates were washed once in buffer I, twice in 20 mM Tris-HCl, pH 8, 150 mM NaCl, 5 mM EDTA, 0.5% Triton X-100, 0.1% SDS and 0.2% BSA, twice in 20 mM Tris-HCl, pH 8, 500 mM NaCl, 0.5% Triton X-100 and 0.2% BSA, once in 50 mM Tris-HCl, pH 8, and then boiled for 5 min

in Laemmli's buffer (Laemmli, 1970). To obtain total amount of SEAP, cells and media were not separated and directly lysed in 2× buffer I.

Proteins were separated on 6–12% polyacrylamide-SDS gradient gels. After migration, gels were fixed for 45 min in 10% acetic acid, 40% ethanol, soaked for 1 h in 1 M salicylate, and then processed for autoradiography. Bands corresponding to SEAP were quantified with a Phosphorimager (Molecular Dynamics, Sunnyvale, CA) equipped with the Image Quant software.

Transport of HA

4 h 15 min after transfection, HeLa cells were incubated for 15 min in medium without methionine and cysteine, metabolically labeled for 10 min with 100 μCi Express ^{35}S-labeling mix and washed once in serum free medium.

To monitor the appearance of HA at the plasma membrane, we took advantage of the fact that HA from the strain we used (Japan) is not cleaved by endogenous proteases. However, HA is susceptible to cleavage by exogenous trypsin when expressed at the cell surface (Lazarowitz and Choppin, 1975; Klenk et al., 1975). This generates two polypeptides, HA1 and HA2, which remain associated by disulfide bonds and can be coimmunoprecipitated. Upon reduction in sample buffer, cell surface cleaved HA is then resolved in two distinct bands (HA1 and HA2). Cells were chased for 75 min in serum free medium supplemented with 2.5 mM methionine and cysteine and consecutively incubated for 5 min at 37°C with 20 μg/ml of TPCK-treated trypsin (Sigma Chem. Co.). They were then placed on ice and trypsin was inhibited by adding a solution of STI (10 mg/ml in PBS) for 2 min. The media were discarded and the cells carefully washed once with PBS and lysed in buffer I containing 2 mg/ml STI. HA was immunoprecipitated overnight with a specific monoclonal antibody (H189) and protein A-Sepharose. Immune precipitates were treated as described above and resolved on 10% polyacrylamide-SDS gels. Bands corresponding to HA were quantified with the Phosphorimager.

To monitor intracellular transport, cells were chased for various lengths of time in serum free medium supplemented with 2.5 mM methionine and cysteine. Chase media were then discarded and cells were directly lysed in buffer I (without trypsination). HA was immunoprecipitated overnight with a mixture of two specific monoclonal antibodies (H189 and H265). Immune precipitates were treated as described above. Proteins were separated on 6–12% polyacrylamide-SDS gradient gels.

Steady-State Labeling of Intracellular SEAP and HA

L cells and HeLa cells were infected and transfected as described above. 4 h 15 min after transfection, cells were incubated for 15 min in medium without methionine and cysteine, labeled for 4 h with 100 μCi Express ^{35}S-labeling mix, and then lysed in buffer I. SEAP and HA present in the cell lysates were then immunoprecipitated as described above.

Endoglycosidase H, Neuraminidase, and N-glycanase Digestion

For endoglycosidase H (endo H) (Sigma Chem. Co.) digestion, immune precipitates were boiled for 3 min in 50 mM Tris HCl, pH 6.8, 1% SDS, 5 mM DTT. 6 μl of sodium citrate 1 M, pH 5.5, and 5 μl of endo H (1 U/ml in 150 mM sodium citrate pH 5.5) were added to one half of the supernatant (35 μl). The other half of the supernatant was taken as undigested control. Samples were then incubated for 6 h at 37°C. For neuraminidase digestion, immune precipitates were divided in two equal parts. To one part were added 30 μl of digestion buffer (100 mM sodium acetate, pH 5, 1 mM CaCl₂ and protease inhibitors) and 10 μl of a stock solution of neuraminidase (10 U/ml) (Sigma Chem. Co.). The other part (control) was resuspended in 40 μl of digestion buffer. Digestion was then carried out for 6 h at 37°C. For N-glycanase digestion, immune precipitates were boiled for 3 min in 20 mM sodium phosphate, pH 7.5, 50 mM EDTA, 0.1 M β-mercaptoethanol. 5 μl of 10% Triton X-100 and 0.4 U of N-glycanase (PNGase F, Boehringer) were added to one half of the supernatant (25 μl). The other half was complemented with 5 μl of 10% Triton X-100 and taken as undigested control. Digestion was carried out for 6 h at 37°C.

Partitioning in Triton X-114 and Immunoblotting

To measure overexpression of rab6 constructs, HeLa cells were lysed in 1% Triton X-100 or 1% Triton X-114. Aqueous and detergent phases were separated as previously described (Roa et al., 1993). Samples were then boiled in Laemmli's buffer, separated by 12% SDS-PAGE, and transferred onto

nitrocellulose. Rab6 was detected using a specific polyclonal antibody (Goud et al., 1990) and either a HRP-conjugated anti-rabbit IgG antibody (ECL protocol, Amersham) or an anti-rabbit IgG antibody coupled to alkaline phosphatase (Promega). To quantitate rab6 bands, immunoblots were incubated with ^{125}I-protein A (Amersham Corp.) as previously described (Goud et al., 1990) and scanned on the phosphorimager.

Sulphatation of SEAP

4 h 30 min after transfection, L cells were washed once with PBS and incubated for 30 min in PBS containing 1 mM CaCl₂, 1 mM MgCl₂, and 0.6% glucose (sulphate depletion). They were then pulse labeled for 5 min with ^{35}[SO₄²⁻] (1 mCi/ml in PBS containing 0.6% glucose) (25–40 Ci/mg, Amersham), washed once in serum free medium supplemented with 1.6 mM MgSO₄ and chased in complete medium containing 1.6 mM MgSO₄ and 10 mM hydroxyurea. After different periods of time, SEAP present in extracellular media or cell lysates was immunoprecipitated as described above. Immune precipitates were washed four times in buffer I, boiled 5 min in Laemmli's buffer and resolved on 8% polyacrylamide-SDS gels. The bands of sulphated SEAP were quantified with the Phosphorimager.

Immunofluorescence

6 h after transfection, HeLa and BHK-21 cells grown on 12-mm round glass coverslips were processed for immunofluorescence as previously described (Roa et al., 1993). HeLa cells were then double labeled with an affinity-purified rabbit anti-rab6 antibody (diluted to 1:200 in order to recognize only the overexpressed rab6 proteins) (Goud et al., 1990) and the mouse monoclonal CTR 433 antibody (Jasmin et al., 1989). As secondary antibodies, we used anti-immunoglobulin antibodies coupled to fluorescein or Texas red (Amersham). Confocal laser scanning microscopy was performed using a Leica CLSM (Leica, Germany) instrument based on a Leitz Diaplan microscope with a plan-Neofluar objective 63×. A focal series of four horizontal planes of section spaced by 0.75 μm have been monitored simultaneously for both fluorescein and Texas-red signals. The two 8 bits encoded 512–512 pixels images from registered horizontal planes of section were combined and visualized with a computer generated red and green pseudo-color scale and printed using a sublimation color printer UP3000 (Sony, Japan).

BHK-21 cells were double labeled with a monoclonal antibody against human placental alkaline phosphatase (Dako, Denmark) and a rabbit antibody against TGN38 (a gift of Dr. George Banting) (Luzio et al., 1990). Cells were examined with a Zeiss epifluorescence microscope.

Results

We employed the T7 polymerase recombinant vaccinia system to transiently express wild-type and mutant forms of rab6 together with transport marker proteins. This expression system has been successfully used to unravel the role of several rab proteins acting along both the secretory (Tisdale et al., 1992; Nuoffer et al., 1994) and endocytic pathways (Bucci et al., 1992). Mouse L cells and human HeLa cells were infected with a recombinant vaccinia virus (Fuerst et al., 1986), and then cotransfected with a plasmid encoding wild-type or mutant rab6 and a plasmid encoding either a luminal or a membrane protein. As a luminal protein, we chose a truncated form of human placental alkaline phosphatase (SEAP) lacking its carboxy-terminal glycosyl-phosphatidylinositol (GPI) anchor (Low, 1989). As a consequence, SEAP can not be anchored into membranes and is released in the extracellular medium (Millàn, 1986; Berger et al., 1988) (Fig. 2 A). As a membrane protein, we followed the intracellular transport and the appearance at the cell surface of the hemagglutinin of the influenza virus (HA) (Gething and Sambrook, 1981).

Various mutants of rab6 were constructed by site-directed mutagenesis. Taking advantage of the remarkable conservation of the GTP/GDP motifs in all small GTPases (Bourne et al., 1991; Valencia et al., 1991), we have introduced into

rab6 a series of point mutations which dramatically affect the guanine nucleotide binding and GTP hydrolysis activities of these proteins. To overexpress a protein preferentially locked in its GDP-bound conformation, we used rab6 T27N which contains a threonine to asparagine substitution at residue 27 in the first GTP/GDP-binding domain. The corresponding mutation in ras and rab proteins greatly diminishes their affinity for GTP, but has a lesser effect on binding of GDP; this mutation also inhibits guanine nucleotide exchange factors (Feig and Cooper, 1988; Ridley et al., 1992; Medema et al., 1993; Stenmark et al., 1994; Nuoffer et al., 1994; Riederer et al., 1994). As expected, rab6 T27N displayed almost undetectable GTP-binding activity by the GTP overlay technique (data not shown). To obtain a rab6 protein locked in its GTP-bound conformation, we have introduced a glutamine to leucine substitution at residue 72 (rab6 Q72L). This mutation reduces both intrinsic and GAP-induced GTPases activities of ras-like GTP-binding proteins (Der et al., 1986; Adari et al., 1988; Tisdale et al., 1992; Walworth et al., 1992; Tanigawa et al., 1993; Stenmark et al., 1994). In some experiments, we have also used rab6 ΔC, in which the last three amino acids at the carboxy-end (CSC) were removed. This protein does not undergo the posttranslational modifications (carboxyl-methylation and geranyl-geranylation) required for the insertion of rab6 (Yang et al., 1992) as well as of other rab proteins into membranes (Zerial and Stenmark, 1993).

Good and comparable levels of expression of the various rab constructs were usually obtained after 4 h 30 min–6 h 30 min of transfection (Fig. 1 A and data not shown). During this period, the biosynthetic/secretory pathway was not markedly altered by the viral infection as judged by the kinetics of appearance of transport markers at the plasma membrane or in the extracellular medium (Figs. 2 A and 3). A significant proportion of WT rab6, rab6 Q72L, and rab6 T27N was isoprenylated (26, 21, and 10% of total, respectively), although the bulk of overexpressed proteins was not processed, as assessed by partitioning experiment using TX-114 (Fig. 1 B). 6 h 30 min after transfection, the increase in the amount of isoprenylated proteins (most of them being membrane bound) was 5–10-fold that of the endogenous level of rab6 (data not shown). These levels of overexpression are comparable to those obtained for other rab proteins using the vaccinia system (Bucci et al., 1992; Tisdale et al., 1992).

