GTP-Binding Mutants of Rab1 and Rab2 are Potent Inhibitors of Vesicular Transport from the Endoplasmic Reticulum to the Golgi Complex

Ellen J. Tisdale*, Jeffrey R. Bourne*‡, Roya Khosravi-Far‡, Channing J. Der‡ and W. E. Balch*

*Departments of Cell and Molecular Biology, The Scripps Research Institute, La Jolla, California 92037; and
‡La Jolla Cancer Research Foundation, La Jolla, California 92037

Abstract. We have examined the role of ras-related rab proteins in transport from the ER to the Golgi complex in vivo using a vaccinia recombinant T7 RNA polymerase virus to express site-directed rab mutants. These mutations are within highly conserved domains involved in guanine nucleotide binding and hydrolysis found in ras and all members of the ras superfamily. Substitutions in the GTP-binding domains of rabla and rablb (equivalent to the ras 17N and 116I mutants) resulted in proteins which were potent *trans* dominant inhibitors of vesicular stomatitis virus glycoprotein (VSV-G protein) transport between the ER and *cis* Golgi complex. Immunofluorescence analysis indicated that expression of rablb$_{121I}$ prevented delivery of VSV-G protein to the Golgi stack, which resulted in VSV-G protein accumulation in pre-Golgi punctate structures. Mutants in guanine nucleotide exchange or hydrolysis of the rab2 protein were also strong *trans* dominant transport inhibitors. Analogous mutations in rab3a, rab5, rab6, and H-ras did not inhibit processing of VSV-G to the complex, sialic acid containing form diagnostic of transport to the *trans* Golgi compartment. We suggest that at least three members of the rab family (rabla, rablb, and rab2) use GTP hydrolysis to regulate components of the transport machinery involved in vesicle traffic between early compartments of the secretory pathway.

MEMBERS of the *rab* family of *ras*-related small GTP-binding proteins are associated with subcellular compartments in both the endocytic and exocytic pathways (reviewed in Balch, 1990; Goud and McCaffrey, 1991). These proteins are likely to be key regulatory components of protein complexes catalyzing the fission and fusion of transport vesicles between distinct subcellular compartments. In support of their proposed function, the rab-related yeast GTP-binding proteins YPT1 and SEC4 have been shown to be required for transport between the ER and the Golgi complex (reviewed in Hicke and Schekman, 1990; Schmitt et al., 1988; Segev et al., 1988; Bacon et al., 1989; Baker et al., 1990; Kaiser and Schekman, 1990), and from the Golgi complex to the cell surface (Salminen and Novick, 1987; Goud et al., 1988; Walworth et al., 1989), respectively. In addition, in vitro assays which efficiently reconstitute ER to Golgi (Beckers et al., 1987) and intra-Golgi transport (reviewed in Waters et al., 1991; Rothman and Orci, 1992) are sensitive to the nonhydrolyzable analog of GTP, GTPγS (Melancon et al., 1987; Beckers and Balch, 1989).

Within the mammalian rab gene family, rabla and rablb share 70–80% identity to YPT1 (Valencia et al., 1991). In fact, mouse rabla can complement mutant yeast which lack YPT1 (Haubruck et al., 1989). Although genetic studies in yeast have shown that the YPT1 gene is required in vesicular transport, there is limited data which demonstrates a similar role of the mammalian rab1 homologue in membrane traffic. We have shown that a peptide homologous to the putative effector domain of the rab1 protein inhibits ER to Golgi transport, presumably by competition for a downstream effector molecule(s) required for vesicle fusion (Plutner et al., 1990). In addition, a neutralizing monoclonal antibody specific for the rablb protein inhibits transport from the ER to the Golgi complex in vitro (Plutner et al., 1991).

All members of the ras superfamily contain highly conserved domains required for guanine nucleotide binding, GTP/GDP[1] exchange, and GTP hydrolysis (Bourne et al., 1990; Bourne et al., 1991). These domains interact with proteins which stimulate guanine nucleotide dissociation (GDS), inhibit GDP exchange (GDI), and promote GTP hydrolysis (GAP) (reviewed in Hall, 1990; Haubruck and McCormick, 1991; Wittinghofer and Pai, 1991; Takai et al., 1992). A host of amino acid substitutions (>100) made in the ras GTP-binding and effector domains have led to the identification of several activation and inhibitory mutations (Barbacid, 1987). Similar substitutions in the SEC4 and YPT1 proteins result in *trans* dominant negative phenotypes

1. *Abbreviations used in this paper:* GAP, GTPase activating protein; GDI, guanine nucleotide dissociation inhibitor; GDS, guanine nucleotide dissociation stimulator; NGS, normal goat serum; VSG-V, vesicular stomatitis virus glycoprotein.

which inhibit vesicular transport (Walworth et al., 1989; Becker et al., 1991).

Using the mutational analysis performed on ras as a model, we have explored the role of rab proteins in ER to Golgi complex transport in mammalian cells using site-directed mutagenesis and a vaccinia based transient expression system. We report that rabla and rablb mutants with altered GDP/GTP binding properties behave as potent *trans* dominant inhibitors of ER to Golgi complex transport in vivo. In addition, we provide evidence that mutations in the GTP-binding domains of rab2, a small GTP-binding protein localized to a post-ER, pre-Golgi intermediate compartment (Chavrier et al., 1990*a*), results in proteins which act as *trans* dominant inhibitors in vesicular traffic. Our results suggest that at least three members of the rab family (rabla, 1b, and 2) are critical for regulating the biochemical machinery involved in vesicle fission and fusion between early compartments of the secretory pathway.