Overexpression of Wild-Type and Mutant Forms of rab6 Inhibits Secretion of SEAP and the Appearance of HA at the Plasma Membrane

As shown in Fig. 2 B, overexpression of WT rab6 in L cells reduced the extent of secretion of SEAP. Typically, after a 60-min chase, the secretion of SEAP was 50% that of control cells. After a longer chase period (3 h) (data not shown), most of the radiolabeled SEAP synthesized in rab6 overexpressing cells was recovered in the medium, indicating that secretion was not impaired, but rather reduced in these cells. A greater effect (70% inhibition as determined by calculating the initial slopes of the release curves) was observed in rab6 Q72L overexpressing cells. On the other hand, overexpression of rab6 T27N had a milder effect than that of WT rab6 (35% inhibition). In contrast, the secretion of SEAP was not affected by overexpression of rab7, a rab protein associated
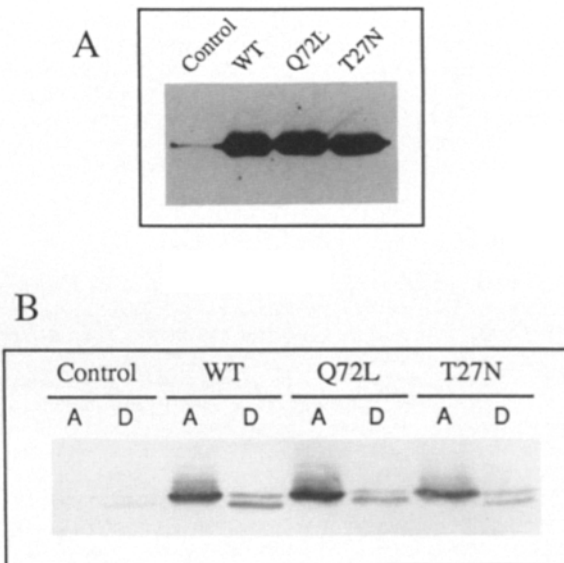
with late endosomes (Fig. 2 B) (Chavrier et al., 1990a). This ruled out the possibility that the rab6-induced inhibition resulted from the titration of general factors regulating rab function, such as GDI (Sasaki et al., 1990). Finally, the overexpression of rab6 ΔC did not alter the secretion of SEAP, indicating that membrane insertion of rab6 is important for affecting intracellular transport of SEAP (Fig. 2 B).

We then monitored the appearance of the HA molecules at the plasma membrane of HeLa cells using its typical cleavage by exogenously added trypsin (see Materials and Methods). Routinely, 50% of the newly synthesized HA had reached the cell surface after a 75-min chase. Overexpression of rab6 and mutants also affected transport of this transmembrane protein. As shown in Fig. 3, the amount of HA present at the plasma membrane was reduced to 25% of the control level in cells overexpressing WT rab6. A more dramatic effect was observed in cells overexpressing rab6 Q72L while a weaker inhibition was measured in rab6 T27N overexpressing cells (Fig. 3). On the other hand, overexpression of rab7 and rab6 ΔC had no or little effect on the appearance of HA at the plasma membrane (Fig. 3), as in the case of rab5 or rab5 N133I (a potent inhibitor of the endocytic process; Bucci et al., 1992; Li and Stahl, 1993) (data not shown). We also found that overexpression of rab8, a Golgi-associated rab protein involved in the transport from the TGN to the
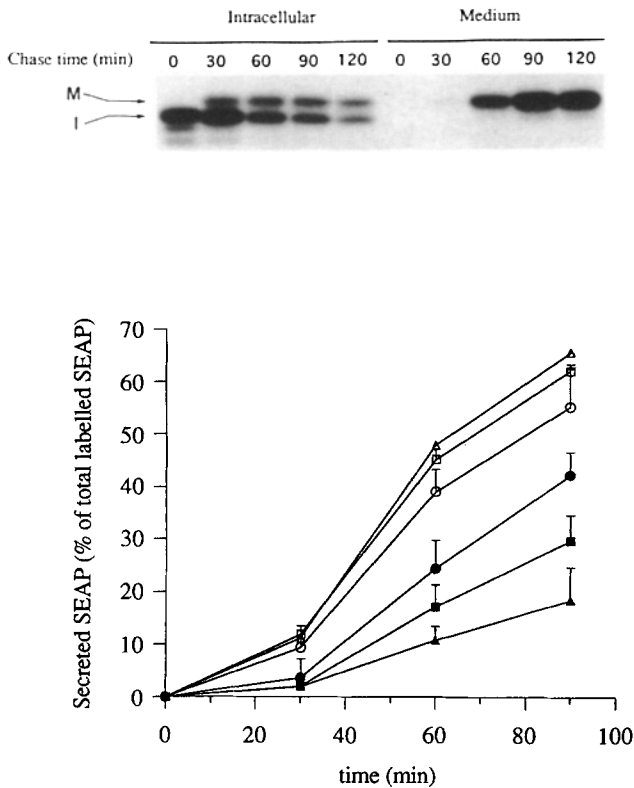
1578

Figure 2. Effects of the overexpression of WT rab6, mutant forms of rab6 and rab7 on the release of SEAP in the extracellular medium. L cells cotransfected with the SEAP plasmid and with pGEM-1 vector (control) (o), rab7 (□), WT rab6 (■), rab6 T27N (●), rab6 Q72L (▲), or rab6ΔC (△) constructs were metabolically labeled for 10 min and chased for various periods of time. At the indicated times, SEAP present in the extracellular medium or inside the cells was immunoprecipitated and quantitated by scanning the specific bands using the Phosphorimager. (*A*) Autoradiogram illustrating the different forms of SEAP detected intracellularly and in the extracellular medium during a pulse/chase experiment in control cells: *I*, immature (endo H sensitive) form; *M*, mature (endo H resistant, neuraminidase sensitive) form (see also Figs. 5 and 7). (*B*) The amount of SEAP present in the extracellular medium was calculated as the percent of total (intracellular + secreted) labeled SEAP at each indicated time. Bars represent standard deviations of three separate experiments.

basolateral plasma membrane in polarized cells (Huber et al., 1993*a,b*), had no effect on HA transport in HeLa cells (Fig. 3).

These results indicate that the overexpression of WT rab6 and rab6 mutants inhibits both the secretion of SEAP and the appearance of HA at the cell surface. Although all the rab constructs (except rab6 ΔC) were inhibitory, the strongest effect was observed with the GTPase mutant rab6 Q72L and the weakest with the GDP-bound mutant rab6 T27N. It has to be noted that transport of a transmembrane protein (HA) appears to be more affected than that of a luminal protein (SEAP) when WT or mutant rab6 are overexpressed.

Overexpression of WT rab6 and Mutants of rab6 Does Not Affect Transport between Golgi and Plasma Membrane or Transport between ER and cis/medial-Golgi
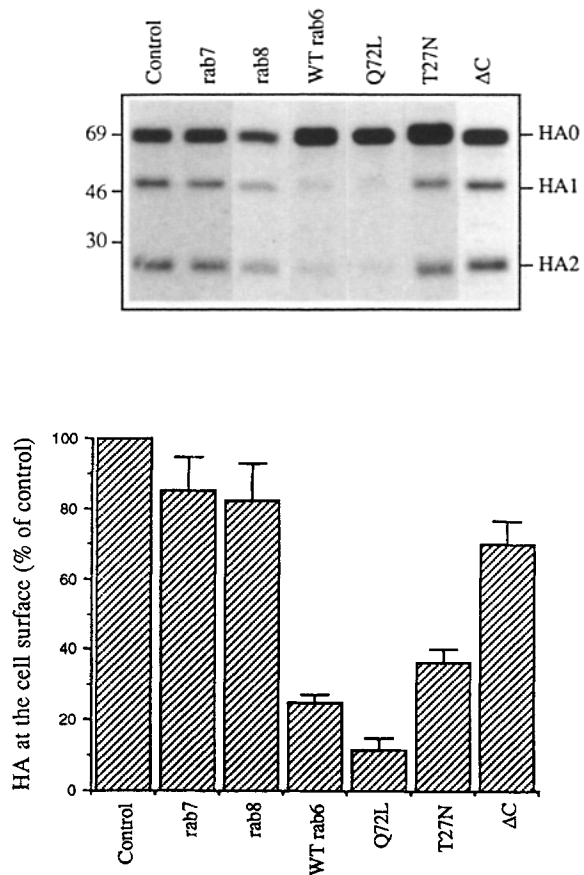
Because rab6 has been detected in the TGN (Antony et al.,

Figure 3. Effects of the overexpression of WT rab6, mutant forms of rab6, rab7, and rab8 on the appearance of HA at the plasma membrane. HeLa cells cotransfected with the HA plasmid and with pGEM-1 vector (*control*) or different rab constructs were metabolically labeled for 10 min, chased for 75 min in serum free medium and trypsinized as described in Materials and Methods. Cell-associated HA was then immunoprecipitated and quantitated. Under these conditions, two trypsin-cleavage products of HA were detected (*HA1* and *HA2*). To estimate the amount of HA molecules present at the cell surface, we calculated the ratio (HA1 + HA2)/(HA0 + HA1 + HA2), HA0 representing uncleaved intracellular HA. In these experiments, the amounts of newly synthesized HA present at the cell surface were: *control*, 50% ± 3.3%; *rab7*, 42.9% ± 7.6%; *rab8*, 40.8% ± 3.4%; *WT rab6*, 12.5% ± 0.9%; *rab6 Q72L*, 5.8% ± 1.2%; *rab6 T27N*, 18.3% ± 0.6%; *rab6ΔC*, 34.7% ± 1.3%. We express, in the figure, the results as percentage of the value found in control cells. Data are the means of three separate experiments ± SD.

1992), we first examined whether overexpression of rab6 and mutants could impair transport between the TGN and the plasma membrane. For that purpose, we took advantage of the fact that SEAP is sulphated, a modification shown to occur in the TGN (Baeuerle and Huttner, 1987; Rosa et al., 1992). L cells coexpressing SEAP and WT rab6 or mutants were pulse labeled for 5 min with ^{35}S-inorganic sulphate, chased for various periods of time and the secretion of the sulphated SEAP was measured (Fig. 4). Cells cotransfected with rab6 Q72L or rab6 T27N displayed the same kinetics of SEAP release as compared with control cells, indicating that these mutants had no effect on transport beyond the TGN. Although we noticed that overexpression of WT rab6
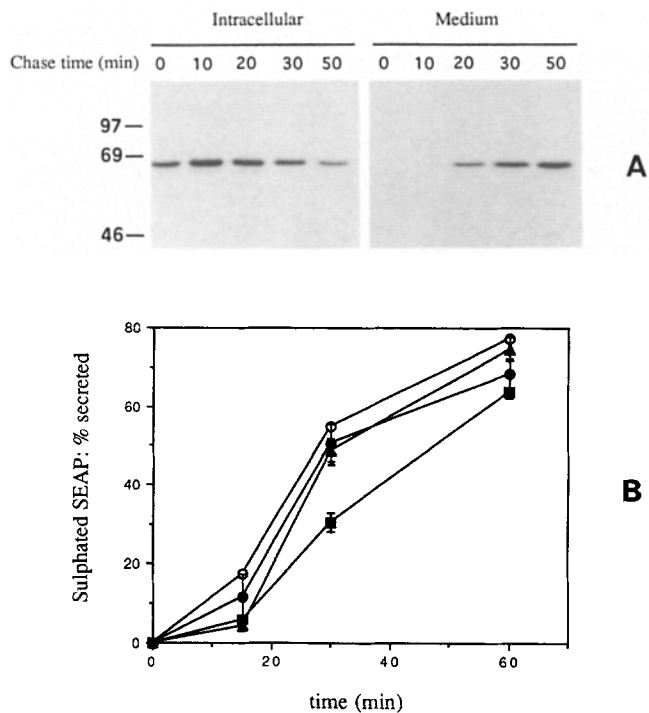
Figure 4. Overexpression of WT rab6, rab6 Q72L, and rab6 T27N does not affect the release of sulphated SEAP into the extracellular medium. L cells cotransfected with pGEM-SEAP and with pGEM-1 (control) (O), WT rab6 (■), rab6 T27N (●), or rab6 Q72L (▲) encoding plasmids were pulsed for 5 min with ^{35}[S]SO$_4$$^{2-}$ and chased for 60 min as described in Materials and Methods. At the indicated times, SEAP present in the extracellular medium or inside the cells was immunoprecipitated and quantitated. (A) Autoradiogram of an experiment performed with control cells which illustrates the release of sulphated SEAP in the extracellular medium. Molecular masses are indicated on the left side of the figure. (B) Sulphated SEAP present in the extracellular medium at each time point is expressed as the percent of total labeled SEAP. This graph represents the means ± SD of three separate experiments.

slowed down the early release of sulphated SEAP (Fig. 4), no difference was found at 60 min when compared to cells transfected with other rab constructs, suggesting that the overall effect of WT rab6 on SEAP secretion cannot be explained by an alteration of trafficking between the TGN and the plasma membrane.

We next examined whether overexpression of WT rab6 and mutants could affect transport from ER to cis/medial-Golgi. It is known that trimming of ER-added high mannose chains by the α-mannosidase II present in cis/medial-Golgi confers endo H resistance to N-linked oligosaccharides (Dunphy et al., 1981; Dunphy and Rothman, 1983; Goldberg and Kornfeld, 1983). We therefore followed in pulse/chase experiments the sensitivity to endo H of the newly synthesized transport markers. Fig. 5 illustrates such an experiment performed with SEAP. Two forms of intracellular SEAP were resolved by SDS-PAGE (see also Fig. 2 A). The lower band was detected after a 10-min pulse, decreased in intensity over the chase period and was fully sensitive to endo H in control cells. It then corresponds to high mannose oligosaccharide-containing molecules still present in compartments before cis/medial-Golgi. The upper band appeared during the chase period and was endo H resistant and

neuraminidase-sensitive (see Fig. 7 A). This form, which migrates at the same level as extracellular SEAP (see Fig. 2 A), and then represents mature SEAP molecules that have passed cis/medial-Golgi compartments. They have acquired all complex sugars, including sialic acids (cleaved by neuraminidase) that are added in trans-Golgi/TGN compartments (Roth et al., 1985). To determine the kinetics of transport to cis/medial-Golgi, we calculated the amounts of endo H sensitive forms of SEAP (marked with filled circles in Fig. 5 A) present over the pulse/chase period in control cells and in cells overexpressing WT rab6, rab6 Q72L, or rab6 T27N. As shown in Fig. 5 B, no difference in the kinetics of decrease of the endo H sensitive forms of SEAP was observed. However, the treatment with endo H generated in cells overexpressing rab6 Q72L a series of bands (marked with filled square and stars in Fig. 5 A) that were not detected in control cells or cells overexpressing WT rab6 and rab6 T27N. We discuss their significance in the next paragraph.

Similar experiments were performed with the HA marker. As in the case of SEAP, no differences in the kinetics of decrease of the endo H sensitive form of HA were measured between control cells and cells overexpressing WT rab6 or rab6 T27N (data not shown). However, it has not been possible to accurately determine the kinetics in cells overexpressing rab6 Q72L, due to the fact that endo H sensitive and partially endo H resistant forms of HA were not enough resolved in these cells by SDS-PAGE (see Fig. 7 B).

We conclude from the above experiments that transport to cis/medial-Golgi was not affected by increased levels of rab6 or mutants.

Overexpression of rab6 Q72L and WT rab6 Affects Transport between cis/medial and Late Golgi/TGN Compartments

We next addressed the question of whether transport between cis/medial and late Golgi compartments was affected in cells overexpressing rab6 constructs. Using pulse/chase experiments, we monitored the appearance of the mature, sialylated form of SEAP. In these experiments, both the intracellular and secreted SEAP were simultaneously immunoprecipitated. Fig. 6 shows that overexpression of rab6 Q72L strongly inhibited the appearance of the sialylated SEAP. Overexpression of WT rab6 had also an inhibitory effect but to a smaller extent than rab6 Q72L. In contrast, the kinetics of SEAP sialylation remained unaffected in rab6 T27N or in rab8 overexpressing cells. Because we previously found that transport between ER and cis/medial-Golgi (α-mannosidase II-positive compartment) and between TGN and the plasma membrane remained normal in cells overexpressing rab6 Q72L or WT rab6, we conclude that intra-Golgi transport was affected in these cells.

A slower transport of glycoproteins within the Golgi apparatus should have an effect on their glycosylation, in particular on the addition of the outer sugar residues onto their N-linked oligosaccharides. Indeed, we could detect in the previously described experiment (Fig. 5 A) unusual endo H resistant forms of SEAP in rab6 Q72L overexpressing cells. To investigate this further, transfected cells were metabolically labeled for 4 h (steady state conditions) and intracellular SEAP was immunoprecipitated. Immune precipitates were then treated with endo H or neuraminidase (Fig. 7 A). In control cells or in cells overexpressing rab6 T27N, only
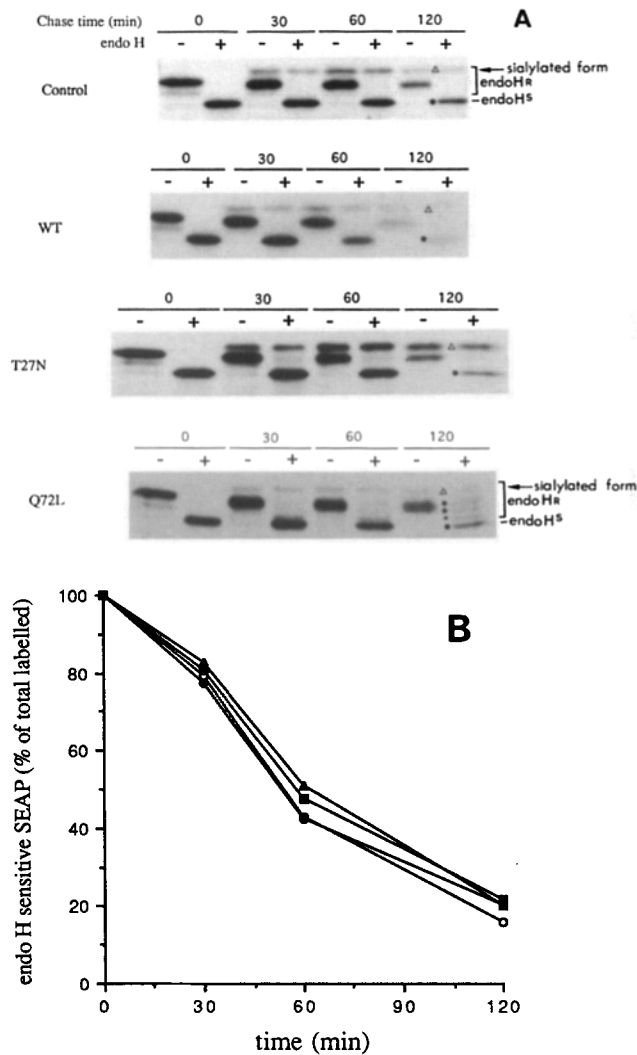
Figure 5. Overexpression of WT rab6, rab6 Q72L, and rab6 T27N does not affect transport of SEAP to *cis*/medial-Golgi. L cells were cotransfected with pGEM-SEAP and with pGEM-1 (control) (○), WT rab6 (■), rab6 T27N (●), or rab6 Q72L (▲) encoding plasmids for 4 h and 15 min, pulsed for 10 min and chased for various lengths of time. (*A*) After pulse (time 0) and chase (times 30, 60, and 120 min), cell-associated SEAP was immunoprecipitated and one half of each immunoprecipitate was either digested (+) or undigested (−) with endo H. In control cells and in cells transfected with WT rab6 and rab6 T27N, two forms of intracellular SEAP were detected before endo H treatment. After treatment with endo H, the lower one displayed a complete shift in mobility. It therefore corresponded to SEAP molecules bearing endo H sensitive oligosaccharides still present in compartments before *cis*/medial-Golgi. The endo H sensitive molecules (endo H^s) obtained after endo H treatment are marked with a filled circle; the upper band (not detected during the pulse) did not shift in mobility after endo H treatment and was sensitive to neuraminidase (see Fig. 7 *A*, *left panel*). It therefore corresponds to molecules which have reached late Golgi/TGN compartments where sialylation takes place. They are marked with an open triangle (sialylated form). The same forms of SEAP as in control cells were detected in cells transfected with rab6 Q72L before endo H treatment. However, the lower one was resolved as a doublet after a 120-min chase. Furthermore, in contrast to control cells or cells overexpressing WT rab6 or rab6 T27N, the treatment with endo H now generated a series of bands becoming clearly visible after a 60-min chase. In addition to the fully endo H sensitive form (*filled circle*), molecules partially endo H resistant
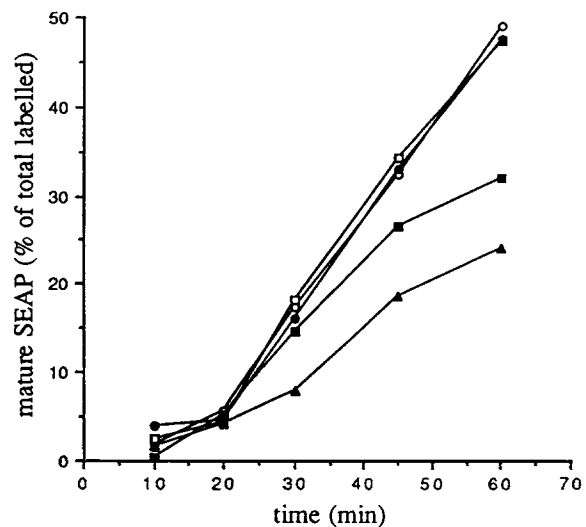