Materials and Methods

Cell Culture and Generation of Mutant Constructs

HeLa cells were cultured in DME (Gibco BRL, Grand Island, NY) supplemented with 10% FBS and penicillin-streptomycin in a 5% humidified CO_2 incubator. Rab expression constructs were generated from canine rabla (Chavrier et al., 1990*a*), rat rablb (Touchot et al., 1987), and human rab2, rab3a, rab5, and rab6 (Zahraoui et al., 1989) cDNA sequences that have been generously provided by M. Zerial (EMBL, Heidelberg, Germany) and A. Tavitian (INSERM, Paris, France). All rab sequences were introduced into the NdeI and BamHI sites of the pET3a vector (Novagen, Madison, WI) for expression (Studier et al., 1990) from the T7 promoter as described previously (Khosravi-Far et al., 1991). To generate structural mutants of each rab protein (see Table 2), oligonucleotide-directed mutagenesis was done on M13 mp18 vector constructs of each rab cDNA sequence using the Polymerase III Mutator protocol (Stratagene Corp., La Jolla, CA) or by TaqI polymerase chain reaction DNA amplification. All mutated rab sequences were verified by dideoxy sequencing. The pET-rab constructs were introduced into *E. coli* strain BL21-DE3 (Novagen) for expression of recombinant rab protein and used for transfection experiments. The pAR-G (pET3a) plasmids encoding VSV-G and tsO45 VSV-G were obtained from J. Rose (Yale University, New Haven, CT) and C. Machamer (Johns Hopkins University, Baltimore, MD).

Transfection Procedure

HeLa cells were plated (5×10^5/35 mm dish) on the day before transfection. The cells were first washed two times with DME, then infected with a vaccinia T7 RNA polymerase recombinant virus (vTF7-3) (Fuerst et al., 1986), at a multiplicity of 10 PFU/cell for 30 min with intermittent rocking in a 37°C incubator. The inoculum was removed and the cells cotransfected with 5 μg pET-rab plasmid DNA encoding for a mutated rab protein as described above, and 5 μg of plasmid encoding for VSV-G protein (pAR-G) that had been premixed for 15 min at room temperature in 1 ml DME and 15 μl of the transfection reagent (TransfectACE [Gibco-BRL]) as outlined by Rose (Rose et al., 1991). 3 h post-transfection, an equal volume of DME/10% FBS was added to the cells and incubated for an additional 3 h to allow synthesis of VSV-G protein and the mutant rab protein. After the transfection period, the media was removed and the cells incubated for 15 min in methionine free MEM (Gibco-BRL), biosynthetically labeled for 10 min with 20 μCi *trans* [^{35}S]-label (spec. act. 1192 Ci/mmol, ICN Biomedicals, Inc., Irvine, CA), then chased for 1 h in DME/10% FBS. The chase was terminated by the addition of lysis buffer (50 mM Tris, pH 8.0, 150 mM NaCl, 1% Triton X-100, 1 mM PMSF) (Sigma Chemical Co., St. Louis, MO) and the lysate precleared for 30 min. with 50 μl Pansorbin (Calbiochem Corp., San Diego, CA). The soluble fraction was incubated overnight at 4°C with a monoclonal antibody to VSV-G (clone 8G5) and pansorbin. Immune precipitates were washed three times with 10 mM Tris, pH 6.8/1% SDS, boiled, then centrifuged. To the supernatant was added 30 μl of 0.2 M citrate, pH 5.5 and 5 μl of endoglycosidase H (endo H) (1 U/ml, Boehringer Mannheim Corp., Indianapolis, IN) and the sample digested overnight at 37°C. Samples were analyzed by SDS-PAGE on 6.75% gels according to the method of Laemmli (Laemmli, 1970). The gel was soaked in 1 M salicylate/30% methanol for 30 min, dried, then exposed to XAR-5 film (Eastman Kodak Corp., Rochester, NY) at −80°C. Quantitation of the VSV-G protein was accomplished using densitometry as described previously (Beckers et al., 1987; Beckers and Balch, 1989; Schwaninger et al., 1991).

Transport Kinetics and Protein Expression of rablb$_{121I}$

HeLa cells (3×10^6/10 cm dish) were infected/transfected at a multiplicity of 10 PFU/cell and 30 μg of mutant rab and 30 μg pAR-G, as described above. 2 h post-transfection, the media was removed and saved, and the cells detached from the culture dish with PBS/50 mM EDTA. The cells were washed one time with DME then replated at equal number in 35-mm dishes with transfection media from the parental dish and equal volume of DME/10% FBS. This would allow a common pool of transfected cells to be analyzed. At 3, 4, 5 and 6 h post-transfection cells were biosynthetically labeled for 10 min and chased for 60 min in complete media, as outlined above.

To determine the level of expression of the rab protein, a comparable number of cells at each time point were detached from the dish with PBS/EDTA and washed one time with 10/18 (10 mM KOAc/18 mM HEPES [pH 7.2]). The cells were resuspended in 10/18 containing protease inhibitors (10 μg/ml leupeptin, 10 μl/ml aprotinin, 10 μM TLCK, 1 μg/ml chymotrypsin, 0.1 μM pepstatin), swollen on ice for 10 min., then homogenized by 10 passes through a 23 gauge needle after which the concentration was adjusted to 25 mM KOAc/125 mM Hepes. The nuclei were removed by centrifugation for 5 min. at 3,000 RPM and the post-nuclear supernatant centrifuged at 30 PSI for 30 min. at 4°C in a Beckman Airfuge. The supernatant was removed and the membrane pellet resuspended in 25/125. Protein determinations were made by the Micro BCA Protein Assay Reagent (Pierce Chem. Co., Rockford, IL). Aliquots of the supernatant and membrane fractions (100 μg total protein) were separated by SDS-PAGE on a 12% gel and transferred to nitrocellulose (Schleicher and Schuell, Keene, NH) in 25 mM Tris pH 8.3, 192 mM glycine, 20% methanol. The membrane was blocked in 5% nonfat dry milk, 0.05% Tween-20 in TBS. Detection of rablb was with the polyclonal antibody p68 (Plutner et al., 1991) followed by 1 μCi ^{125}I Protein A (spec. act 85.6 μCi/μg, ICN). Standard curve was generated from purified recombinant rab protein.