Figure 6. Overexpression of WT rab6 and rab6 Q72L inhibits Golgi maturation of newly synthesized SEAP. L cells were cotransfected with the SEAP plasmid and the following plasmids: pGEM-1 (○), pGEM-rab8 (□), pGEM-WT rab6 (■), pGEM-rab6 T27N (●), or pGEM-rab6 Q72L (▲). After a 10-min pulse, cells were chased for short periods of time. At each time point, cells and media were not separated and directly lysed in lysis buffer. SEAP was immunoprecipitated as described in Materials and Methods. The two bands of SEAP resolved by SDS-PAGE (see Figs. 2 *A* and 5) were measured by scanning with the Phosphorimager. The proportion of mature, sialylated molecules (*upper band*) relative to that of total labeled SEAP (*upper + lower bands*) was then calculated. This graph represents the result of a representative experiment.

fully endo H sensitive and sialylated (neuraminidase sensitive) forms of SEAP were detected (*marked with filled circle and open triangle*, respectively). In contrast, a series of other bands were detected in cells overexpressing rab6 Q72L as well as WT rab6. Some of them were likely the same as those detected in pulse/chase experiments in rab6 Q72L-transfected cells (see Fig. 5 *A* in which they are marked with the same symbols as in Fig. 7). These forms of SEAP were either partially endo H resistant (*filled square*) or endo H resistant, but not sialylated (*stars*). In rab6 Q72L-transfected cells, a smear of neuraminidase-sensitive forms was also detected, indicative of an accumulation of partially sialylated molecules. In addition, only a low amount of mature, fully sialylated form of SEAP was present in these cells (*open triangle into brackets*). Most likely, the forms of SEAP detected in cells transfected with rab6 Q72L and WT rab6 corresponded to molecules incompletely processed by Golgi glycosyltransferases. To confirm this point, immune precipi-

(*marked with filled square*) or endo H resistant, but not sialylated (*marked with stars*) were detected. (*B*) Kinetics of decrease of the endo H sensitive form of SEAP. After a 10-min pulse and various times of chase, cells, and media were not separated, directly lysed in lysis buffer and SEAP was immunoprecipitated. The different bands of SEAP obtained after endo H treatment were quantitated. The proportion of fully endo H sensitive forms of SEAP (corresponding to the *bands marked with filled circles* in Fig. 5 *A*) was then estimated. This graph represents the result of a representative experiment.
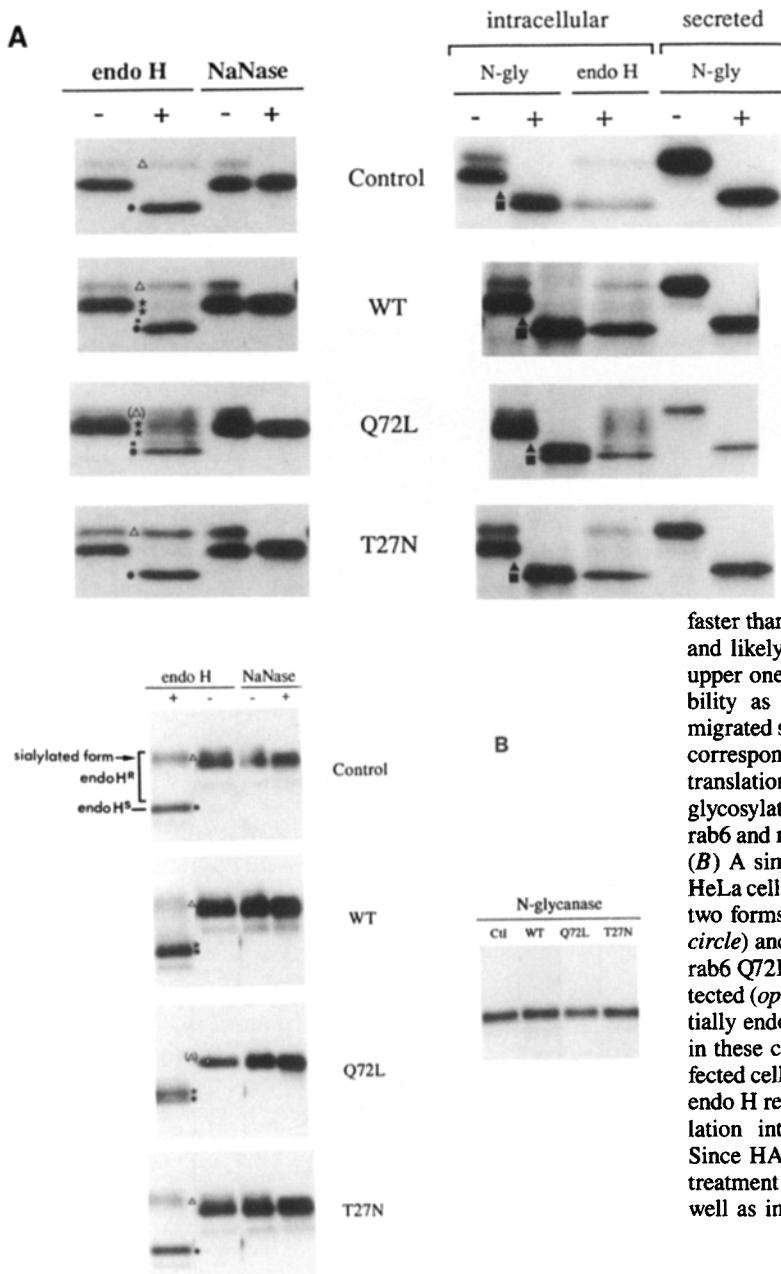
A

Figure 7. Cells overexpressing WT rab6 and rab6 Q72L accumulate glycosylation intermediates of SEAP and HA. (*A*) L cells cotransfected with the SEAP plasmid and with pGEM-1 or pGEM-1 encoding for the different rab6 constructs were incubated for 4 h with ³⁵[S]methionine and cysteine in order to label all the intracellular pool of SEAP. Left and right panels show the results of two separate experiments. Immune precipitates were divided into equal parts and treated with (+) or without (−) the indicated glycosidases as described in Materials and Methods (*NaNase*, neuraminidase; *N-gly*, N-glycanase). (*Left panel*) Cells overexpressing WT rab6 or rab6 Q72L accumulated a series of glycosylation intermediates of SEAP migrating between the fully endo H sensitive form (*filled circle*) and the mature, sialylated form (*open triangle*). Some of them (*filled square and stars*) are likely the same as those detected in pulse/chase experiments (see Fig. 5 *A*). (*Right panel*) The treatment with N-glycanase of immune precipitates of SEAP generated two bands. The lower one (*filled large square*) migrated slightly faster than the fully endo H sensitive form (*filled circle, left panel*) and likely corresponded to ER-associated, immature SEAP. The upper one (*filled triangle*) displayed the same electrophoretic mobility as the N-glycanase–treated secreted form of SEAP, but migrated slightly above the endo H sensitive form of SEAP. It could correspond to molecules which have acquired an early Golgi posttranslational modification, possibly an O-linked glycosylation. The glycosylation intermediates detected in cells overexpressing WT rab6 and rab6 Q72L disappeared after treatment with N-glycanase. (*B*) A similar experiment was performed with the HA marker in HeLa cells. In control cells and in rab6 T27N transfected cells, only two forms of HA were detected: an endo H sensitive form (*filled circle*) and an endo H resistant, sialylated form (*open triangle*). In rab6 Q72L overexpressing cells, no sialylated form of HA was detected (*open triangle into brackets*). A band corresponding to a partially endo H resistant form of HA (*marked with star*) was present in these cells. The same band was also visible in WT rab6-transfected cells. In these cells, a smear of partially endo H resistant and endo H resistant forms of HA was also detected. All these glycosylation intermediates disappeared after N-glycanase treatment. Since HA molecules only contain N-linked oligosaccharides, the treatment with N-glycanase generated a single band in control as well as in WT and mutant rab6 overexpressing cells.

tates of SEAP were treated with N-glycanase, an enzyme which removes all N-linked sugars. As shown in Fig. 7 *A* (*right panel*), all the glycosylation intermediates detected in cells overexpressing rab6 Q72L and WT rab6 disappeared after treatment with N-glycanase.

Similar experiments were performed using HA as a transport marker (Fig. 7 *B*). Two forms of HA, migrating close to each other in SDS gels but better separated after glycosidase treatment, were detected in control cells and in cells overexpressing rab6 T27N. The higher molecular mass form, resistant to endo H but sensitive to neuraminidase corresponds to the mature, sialylated HA (*open triangle*). The lower molecular mass form, sensitive to endo H (*filled circle*) corresponds to the immature, ER-associated HA. Strikingly, in rab6 Q72L-transfected cells, no sialylated form of HA was detected, indicating that transport to late Golgi/ TGN compartments was strongly reduced in these cells. In-

stead, a partially endo H resistant form of HA (*star*), migrating over the fully endo H sensitive form (*filled circle*), was present. The same form was detected in WT rab6-transfected cells. In addition, a smear of endo H resistant (or partially endo H resistant) forms was also visible in these cells. The treatment with N-glycanase (Fig. 7 *B*) confirmed that, as already found for the SEAP marker (Fig. 7 *A*), HA bands detected in WT rab6 and rab6 Q72L-transfected cells corresponded to N-glycosylation intermediates.

Altogether, these results show that overexpression of WT rab6 or rab6 Q72L impairs the addition of the most outer sugars on N-linked oligosaccharides, including sialic acids, thereby allowing the detection of glycosylation intermediates of the transported glycoproteins. These glycosylation intermediates were more visible in rab6 Q72L than in WT rab6-transfected cells, in good agreement with the fact that rab6 Q72L has the strongest inhibitory effect on the kinetics of
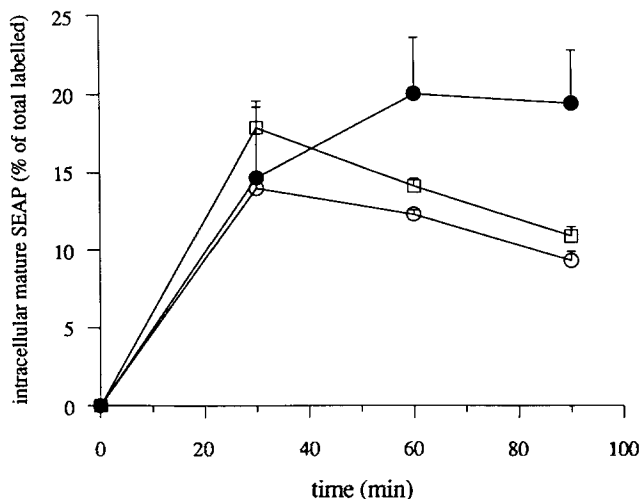
Figure 8. Overexpression of rab6 T27N causes an accumulation of intracellular sialylated SEAP. L cells were cotransfected with pGEM-SEAP and with pGEM-1 (o), rab6T27N (●), or rab7 (□) encoding plasmids for 4 h and 15 min, pulsed for 10 min and chased for 30, 60, and 90 min. Cell-associated SEAP was then immunoprecipitated and the amount of radioactivity present in the different bands (see Figs. 2 *A* and 5) was measured by scanning with the Phosphorimager. The results are expressed as the percent of intracellular mature, sialylated (*upper band*) form of SEAP to that of total labeled SEAP. This graph represents the means ± SD of three separate experiments.

intra-Golgi transport (Fig. 6). Increased levels of WT rab6 and of rab6 Q72L then affect transport between *cis*/medial-(α-mannosidase II positive) and *trans*-Golgi/TGN (sialyltransferase positive) compartments.

Overexpression of rab6 T27N Leads to an Intracellular Accumulation of Sialylated SEAP

The experiments described above indicate that in cells overexpressing rab6 T27N, neither ER to *cis*/medial-Golgi (Fig. 5), nor intra-Golgi (Fig. 6) nor TGN to plasma membrane (Fig. 4) transport was affected. However, the overexpression of this mutant did inhibit the overall secretion of SEAP and the appearance of HA at the plasma membrane (Figs. 2 and 3). One possible explanation is that this inhibition in transport was due to an accumulation of the markers in late Golgi/TGN compartments. To test this hypothesis, we have measured the amount of intracellular mature SEAP in pulse/chase experiments. As shown in Fig. 8, cells overexpressing rab6 T27N progressively accumulated sialylated SEAP during the chase period. After a 90-min chase, these cells contained about twofold more mature SEAP as compared with control cells or rab7 overexpressing cells. Such an accumulation could also be detected when rab6 T27N overexpressing cells were labeled at steady state (Fig. 7 *A*). Quantitation of this experiment showed that the amount of intracellular sialylated SEAP was increased to threefold control level in cells overexpressing rab6 T27N.

These data suggest that, in contrast to WT rab6 or rab6 Q72L, the inhibition of secretion induced by overexpression of rab6 T27N is not due to a reduced transport between the different compartments of the secretory pathway, but rather

to an accumulation of the transport markers in late Golgi compartments.

Morphological Alterations of the Golgi Complex

Dramatic changes in the morphology of transport organelles have frequently been observed upon overexpression of several rab proteins (Bucci et al., 1992; Van der Sluijs et al., 1992; Wilson et al., 1994). Therefore, we examined whether overexpression of WT rab6 and of various constructs could alter the morphology of the Golgi apparatus. Transfected HeLa cells were double labeled with a polyclonal anti-rab6 antibody and a monoclonal antibody (CTR 433) directed against a medial-Golgi antigen (Jasmin et al., 1989). We have previously shown that these two proteins colocalized (Goud et al., 1990; Roa et al., 1993). Fig. 9, *a*, *d*, and *g* show cells overexpressing the rab constructs. The staining appeared diffuse due to the fact that most of the overexpressed proteins were cytosolic (Fig. 1 *B*). In addition, the anti-rab6 antibody was diluted in order to avoid detection of the endogenous rab6. Analysis by confocal microscopy strikingly revealed that typical Golgi structures could not be observed in cells overexpressing rab6 Q72L. The staining of the Golgi antigen was instead weak, diffuse, and somewhat reticular (Fig. 9, *d*, *e*, and *f*). Similar results were obtained when antibodies directed against MG-160, a transmembrane medial-Golgi resident protein and against α-mannosidase II were used (data not shown). In contrast, Golgi staining appeared more prominent in cells overexpressing rab6 T27N than in control cells, strongly suggesting that Golgi structures were enlarged (Fig. 9, *g*, *h*, and *i*). Although no apparent modification of the Golgi staining was detectable in most of the cells overexpressing WT rab6, we noticed however that some cells, probably those expressing high levels of rab6, exhibited a similar alteration of the Golgi staining as seen in cells transfected with rab6 Q72L (Fig. 9, *a*, *b*, and *c*).

The distribution of TGN 38, a marker of the TGN (Luzio et al., 1990), as well as that of SEAP was also examined in cells overexpressing WT rab6 and mutants. Because of the restricted specificity of the antibody against TGN 38, these experiments were performed on BHK-21 cells instead of HeLa cells. In cells cotransfected with the SEAP plasmid and empty pGEM-1 (control), intracellular SEAP was detected in several structures of the secretory pathway, but often concentrated in the Golgi area, as assessed by the overlapping with TGN 38 staining (Fig. 10, *a* and *b*). In most of the cells cotransfected with rab6 Q72L, the localization of SEAP was much more punctate and reticular than in control cells (Fig. 10, *e* and *f*). Concomitantly, these cells lacked recognizable TGN structures. In contrast, cells cotransfected with rab6 T27N display an intense Golgi staining with both anti-SEAP and anti-TGN 38 antibodies (Fig. 10, *g* and *h*). In many cells, the labeled structures appear larger than in controls. Finally, a staining pattern close to the one found in control cells was observed in cells cotransfected with WT rab6 (Fig. 10, *c* and *d*). However, a significant percentage of cells displayed a staining pattern similar to the one observed in rab6 Q72L overexpressing cells, the SEAP marker being present in punctate and reticular structures and TGN 38 mostly dispersed (*marked with an arrowhead*).

The above observations indicate that overexpression of rab6 Q72L and rab6 T27N have distinct and opposite effects
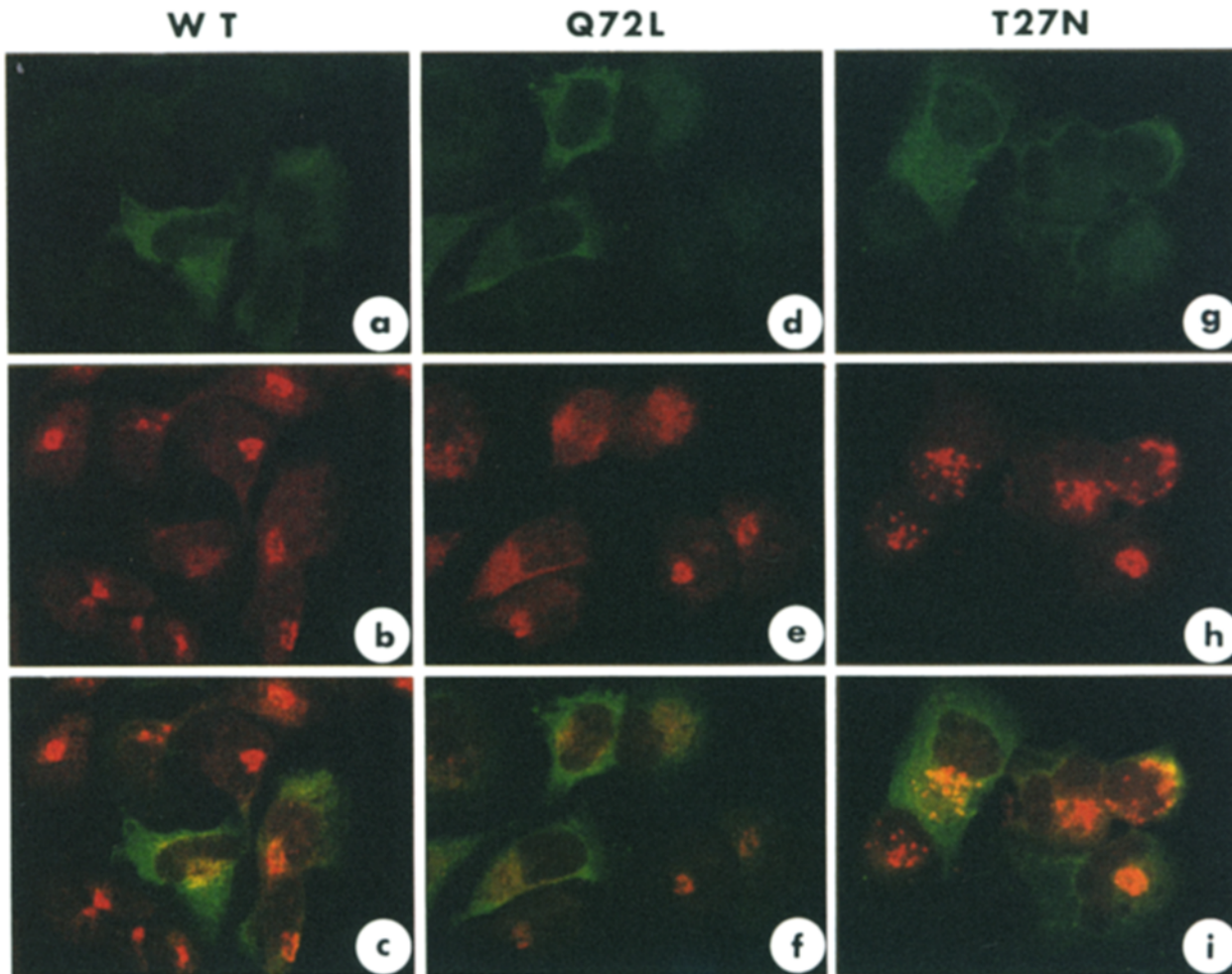
Figure 9. The distribution of a medial-Golgi marker is altered in HeLa cells overexpressing rab6 constructs. HeLa cells transfected with WT rab6 (*a*, *b*, and *c*), rab6 Q72L (*d*, *e*, and *f*), or rab6 T27N (*g*, *h*, and *i*) were fixed in 4% paraformaldehyde 6 h after transfection. They were then permeabilized with saponin and double labeled with an affinity purified anti-rab6 antibody (*a*, *d*, and *g*) and a monoclonal antibody directed against a medial-Golgi antigen (CTR433) (*b*, *e*, and *h*), as described in Materials and Methods. Pictures *c*, *f*, and *i* show the superposition of rab6 and CTR433 labelings. Here are shown single representative optical slices obtained from the confocal microscope.

on the morphology of the Golgi complex, as assessed by the distribution of Golgi and TGN markers. In addition, these mutants modify the pattern of distribution of the SEAP marker. Likely, these morphological changes are correlated to alterations in the transport events that we have seen in our experiments.