Western Analysis of Wild Type and Mutant Rab Proteins

HeLa cells (5×10^5/35 mm dish) were infected/transfected, as outlined above. 6 h post-transfection, the cells were detached from the culture dish with PBS/EDTA, washed two times with PBS, then lysed in 10% TCA for 2 h on ice. TCA-precipitated material from the cells was washed one time with acetone and dissolved in 100 μl of sample buffer (0.1% SDS, 0.5 M Tris-HCl, pH 6.8, 10% glycerol, 5% β-mercaptoethanol, and 0.1% bromphenol blue). Samples were separated by SDS-PAGE using 12% gels and transferred to nitrocellulose as described above. After blocking, the membrane was incubated with the appropriate anti-rab antibody, washed then incubated with an alkaline phosphatase-conjugated second antibody (Pierce Chemical Co.) and developed with 5-bromo-4-chloro-3-indoyl phosphate and nitroblue tetrazolium (Pierce Chemical Co.). HeLa cell (5×10^5) lysate of non-transfected cells was coanalyzed to determine the endogenous level of rab protein. Purified recombinant rab proteins were included as controls. Overexpression of rab proteins was detected by antibody reagents specific for rablb (m5C6b) (Plutner et al., 1991), rab2 (W. E. Balch, unpublished data), rab5 (generously provided by M. Zerial, EMBL) and a polyclonal antibody which cross-reacts with rabla, 1b, 3 and 6.

Indirect Immunofluorescence

Hela cells (5×10^5) were plated on coverslips in 35-mm tissue culture dishes on the day before transfection. Cells were infected/co-transfected as above with 5 μg pAR tsO45-G, 5 μg pET rablb and/or 5 μg of pET rablb$_{121I}$. Cells were incubated at 40°C for 4 h followed by incubation at 32°C for 2 h. To terminate transport, the cells were transferred to ice, fixed in 2% paraformaldehyde/PBS for 10 min at room temperature. Fixation was quenched with 10 mM ammonium chloride in PBS for 10 min then blocked in PBS/5% normal goat serum (NGS) for 10 min at room temperature. To detect intracellular VSV-G, cells were permeabilized with 0.05% saponin in PBS/NGS for 10 min, washed with PBS, then incubated for 30 min with a monoclonal antibody specific for the VSV-G protein cytoplasmic tail

(P5D4). Cells were washed, costained for 30 min with Texas Red goat α-mouse antibody (Molecular Probes, Inc., Eugene, OR) and 10 μg/ml FITC-conjugated Lens culinaris lectin (E-Y Laboratories, San Mateo, CA) in PBS/NGS to label Golgi compartments containing terminal N-acetylglucosamine. For cell surface labeling, VSV-G was detected by an antibody specific for the extra-cellular domain of VSV-G (clone 8AG) followed by a Texas red secondary reagent, as described above. Coverslips were mounted in Moviol (Calbiochem Corp., San Diego, CA) and viewed under a Zeiss Axiovert fluorescence microscope.

Results

A Transient Expression System to Study rab Protein Function In Vivo

To study the in vivo consequences of mutated forms of the rab proteins on vesicular traffic, we employed a transient expression system in which HeLa cells are first infected with vaccinia T7 RNA polymerase recombinant virus (vTF7-3) then cotransfected with a mutant rab plasmid DNA and a plasmid encoding for vesicular stomatitis virus glycoprotein (VSV-G) (pAR-G) which serves as the reporter molecule (Fuerst et al., 1986; Moss, 1989). VSV-G protein is a type I integral membrane protein which acquires two *N*-linked oligosaccharides during cotranslational insertion into the ER. Transport of VSV-G protein from the ER to the *cis*/medial Golgi compartments results in processing of the high mannose (man_9) endoglycosidase H sensitive (endo H_S) oligosaccharides found in the ER to the complex, endo H resistant (endo H_R) forms present in the Golgi stack and on the plasma membrane due to trimming by Golgi associated mannosidases and glycosyl transferases. Appearance of the various oligosaccharide processing intermediates can be used to follow the transport of VSV-G through the *cis*, medial, and *trans* Golgi compartments. These intermediate forms of VSV-G can be distinguished by their unique mobilities after SDS-PAGE (Schwaninger et al., 1991).

To establish conditions to study transport, Hela cells infected with vTF7-3 were transfected with the VSV-G protein encoding plasmid at 60 min intervals, biosynthetically radiolabeled for 10 min, then chased for 1 h in the absence of radiolabel. Immunoprecipitated G protein was first detected 3 h post-transfection and continued to increase in concentration in a linear fashion with time (data not shown). We selected the 4–6 h post-transfection period to study transport because both rab and VSV-G proteins were expressed at significant levels without adversely affecting the secretory pathway. The rate of transport of VSV-G from the ER to the Golgi complex in vaccinia infected cells transfected with the VSV-G plasmid after 5 h post-transfection is shown in Fig. 1. For each time point, immunoprecipitated VSV-G protein was digested with endo H, then analyzed by SDS-PAGE and fluorography to determine the extent of VSV-G processing from the endo H_S to the endo H_R form. As shown in Fig. 1, the appearance of the endo H_R form of VSV-G, coincident with transport to the *cis*/medial Golgi compartments, occurred with a half time of ~30 min. VSV-G protein was quantitatively (90%) converted to the endo H_R form after a 60–75 min chase period (Fig. 1). The efficiency of transport in different experiments was found to vary from 70–90%. The kinetics of VSV-G transport were identical to those observed in the presence of rifampicin or hydroxyurea, reagents which inhibit maturation of vaccinia particles (Vos and Stunnenberg, 1988; Moss et al., 1991).

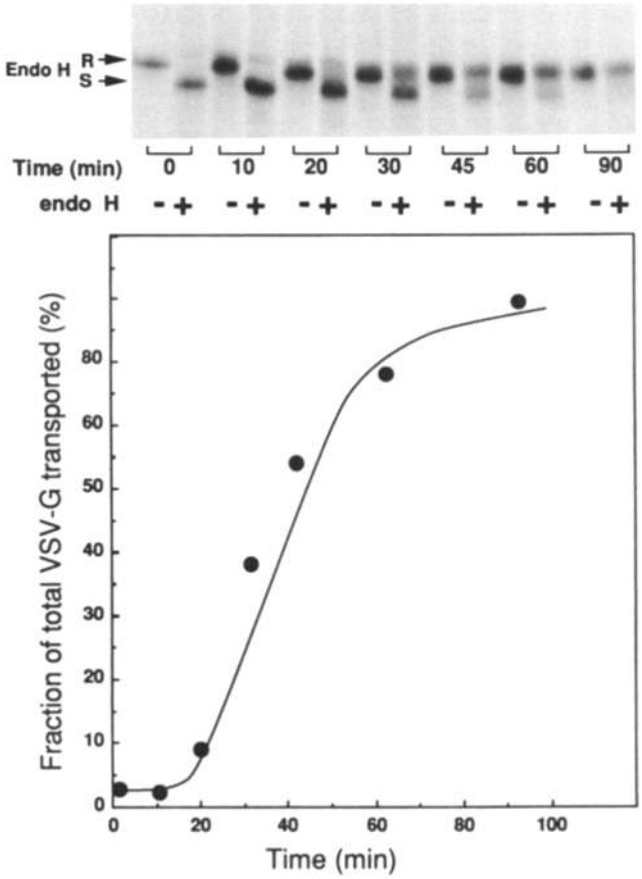


Figure 1. Kinetics of transport of VSV-G from the ER to the Golgi complex in vaccinia infected/transfected HeLa cells. Vaccinia infection and pAR-G (a plasmid encoding the VSV-G protein) transfection were performed as described in Materials and Methods. 6 h post-transfection, cells were biosynthetically labeled with *trans* (^{35}S) for 10 min, then chased for the indicated time in radioactive free media. VSV-G protein was immunoprecipitated and incubated in the absence (−) or presence (+) of endoglycosidase H (endo H) as described in Materials and Methods. The endoglycosidase sensitive (endo H_S, lower band) forms and endoglycosidase H resistant (endo H_R, upper band) form were separated by SDS-PAGE. The fraction (%) of VSV-G protein transported to the endo H resistant form (lower panel) was quantitated as described in Materials and Methods. VSV-G protein is converted to the endo H_R form with a half-time of 30 min.

Mutations in GTP-Binding Domains of rab1 are Trans Dominant Inhibitors of ER to Golgi Transport

To identify rab proteins essential for vesicular transport between the ER and the Golgi complex, we performed site-directed mutagenesis within domains involved in guanine nucleotide interactions. Ras and rab proteins contain four highly conserved GTP-binding domains (Bourne et al., 1991; Valencia et al., 1991). Mutational and structural analysis of the ras protein have determined essential amino acid residues in these domains for binding and hydrolysis of GTP (reviewed in Barbacid, 1987; Valencia et al., 1991) (Table 1). Amino acid substitutions at specific sites in the guanine nucleotide binding domains of ras alter guanine nucleotide binding affinity for GDP and GTP (Table 2, 17 S→A, 116

Table I. Comparison of Regions of Ras-related rab Proteins Involved in GTP-binding and Regulation of GTP Hydrolysis

	10	30	57	113	187
H-ras	NH_2--GAGGVGKS----DEYDPTIEDSYRKQV-----		DTAGQEE	----LVGNKCDL	----CVLS-COOH
SEC4	-GDSGVGKS----PSFITTIGIDFKIKT-----		DTAGQER	----LVGNKSDM	----CC
YPT1	-GNSGVGKS----NDYISTIGVDFKIKT-----		DTAGQER	----LVGNKCDL	----CC
rab1a(rat)	-GDSGVGKS----ESYISTIGVDFKIRT-----		DTAGQER	----LVGNKCDL	----CC
rab1b(rat)	-GDSGVGKS----ESYISTIGVDFKIRT-----		DTAGQER	----LVGNKSDL	----CC
rab2	-GDTSGVKS----PVHDLTMGVEFGARM-----		DTAGQER	----LIGNKSDL	----CC
rab3	-GNSSGVKS----PAFVSTVGIDFKVKT-----		DTAGQER	----LVGNKCDL	----CAC
rab5	-GESAVGKS----EFQESTIGAAFLTQT-----		DTAGQER	----LSGNKADL	----CCSN
rab6	-GEQSVGKS----NTYQATIGIDFLSKT-----		DTAGQER	----LVGNKTDL	----CSC
	Phosphoryl-binding site	Effector domain	Phosphoryl-binding site	Guanine ring contact site	Prenylation site

* The amino acid sequences of rab-related proteins which make up the functional GTP-binding and effector domains are compared to H-ras (human). The numbering is that of H-ras. The functional designations are based on the crystal structure of ras. Mutations used in this study which alter GTP-binding or hydrolysis for ras are indicated by the asterisk although essentially mutations at all positions in these domains alter GTP-binding or hydrolysis (see Valencia et al., 1991 for review).