Discussion

Rab6 Is Involved in intra-Golgi Transport

In this study, we have overexpressed WT rab6 and corresponding mutants and followed transport of a luminal (SEAP) and a transmembrane (HA) protein. We have shown that both WT rab6 and the GTPase mutant rab6 Q72L reduce transport of these markers to the plasma membrane and the extracellular medium, the strongest effect being observed with rab6 Q72L. The GDP-bound mutant rab6 T27N also inhibits transport, but less than WT rab6 and rab6 Q72L. More precisely, we found that overexpression of rab6 and rab6 Q27L affects transport of both membrane and soluble proteins between a α-mannosidase II positive and a sialyltrans-

ferase positive compartment. We believe that these effects are specific because neither transport between the ER and a α-mannosidase II-rich compartment nor transport between a sulfotransferase-rich compartment and plasma membrane were affected. Furthermore, although overexpression of rab6 T27N did not affect the kinetics of transport within the secretory pathway, it resulted in a significant intracellular accumulation of the sialylated forms of the markers. The topological distribution of the different processing enzymes (α-mannosidase II, sialyl-, and sulfotransferases) in the secretory pathway allows us to conclude that rab6 plays an important role in transport between the *cis*/medial-Golgi and the *trans*-Golgi/TGN. This is consistent with our previous morphological studies showing that rab6 localizes to the medial and *trans*-Golgi cisternae as well as to the TGN (Goud et al., 1990; Antony et al., 1992). In addition, deletion of the yeast homolog of *rab6*, *YPT6* (*Ryh1*), affects the late glycosylation of a secretory marker, indicating that YPT6 also functions at the Golgi level (Hengst et al., 1990). Recently, the addition of anti-rab6 antibodies was found to inhibit the in vitro formation of exocytic vesicles from rat liver Golgi membranes, suggesting that rab6 could play a

SEAP · TGN 38

control
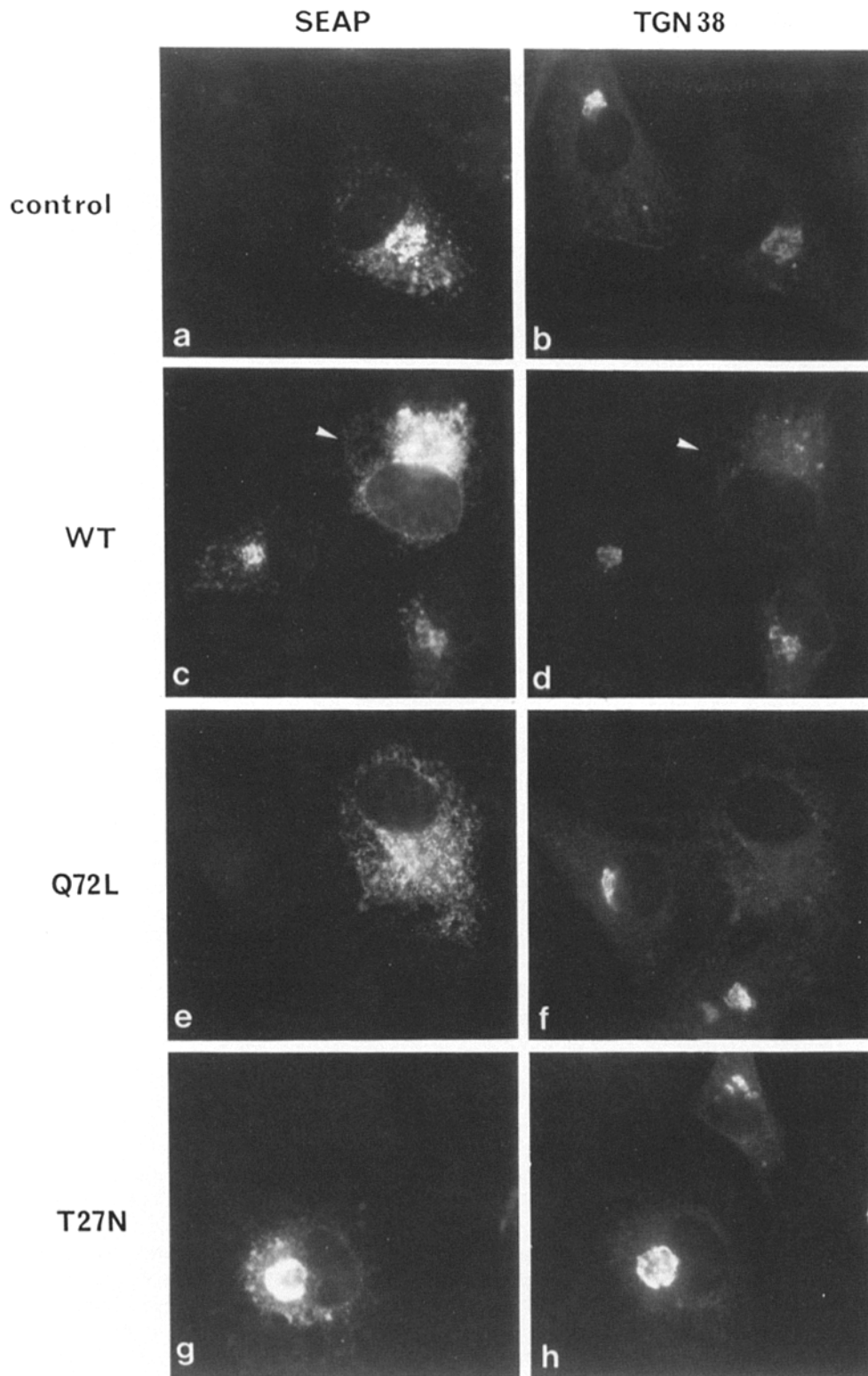a · b

WT
c · d

Q72L
e · f

T27N
g · h

Figure 10. Distribution of the SEAP marker and of TGN 38 in BHK-21 cells overexpressing rab6 constructs. BHK-21 cells cotransfected with pGEM-SEAP and with pGEM-1 (*control*, *a* and *b*), WT rab6 (*c* and *d*), rab6 Q72L (*e* and *f*), or rab6 T27N (*g* and *h*) were fixed in 4% paraformaldehyde 6 h after transfection. After permeabilization with saponin, they were double labeled with a monoclonal antibody against human placental alkaline phosphatase (*a, c, e,* and *g*) and a rabbit polyclonal antibody directed against TGN 38 (*b, d, f,* and *h*). The arrowhead in *d* points to a cell with a dispersed TGN in cells transfected with WT rab6.

role in vesicle budding for the TGN (Jones et al., 1993). Although rab6 is predominantly associated with Golgi stacks in rat liver cells (Feldmann, G., and B. Goud, unpublished results), rab6 is also found associated with post-Golgi organelles in some specialized cell types such as neurons or photo-receptors (Jasmin et al., 1992; Tixier-Vidal et al., 1993; Deretic and Papermaster, 1993). It is then possible that rab6 exhibits additional functions at the exit of the TGN that we have not detected under our experimental conditions. Further experiments will be necessary to clarify this issue. Finally, the fact that overexpression of WT rab6 produces significant effects on intra-Golgi transport strongly suggests that rab6, as also observed for some other rab proteins, is a rate-limiting component of the transport machinery.

Possible Role of rab6 in intra-Golgi Transport

The function of several rab proteins associated with organelles of the secretory and the endocytic pathways have been recently addressed using in most cases, a similar approach as that developed in this study. Overexpression of wild-type proteins generally results in a gain of function, most likely due to an increase in the level of the GTP-bound form of the protein inside the cells. This is the case for rab5 whose overexpression accelerates the uptake of endocytic markers (Bucci et al., 1992; Li and Stahl, 1993). Cells stably overexpressing rab4a display an increased recycling rate from early endosomes (Van der Sluijs et al., 1992) and cytosol prepared from cells overexpressing rab9 stimulates endosome to TGN transport in vitro (Lombardi et al., 1993). It should be pointed out, however, that overexpression of some WT rab proteins, such as rab1b and rab2, does not apparently modify the kinetics of transport (Tisdale et al., 1992). On the other hand, overexpression of rab proteins in their GDP-bound conformation results in a strong inhibition of transport reactions, as exemplified by studies with rab1b S22N, rab1a S25N, or rab5 S34N (Tisdale et al., 1992; Li and Stahl, 1993; Nuoffer et al., 1994; Stenmark et al., 1994). These mutants could act as dominant inhibitors because they decrease the level of wild-type rab proteins in their GTP-bound conformation in membranes, possibly by saturating exchange factors needed for normal rab function (Pfeffer, 1994). The overexpression of GTPase mutants has been more difficult to interpret. Expression of Sec4 Q79L and rab2 Q65L for example has been shown to inhibit transport (Tisdale et al., 1992; Walworth et al., 1992), consistent with the hypothesis that GTP hydrolysis serves as a kinetic proofreading device before docking and/or fusion of transport vesicles with their acceptor membranes (Bourne, 1988). However, overexpression of rab1b Q61L remained without any apparent effect on transport (Tisdale et al., 1992). In addition, rab5 Q79L strongly increases the rate of endocytosis and in vitro early endosome fusion, suggesting that the primary role of GTP hydrolysis is to convert the active rab6-GTP into the inactive rab5-GDP (Stenmark et al., 1994).

Our results clearly indicate that overexpression of WT rab6 has an inhibitory effect on the secretory pathway. We found that the GDP-bound mutant rab6 T27N has a less pronounced effect on secretion than WT rab6, whereas the GTPase mutant rab6 Q72L exhibits a stronger effect. Two possibilities could be envisaged to explain these effects. First, rab6 could act directly as an inhibitor of anterograde transport. As discussed above, most rab proteins studied so far are active under their GTP-bound form and are usually rate-limiting components of the transport machinery. However, there is at least one example of a rab protein that appears to act as a negative regulator in a transport reaction. It has been recently shown that GTP-bound rab3a, a protein associated with synaptic vesicles and chromaffin granules, inhibits exocytosis in PC12 and chromaffin cells (Holz et al., 1994; Johannes et al., 1994), suggesting that the function of rab3a would be to prevent fusion of granules or vesicles with the plasma membrane. According to this model, rab6 in its GTP-bound conformation could prevent either the formation or/and the fusion of transport vesicles within Golgi cisternae, thereby preventing secretion. GTP hydrolysis would be required for normal progression of secretory proteins through Golgi compartments, as illustrated by the strong in-

hibitory effect of rab6 Q72L. Finally, overexpression of rab6 T27N could at least partially release the inhibitory effect of endogenous rab6 by increasing the level of rab6-GDP in membranes. We have not seen, however, any effect of this mutant on the kinetics of transport between cis/medial- and trans-Golgi compartments. Second, rab6 could indirectly affect the normal progression of secretory components through the secretory pathway by acting on the putative retrograde intra-Golgi transport. Overexpression of WT rab6 would increase the rate of retrograde transport thereby decreasing the apparent rate of secretion. The GDP-bound mutant rab6 T27N inhibiting only retrograde transport could have a less pronounced effect on anterograde transport. Along with this hypothesis, the GTPase mutant of rab6 would be a dominant activator of the retrograde transport. So far, such a retrograde transport from late Golgi to early Golgi compartments has not been well documented, although it has been postulated to explain the dynamics of the Golgi apparatus and the retrieval of proteins of the transport machinery (Rothman, 1981; Rothman and Warren, 1994). The fact that some ER resident proteins can be posttranslationally modified by Golgi enzymes, including the sialyltransferases, strongly suggests that they are retrieved back to the ER after reaching Golgi compartments (Pelham, 1989; Pelham and Munro, 1993; Jackson et al., 1993). Such a pathway, as many others, could be controlled by GTP-binding proteins, rab6 being potentially one of them.