N→I) (Der et al., 1988; Feig and Cooper, 1988), abolish interaction with GTPase activating protein (GAP) (Table 2, 35 T→A) (Haubruck and McCormick, 1991) and impair GTP hydrolytic activity (Table 1, 12 G→S, 61 Q→L) (Haubruck and McCormick, 1991). The prominent effects of these mutations on ras biological function and the conservation of these domains among all ras related proteins (Bourne et al., 1991; Valencia et al., 1991), suggest that these regions are likely to contain key residues critical for rab function.

To study the in vivo effect of overexpression of mutant forms of rab proteins on vesicular transport, vTF7-3 infected HeLa cells were cotransfected with a rab mutant and the VSV-G expression constructs for 5–6 h, pulse-labeled for 10 min, then chased for 1 h in nonradioactive medium to promote transport to the Golgi complex. The first mutation we generated in rablb and rabla was a substitution of N to I at position 121. The main function of this highly conserved ASN is to hold together the three elements which are involved in guanine nucleotide binding. In the case of ras, mutations in the consensus guanine nucleotide binding sequence NKXD (Table 1, ras residues 116–119) have reduced guanine nucleotide binding affinity. As a consequence of enhanced GDP/GTP exchange, ras_{116I} is a constitutively activated, transforming protein. The N to I substitution in the corresponding NKXD motif of both YPT1 and SEC4 result in mutant proteins which do not bind GTP and produce dominant lethal phenotypes and secretory defects when expressed in yeast (Schmitt et al., 1986; Schmitt et al., 1988; Walworth et al., 1989).

As shown in Fig. 2, overexpression of $rablb_{121I}$ (~10-fold)

Table II. Summary and Properties of Rab Mutant Proteins

Mutant	Substitution*	Ras‡	Inhibition of ER-Golgi transport
$rab1b_{22N}$	Ser(TCA) - Asn(AAC)	17N	+
$rab1b_{40A}$	Thr(ACC) - Ala(GCC)	35A	−
$rab1b_{41M}$	Ile(ATT) - Met(ATG)	36M	−/+
$rab1b_{44N}$	Asp(GAC) - Asn(AAC)	39N	−
$rab1b_{67L}$	Gln(CAG) - Leu(CTG)	61L	−
$rab1b_{121I}$	Asn(AAC) - Ile(ATC)	116I	+
$rab1b_{121I}$,ΔCC	Asn(AAC) - Ile(ATC), Cys(TGC) - ter(TGA)	116I/ ΔCAAX	+
$rab1b_{121I}$/rab5	Asn(AAC) - Ile(ATC): rab1b(1-150)/rab5(163-215)	None	−
$rab1a_{121I}$	Asn(AAC) - Ile(ATC)	116I	+
$rab2_{20N}$	Ser(TCA) - Asn(ACC)	17N	−
$rab2_{38A}$	Thr(ACT) - Ala(GCT)	35A	−
$rab2_{65L}$	Gln(CAA) - Leu(CTA)	61L	+
$rab2_{119I}$	Asn(AAT) - Ile(ATT)	116I	+
$rab3a_{135I}$	Asn(AAC) - Ile(ATC)	116I	−
$rab5_{133I}$	Asn(AAC) - Ile(ATC)	116I	−
$ra6_{126I}$	Asn(AAT) - Ile(ATT)	116I	−
$H\text{-}ras_{116I}$	Asn(AAC) - Ile(ATC)	116I	−

* Nucleotide substitutions are underlined.
‡ Analogous ras mutant protein.

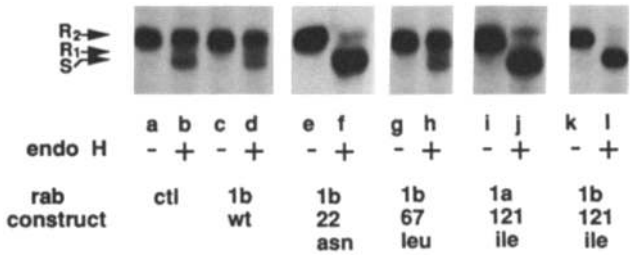


Figure 2. Mutations in GTP-binding domains of rabla and rablb are *trans* dominant inhibitors of transport from the ER to the Golgi complex. Vaccinia infection and cotransfection protocols with pAR-G and mutant (pET) plasmids were as described in Materials and Methods. Briefly, 5 h post-transfection cells were labeled with *trans* (^{35}S) for 10 min and chased for 60 min in the absence of radiolabel. VSV-G protein was immunoprecipitated and digested in the absence (−) or presence (+) of endo H, and the endo H sensitive (S) or resistant (R_1 and R_2) forms analyzed by SDS-PAGE as described in Materials and Methods. R_1 is a processing intermediate with one of the two oligosaccharide chains processed to the endo H resistant form (Schwaninger et al., 1991). R_2 is the form of VSV-G containing two endo H resistant oligosaccharides fully processed and containing sialic acid. Transport of VSV-G protein from the ER to the Golgi complex is totally inhibited in cells overexpressing $rablb_{12II}$, and $rabla_{12II}$, and $rablb_{22N}$.

resulted in a complete block of VSV-G protein transport (Fig. 2, lane *l*). No processing of VSV-G protein to endo H_R forms was observed after the 60 min chase period compared to the control in which 75% of the VSV-G protein was processed to the endo H resistant form (Fig. 2, compare lanes *b* and *l*). VSV-G neither accumulated in the Man_5 endoglycosidase D sensitive form (data not shown), a processing reaction diagnostic of protein transport to the *cis* Golgi compartment in the mutant CHO cell line clone 15B (Beckers et al., 1987), nor in an early endo H_R processing intermediate observed in the control (R_1 in Fig. 2, lanes *b* or *d*) diagnostic of transport from the ER to the *cis* Golgi compartment in wild-type CHO cells (Schwaninger et al., 1991). Identical results were obtained for $rabla_{121I}$ (95% identity with rablb) (Fig. 2, lane *j*). As expected, recombinant $rabla_{121I}$ (data not shown) or $rablb_{121I}$ did not bind detectable levels of GTP (<0.1%) compared to the wild-type proteins measured in solution or by Western blotting (Fig. 3, lanes *a* and *e*). In contrast, VSV-G protein was efficiently (>85%) transported and processed to the fully sialylated, complex form (R_2 in Fig. 2, lane *d*) in the presence of overexpressed (~10-fold) wild-type rablb or wild-type rabla (data not shown). We have been unable to detect any statistically significant change in the extent or rate of processing of VSV-G to endo H_R forms in cells overexpressing (10-fold) wild-type rabla or 1b (data not shown), suggesting that the endogenous rabl protein is not rate limiting for transport of VSV-G protein from the ER to the terminal compartments of the Golgi complex.

To provide evidence that expression of the $rablb_{121I}$ protein imposes a transport block between the ER and the Golgi stack, we analyzed the distribution of VSV-G in the presence of $rablb_{121I}$ by indirect immunofluorescence. For this purpose, Hela cells were transfected with a plasmid (pAR-tsO45-G protein) expressing a temperature-sensitive form of VSV-G which misfolds at the restrictive temperature (39.5°C) and fails to be exported from the ER (Lafay, 1974; Balch et al., 1986) (Fig. 4, *A* [VSV-G] and *B* [Golgi complex]). TsO45-VSV-G is rapidly mobilized to the Golgi membranes when shifted to the permissive temperature (32°C) (Fig. 4, *C* [VSV-G] and *D* [Golgi complex]). When cells cotransfected with pAR-tsO45-G and wild-type rablb and were held at the restrictive temperature for 4 h followed by a 90 min chase at the permissive temperature, VSV-G protein was found to be efficiently transported to the Golgi complex (Fig. 4, *E*, *E'* [VSV-G] and *F*, *F'* [Golgi complex]). In contrast, expression of $rablb_{121I}$ resulted in the inability of VSV-G to enter the Golgi stack. The tsO45-G protein was partially transported to punctate structures scattered throughout the cytoplasm (Fig. 4, *G*, *G'* [VSV-G] and *H*, *H'* [Golgi complex]), similar to the distribution of pre-Golgi intermediates in BHK cells (Saraste, 1984; Saraste et al., 1991). Furthermore, cells expressing tsO45 VSV-G and $rablb_{121I}$ failed to transport VSV-G protein to the surface (Fig. 5, *B*) indicative of a block in transport.

The striking inhibition of protein transport produced by expression of $rablb_{121I}$ prompted us to examine the threshold of this mutated protein required to inhibit vesicular traffic. Cells cotransfected with the VSV-G protein encoding plasmid and pET $rablb_{121I}$ were radiolabeled at 3, 4, 5, and 6 h post-transfection for 10 min, chased for 1 h, then transport assessed as described above. In addition, the level of expression and distribution of rablb between membrane and cytosol was quantitated by Western blotting (Fig. 6). The endogenous pool of rablb in Hela cells is distributed between membrane-associated and soluble forms (Fig. 6, *B*). Expression of $rablb_{121I}$ after a 3 h post-transfection period resulted in ~2 fold increase in the total rab pool (Fig. 5, *B*). Even at this level of expression of the mutant protein, VSV-G protein transport to the Golgi complex was reduced by ~25% (Fig. 5, *A*). From 4 to 6 h post-transfection, a 5- to 10-fold increase in $rablb_{121I}$ was observed with $rablb_{121I}$ now being detected increasingly in the membrane fraction (Fig. 3, *B*). At 6 h post-transfection transport was inhibited >80% (Fig. 5, *A*). In general, overexpression of $rablb_{121I}$ in the range of four to eightfold results in the total arrest of VSV-G protein transport and processing to the endo H_R form as early as 3–4 h post-transfection. We have been unable to reverse inhibition by cotransfection with pET wild-type rablb suggesting that the $rablb_{121I}$ mutant irreversibly inhibits transport (data not shown).