Morphological Alterations of the Golgi Complex

The other interesting finding of this study is that overexpression of WT rab6 and mutants induce striking morphological changes of the Golgi apparatus. At the immunofluorescence level, this organelle appears diffuse in cells overexpressing the GTPase mutant rab6 Q72L while it appears expanded in cells overexpressing the GDP-bound mutant rab6 T27N. Such alterations have been seen with several Golgi markers, including the α-mannosidase II and TGN 38, a marker of the TGN (Luzio et al., 1990). Preliminary electron microscopy studies corroborate these observations and indicate that the Golgi apparatus is dispersed in small vesicles in rab6 Q72L-transfected cells, while cells overexpressing rab6 T27N display an accumulation of Golgi membranes, possibly at the trans-side of this organelle (Antony, C., O. Martinez, and B. Goud, unpublished results). Profound alterations in the morphology of intracellular organelles have also been documented in rab overexpressing cells, pointing out that rab proteins play a role in both vesicular traffic and dynamics of organelles. The morphology of transport organelles is probably maintained by a fine tuning between the entry and the exit from these organelles of transport vesicles. An alteration of vesicular traffic caused by dominant negative mutants of rab proteins could disrupt their integrity. For instance, the GDP-bound mutant rab1a S25N which reduces ER to Golgi transport, also promotes the disassembly of the Golgi apparatus (Wilson et al., 1994). Similarly, overexpression of WT rab6 (Bucci et al., 1992) or of its GTPase mutant, rab5 Q79L, (Stenmark et al., 1994) which modify the kinetics of endocytosis, increases the size of early endosomes while overexpression of the GDP-bound mutant induces the accumulation of small endocytic structures (Stenmark et al., 1994). However, rab proteins have also been involved in the control of fusion events between identical organelles (also referred to

as homotypic fusion), as exemplified by rab5 implicated in in vitro early endosome fusion (Gorvel et al., 1991). If rab6 was controlling homotypic fusion between Golgi stacks, for instance medial- and *trans*-compartments, it could be expected that overexpression of the "active" GTP-bound forms of rab6 would result in the enlargement or at least in the maintenance of Golgi structures and not in the fragmentation of the organelle. In contrast, the GDP-bound mutant rab6 T27N would fragment Golgi structures and not lead to a more compact organelle. Clearly, the opposite morphological effects that we observed do not support this possibility and would rather favor the proposal that the morphological alterations of the Golgi apparatus induced by the overexpression of WT rab6 and mutants are due to modifications of vesicular traffic throughout this organelle.

If the experiments described above illustrate the importance of rab6 in controlling transport of secretory proteins within Golgi compartments, further work is necessary to pinpoint its precise site of action. In particular, it will be of importance to discriminate between retrograde and anterograde transport within the Golgi apparatus. Clearly, this will require the design and the use of new functional assays directly measuring the putative retrograde transport of membrane proteins. Finally, a more complete morphological study of cells overexpressing rab6 mutants, based on electron microscopy and the use of different Golgi markers, will also provide valuable pieces of information and shed some more light on rab6 function in the secretory pathway.

References

Adari, E., D. R. Lowy, B. M. Willumsen, C. J. Der, and F. McCormick. 1988. Guanosine triphosphatase activating protein (GAP) interacts with the p21 *ras* effector binding domain. *Science (Wash. DC).* 240:518–521.

Antony, C., C. Cibert, G. Géraud, A. Santa Maria, B. Maro, V. Mayau, and B. Goud. 1992. The small GTP-binding protein rab6p is distributed from medial Golgi to the *trans*-Golgi network as determined by a confocal microscopic approach. *J. Cell Sci.* 103:785–796.

Bacon, R., A. Salminen, H. Ruohola, P. Novick, and S. Ferro-Novick. 1989. The GTP-binding protein YPT1 is required for transport *in vitro*: the Golgi apparatus is defective in YPT1 mutants. *J. Cell Biol.* 109:1015–1022.

Baeuerle, P. A., and W. B. Huttner. 1987. Tyrosine sulfation is a *trans*-Golgi-specific protein modification. *J. Cell Biol.* 105:2655–2664.

Bednarek, S. Y., T. L. Reynolds, M. Schroeder, R. Grabowski, L. Hengst, D. Gallwitz, and N. Raikhel. 1994. A small GTP-binding protein from *Arabidopsis thaliana* functionally complements the yeast YPT6 null mutant. *Plant Physiol.* 104:591–596.

Berger, J., J. Hauber, R. Hauber, R. Geiger, and B. R. Cullen. 1988. Secreted placental alkaline phosphatase: a powerful new indicator of gene expression in eukaryotic cells. *Gene (Amst.).* 66:1–10.

Boguski, M. S., and F. McCormick. 1993. Proteins regulating ras and its relatives. *Nature (Lond.).* 366:643–654.

Bourne, H. R. 1988. Do GTPases direct membrane traffic in secretion? *Cell.* 55:59–61.

Bourne, H. R., D. A. Sanders, and F. McCormick. 1991. The GTPase super-

family: conserved structures and molecular mechanisms. *Nature (Lond.).* 349:117–127.

Bucci, C., R. Parton, I. Mather, H. Stunnenberg, K. Simons, B. Hoflack, and M. Zerial. 1992. The small GTPase Rab5 functions as a regulatory factor in the endocytic pathway. *Cell.* 70:715–728.

Chavrier, P., R. G. Parton, H. F. Hauri, K. Simons, and M. Zerial. 1990a. Localization of low molecular weight GTP binding proteins to exocytic and endocytic compartments. *Cell.* 62:317–329.

Chavrier, P., M. Vingron, C. Sander, K. Simons, and M. Zerial. 1990b. Molecular cloning of YPT1/SEC4-related cDNAs from an epithelial cell line. *Mol. Cell Biol.* 10:6578–6585.

Der, C., T. Finkel, and G. Cooper. 1986. Biological and biochemical properties of human ras genes mutated at codon 61. *Cell.* 44:167–176.

Deretic, D., and D. S. Papermaster. 1993. Rab6 is associated with a compartment that transports rhodopsin from the *trans*-Golgi to the site of rod outer segment disk formation in retinal photoreceptors. *J. Cell Sci.* 106:803–813.

Dunphy, W. G., E. Fries, L. J. Urbani, and J. E. Rothman. 1981. Early and late functions associated with the Golgi apparatus reside in distinct compartments. *Proc. Natl. Acad. Sci. USA.* 78:7453–7457.

Dunphy, W. G., and J. E. Rothman. 1983. Compartmentation of asparagine-linked oligosaccharide processing in the Golgi apparatus. *J. Cell Biol.* 97:270–275.

Elferink, L. A., K. Anzai, and R. H. Scheller. 1992. Rab15, a novel low molecular weight GTP-binding protein specifically expressed in rat brain. *J. Biol. Chem.* 267:5768–5775.

Feig, L., and G. Cooper. 1988. Inhibition of NIH 3T3 cell proliferation by mutant ras protein with preferential affinity for GDP. *Mol. Cell. Biol.* 8:3235–3243.

Fuerst, T., E. Niles, F. Studier, and B. Moss. 1986. Eukaryotic transient-expression system based on recombinant vaccinia virus that synthesizes bacteriophage T7 RNA polymerase. *Proc. Natl. Acad. Sci. USA.* 83:8122–8126.

Gething, M. J., and J. Sambrook. 1981. Cell-surface expression of influenza haemagglutinin from a cloned DNA copy of the RNA gene. *Nature (Lond.).* 293:620–625.

Goldberg, D. E., and S. Kornfeld. 1983. Evidence for extensive subcellular organization of asparagine-linked oligosaccharide processing and lysosomal enzyme phosphorylation. *J. Biol. Chem.* 258:3159–3165.

Gorvel, J., P. Chavrier, M., Zerial, and J. Gruenberg. 1991. Rab5 controls early endosome fusion in vitro. *Cell.* 64:915–925.

Goud, B., and M. McCaffrey. 1991. Small GTP-binding proteins and their role in transport. *Curr. Opin. Cell Biol.* 3:626–633.

Goud, B., A. Salminen, N. C. Walworth, and P. J. Novick. 1988. A GTP binding protein required for secretion rapidly associates with secretory vesicles and the plasma membrane in yeast. *Cell.* 53:753–768.

Goud, B., A. Zahraoui, A. Tavitian, and J. Saraste. 1990. Small GTP-binding protein associated with Golgi cisternae. *Nature (Lond.).* 345:553–556.

Hengst, L., T. Lehmeier, and D. Gallwitz. 1990. The ryh1 gene in the fission yeast *Schizosaccharomyces pombe* encoding a GTP-binding a protein related to ras, rho and ypt: structure, expression and identification of its human homologue. *EMBO (Eur. Mol. Biol. Organ.) J.* 9:1949–1955.

Holz, R. W., W. H. Brondyk, R. A. Senter, L. Kuizon, and I. G. Macara. 1994. Evidence for the involvement of rab3A in Ca^{2+}-dependent exocytosis from adrenal chromaffin cells. *J. Biol. Chem.* 269:10229–10234.

Huber, L. A., S. Pimplikar, R. G. Parton, H. Virta, M. Zerial, and K. Simons. 1993a. Rab8, a small GTPase involved in vesicular traffic between the TGN and the basolateral plasma membrane. *J. Cell Biol.* 123:35–45.

Huber, L. A., M. J. de Hoop, P. Dupree, M. Zerial, K. Simons, and C. Dotti. 1993b. Protein transport to the dendritic plasma membrane of cultured neurons is regulated by rab8p. *J. Cell Biol.* 123:47–55.

Jackson, M. R., T. Nilsson, and P. A. Peterson. 1993. Retrieval of transmembrane proteins to the endoplasmic reticulum. *J. Cell Biol.* 121:317–333.

Jasmin, B. J., J. Cartaud, M. Bornens, and J. P. Changeux. 1989. Golgi apparatus in chick skeletal muscle: changes in its distribution during end plate development and after denervation. *Proc. Natl. Acad. Sci. USA.* 86:7218–7222.

Jasmin, B., B. Goud, G. Camus, and J. Cartaud. 1992. The low molecular weight guanosine triphosphate-binding protein Rab6p associates with distinct post-Golgi vesicles in *Torpedo marmorata* electrocytes. *Neuroscience.* 49:849–855.

Johannes, L., P.-M., Lledo, M. Roa, J.-D. Vincent, J.-P. Henry, and F. Darchen. 1994. The GTPase Rab3a negatively controls calcium-dependent exocytosis in neuroendocrine cells. *EMBO (Eur. Mol. Biol. Organ.) J.* 13:2029–2037.