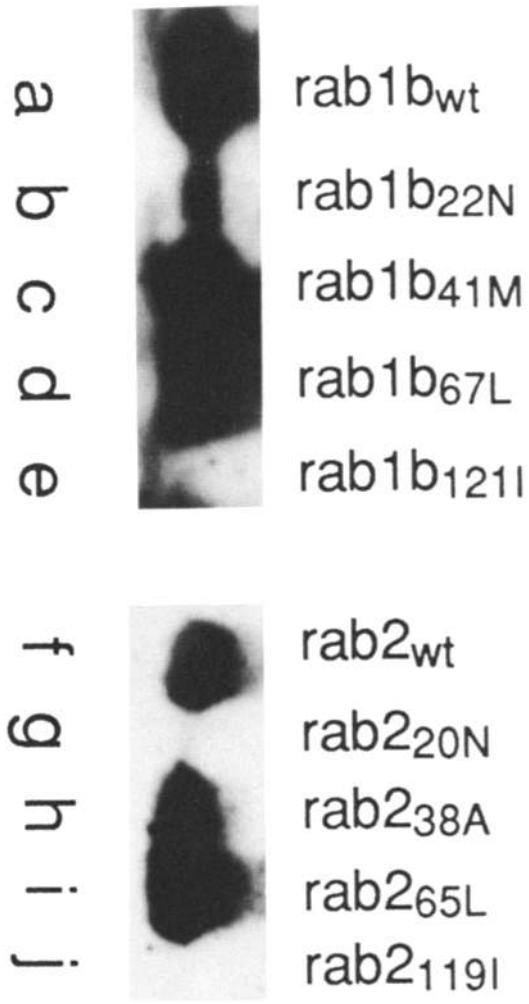


Figure 3. [$\gamma^{35}\Sigma$]GTP-binding to rabl and rab2 wild-type and mutant proteins after Western blotting. Equivalent amounts of the indicated wild-type and mutant proteins were bacterially expressed and separated by SDS-PAGE, then transferred to nitrocellulose filters. The filters were then incubated with 1 μM [γ^{35}S]GTP in 50 mM Tris, 0.3% Tween, 0.5 M EDTA, 0.5 M $MgCl_2$ and 10 μM of cold GTP (Schmitt et al., 1986). The bound [^{35}S]GTP was visualized by fluorography.

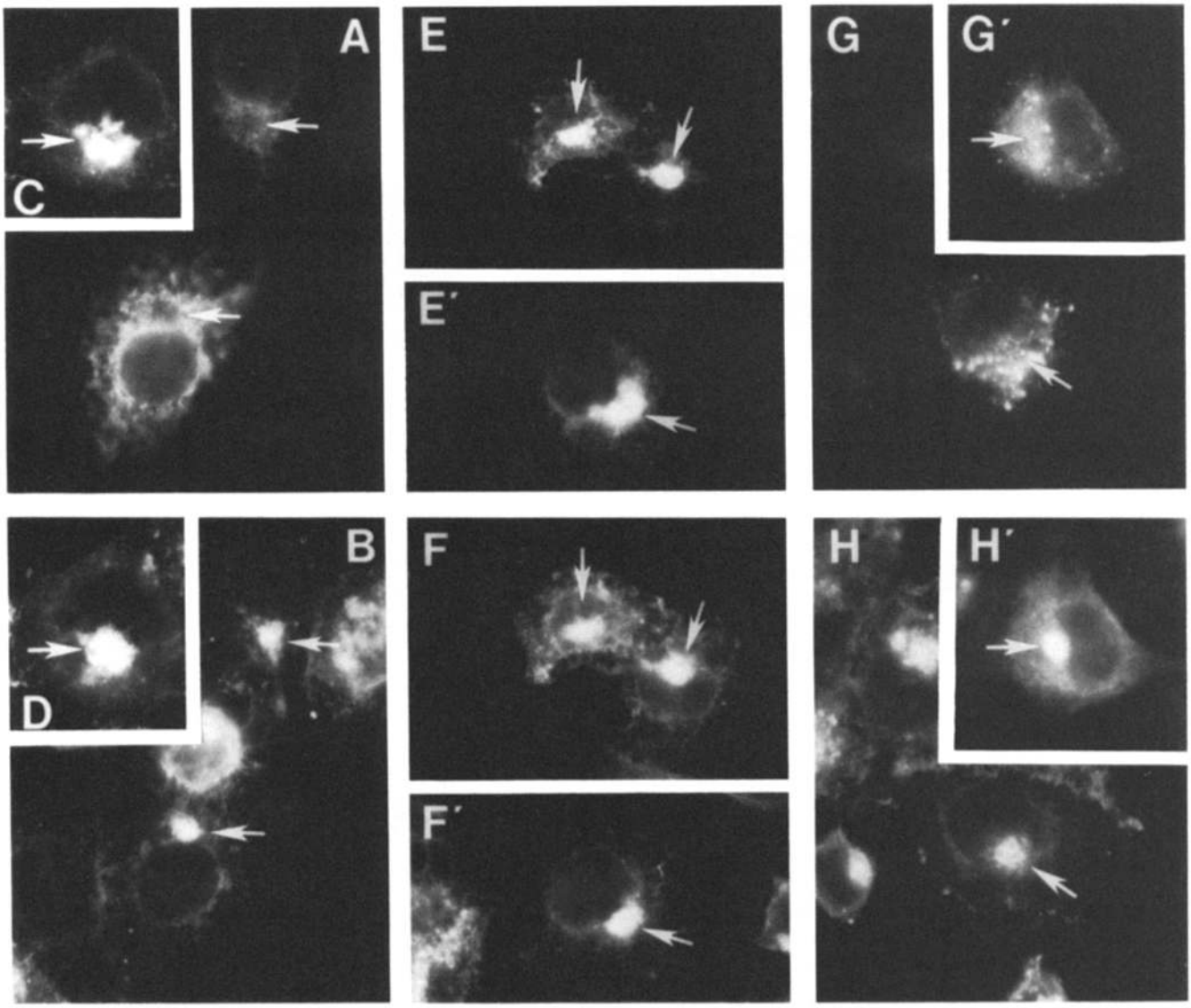
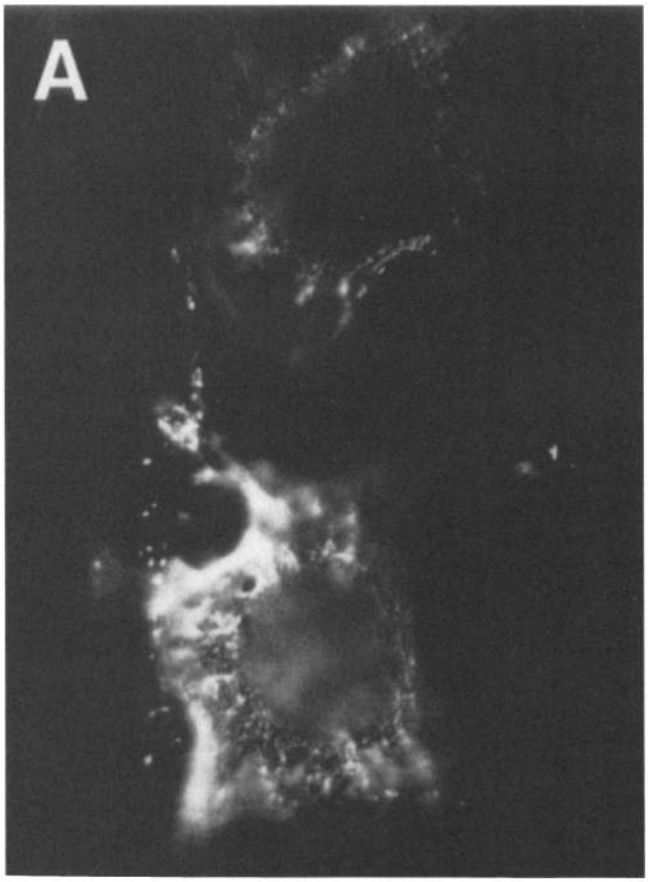
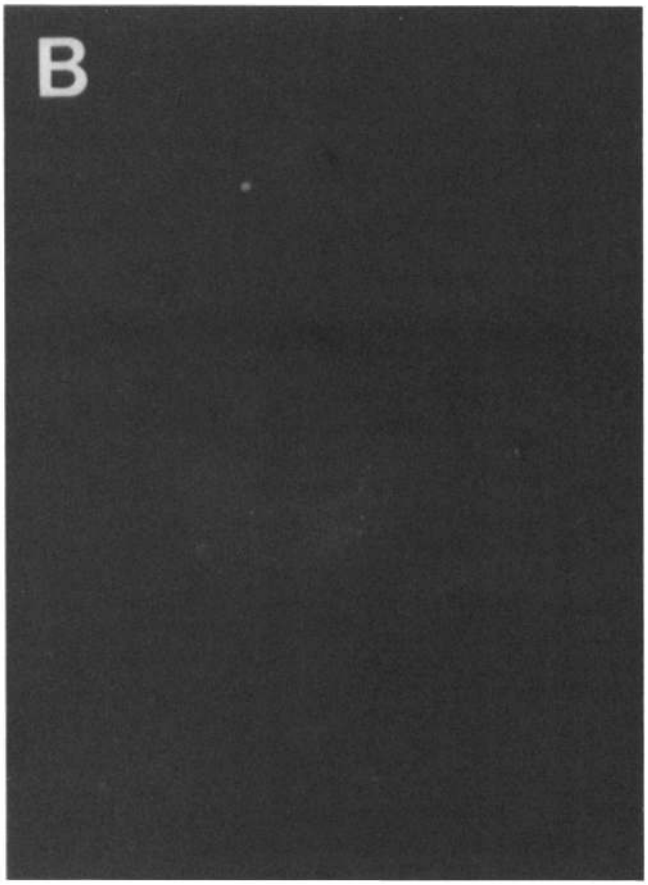