Jones, S. M., J. R. Crosby, J. Salamero, and K. E. Howell. 1993. A cytosolic complex of p62 and rab6 associates with TGN38/41 and is involved in budding of exocytic vesicles from the *trans*-Golgi network. *J. Cell Biol.* 122:775–788.

Klenk, H. D., R. Rott, M. Ohlich, and J. Blödom. 1975. Activation of influenza A viruses by trypsin treatment. *Virology.* 68:426–439.

Laemmli, U. K. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature (Lond.).* 227:680–685.

Landt, O., H.-P. Grunert, and U. Hahn. 1990. A general method for rapid site-directed mutagenesis using the polymerase chain reaction. *Gene (Amst.).* 96:125–128.

Lazarowitz, S. G., and P. W. Choppin. 1975. Enhancement of the infectivity of influenza A and B viruses by proteolytic cleavage of the hemagglutinin polypeptide. *Virology.* 68:440–454.

Leyte, A., F. A. Barr, R. H. Kehlenbach, and W. B. Huttner. 1992. Multiple trimeric G-proteins on the *trans*-Golgi network exert stimulatory effects on secretory vesicles formation. *EMBO (Eur. Mol. Biol. Organ.) J.* 11: 4795–4804.

Li, G. P., and P. D. Stahl. 1993. Structure-function relationship of the small GTPase rab5. *J. Biol. Chem.* 268:24475–24480.

Lledo, P.-M., P. Vernier, J.-D. Vincent, W. T. Mason, and R. Zorec. 1993. Inhibition of Rab3B expression attenuates Ca²⁺-dependent exocytosis in rat anterior pituitary cells. *Nature (Lond.).* 364:540–544.

Lombardi, D., T. Soldati, M. A. Riederer, Y. Goda, M. Zerial, and S. R. Pfeffer. 1993. Rab9 functions in transport between late endosomes and the *trans*-Golgi network. *EMBO (Eur. Mol. Biol. Organ.) J.* 12:677–682.

Low, M. G. 1989. The glycosyl-phosphatidylinositol anchor of membrane proteins. *Biochem. Biophy. Act.* 988:427–454.

Luzio, J. P., B. Brake, G. Banting, K. E. Howell, P. Braghetta, and K. K. Stanley. 1990. Identification, sequencing and expression of an integral membrane protein of the *trans*-Golgi network (TGN 38). *Biochem. J.* 270:97–102.

Medema, R. H., A. M. M. de Vries-Smits, G. C. M. van der Zon, J. Antonie Maassen, and J. L. Bos. 1993. Ras activation by insulin and epidermal growth factor through enhanced exchange of guanine nucleotides on p21ʳᵃˢ. *Mol. Cell. Biol.* 13:155–162.

Millàn, J. L. 1986. Molecular cloning and sequence analysis of human placental alkaline phosphatase. *J. Biol. Chem.* 261:3112–3115.

Nuoffer, C., H. W. Davidson, J. Metteson, J. Meinkoth, and W. E. Balch. 1994. A GDP-bound form of rab1 inhibits protein export from the endoplasmic reticulum and transport between Golgi compartments. *J. Cell Biol.* 125:225–237.

Ostermann, J., L. Orci, K. Tani, M. Amherdt, M. Ravazzola, Z. Elazar, and J. E. Rothman. 1993. Stepwise assembly of functionally active transport vesicles. *Cell.* 75:1015–1025.

Pelham, H. R. B. 1989. Control of protein exit from the endoplasmic reticulum. *Annu. Rev. Cell Biol.* 5:1–23.

Pelham, H. R. B., and S. Munro. 1993. Sorting of membrane proteins in the secretory pathway. *Cell.* 75:603–605.

Pfeffer, S. R. 1994. Rab GTPases: master regulators of membrane trafficking. *Curr. Opin. Cell. Biol.* 6:522–526.

Plutner, H., A. D. Cox, S. Pind, R. Khovravi-Far, J. R. Bourne, R. Schwaninger, C. J. Der, and W. E. Balch. 1991. Rab1b regulates vesicular transport between the endoplasmic reticulum and successive Golgi compartments. *J. Cell Biol.* 115:31–43.

Ridley, A. J., H. F. Paterson, C. L. Johnston, D. Diekmann, and A. Hall. 1992. The small GTP-binding protein rac regulates growth factor-induced membrane ruffling. *Cell.* 70:401–410.

Riederer, M. A., T. Soldati, A. D. Shapiro, J. Lin, and S. R. Pfeffer. 1994. Lysosome biogenesis requires rab9 function and receptor recycling from endosomes to the *trans*-Golgi network. *J. Cell Biol.* 125:573–582.

Roa, M.,V. Cornet, C. -Z. Yang, and B. Goud. 1993. The small GTP-binding protein rab6p is redistributed in the cytosol by brefeldin A. *J. Cell Sci.* 106:782–802.

Rodriguez, J. F., R. Janeczko, and M. Esteban. 1985. Isolation and characterization of neutralizing monoclonal antibodies to vaccinia virus. *J. Virol.* 56:482–488.

Rosa, P., S. Mantovani, R. Rosboch, and W. B. Huttner. 1992. Monensin and brefeldin A differentially affect the phosphorylation and sulfation of secretory proteins. *J. Biol. Chem.* 267:12227–12232.

Roth, J., D. J. Taatjes, J. M. Lucocq, J. Weinstein, and J. C. Paulson. 1985. Demonstration of an extensive *trans*-tubular network continuous with the Golgi apparatus stack that may function in glycosylation. *Cell.* 43:287–295.

Rothman, J. E. 1981. The Golgi apparatus: two organelles in tandem. *Science (Wash. DC).* 213:1212–1219.

Rothman, J. E., and G. Warren. 1994. Implications of the SNARE hypothesis for intracellular membrane topology and dynamics. *Curr. Biol.* 4:220–233.

Salminen, A., and P. Novick. 1987. A ras like protein is required for a post-Golgi event in yeast secretion. *Cell.* 47:527–538.

Sasaki, T., A. Kikuchi, S. Araki, Y. Hata, M. Isomura, S. Kuroda, and Y. Takai. 1990. Purification and characterization from bovine brain cytosol of a protein that inhibits the dissociation of GDP from and the subsequent binding of GTP to *smg* p25A, a *ras* p21-like GTP-binding protein. *J. Biol. Chem.* 265:2333–2337.

Segev, N. 1991. Mediation of the attachment or fusion step in vesicular transport by the GTP-binding YPT1 protein. *Science (Wash. DC).* 252: 1553–1556.

Segev, N., J. Mulholland, and D. Botstein. 1988. The yeast GTP-binding YPT1 protein and a mammalian counterpart are associated with the secretion machinery. *Cell.* 52:915–924.

Söllner, T., S. W. Whiteheart, M. Brunner, H. Erdjument-Bromage, S. Geromanos, P. Tempst, and J. E. Rothman. 1993. SNAP receptors implicated in vesicle targeting and fusion. *Nature (Lond.).* 362:318–324.

Stamnes, M. A., and J. E. Rothman. 1993. The binding of AP-1 clathrin adaptor particles to Golgi membranes requires ADP-ribosylation factor, a small GTP-binding protein. *Cell.* 73:999–1005.

Stenmark, H., R. G. Parton, O. Steele-Mortimer, A. Lütcke, J. Gruenberg, and M. Zerial. 1994. Inhibition of rab5 GTPase activity stimulates membrane fusion in endocytosis. *EMBO (Eur. Mol. Biol. Organ.) J.* 13:1287–1296.

Tanigawa, G., L. Orci, M. Amherdt, M. Ravazzola, J. B. Helms, and J. E. Rothman. 1993. Hydrolysis of bound GTP by ARF protein triggers uncoating of Golgi-derived COP-coated vesicles. *J. Cell Biol.* 123:1365–1371.

Tisdale, E., J. Bourne, R. Khosravi-Far, C. Der, and W. Balch. 1992. GTP-binding mutants of Rab1 and Rab2 are potent inhibitors of vesicular transport from the endoplasmic reticulum to the Golgi complex. *J. Cell Biol.* 119:749–761.

Tixier-Vidal, A., A. Barret, R. Picart, V. Mayau, D. Vogt, B. Wiedenman, and B. Goud. 1993. The small GTP-binding protein rab6p is associated with both Golgi and post-Golgi synaptophysin-containing membranes during synaptogenesis of hypothalamic neurones in culture. *J. Cell Sci.* 105:935–947.

Touchot, N., P. Chardin, and A. Tavitian. 1987. Four additional members of the ras gene superfamily isolated by an oligonucleotide strategy: molecular cloning of YPT-related cDNAs from a rat brain library. *Proc. Natl. Acad. Sci. USA.* 84:8210–8214.

Valencia, A., P. Chardin, A. Wittinghofer, and C. Sander, 1991. The ras protein family: evolutionary tree and role of conserved amino acids. *Biochemistry.* 30:4637–4648.

Van der Sluijs, P., M. Hull, P. Webster, P. Mâle, B. Goud, and I. Mellman. 1992. The small GTP-binding protein rab4 controls an early sorting event on the endocytic pathway. *Cell.* 70:729–740.

Walworth, N. C., P. Brennwald, A. K. Kabcenell, M. Garrett, and P. Novick. 1992. Hydrolysis of GTP by Sec4 protein plays an important role in vesicular transport and is stimulated by a GTPase-activating protein in *Saccharomyces cerevisiae. Mol. Cell. Biol.* 12:2017–2028.

Wilson, B. S., C. Nuoffer, J. L. Meinkoth, M. McCaffery, J. R. Feramisco, W. E. Balch, and M. G. Farquhar. 1994. A rab1 mutant affecting guanine nucleotide exchange promotes disassembly of the Golgi apparatus. *J. Cell Biol.* 125:557–571.

Yang, C.-Z., V. Mayau, F. Godeau, and B. Goud. 1992. Characterization of the unprocessed and processed forms of rab6 expressed in baculovirus/insect cell systems. *Biochem. Biophys. Res. Commun.* 182:1499–1505.

Zahraoui, A., N. Touchot, P. Chardin,and A. Tavitian. 1989. The human rab genes encode a family of GTP-binding proteins related to yeast YPT1 and SEC4 products involved in secretion. *J. Biol. Chem.* 264:12394–12401.

Zahraoui, A., G. Joberty, M. Arpin, J.-J. Fontaine, R. Hellio, A. Tavitian, and D. Louvard. 1994. A small rab GTPase is distributed in cytoplasmic vesicles in non polarized cells but colocalizes with the tight junction marker ZO-1 in polarized epithelial cells. *J. Cell Biol.* 124:101–115.

Zerial, M., and H. Stenmark. 1993. Rab GTPases in vesicular transport. *Curr. Opin. Cell Biol.* 5:613–620.