Figure 4. Rablb$_{12II}$ inhibits transport to the Golgi complex. Vaccinia infected cells (see Materials and Methods) were transfected with rablb or rablb$_{12II}$ cDNA, and a plasmid expressing the temperature-sensitive mutant form of VSV-G protein (pAR-tsO45-G) for 4 h at 39.5°C to retain tsO45 VSV-G in the ER. 4 h post-transfection, cells were shifted to the permissive temperature (32°C) and incubated for 2 h. Cells were fixed, permeabilized, and the distribution of tsO45 and VSV-G protein (*A*, *C*, *E*, *E'*, *G*, *G'*) and Golgi membranes (*B*, *D*, *F*, *F'*, *H*, *H'*) viewed by immunofluorescence microscopy as described in the Materials and Methods. (*A*, *B*) Distribution of tsO45 VSV-G (*A*) or Golgi membranes (*B*) after 6 h continuous incubation at 39.5°C. (*C*, *D*) Distribution of tsO45 VSV-G (*C*) or Golgi membranes (*D*) after incubation for 2 h at 32°C. (*E*, *F*) Distribution of tsO45 VSV-G (*E*, *E'*) or Golgi membranes (*F*, *F'*) after incubation for 2 h at 32°C in the presence of rablb protein. (*G*, *H*) Distribution of tsO45 VSV-G (*G*, *G'*) or Golgi membranes (*H*, *H'*) after incubation for 2 h at 32°C in the presence of rablb$_{12II}$ protein.

To further characterize the role of GTP-binding in the regulation of rabl function in ER to Golgi traffic, mutations were generated in two domains involved in either interaction with the phosphates or the guanine ring (Table 1). Rablb$_{22N}$ (Table 2) is equivalent to the ras$_{17N}$ mutation which exhibits a 20-fold reduced affinity for GTP but not GDP. It is thought that ras$_{17N}$ is locked in the GDP-bound form and to be associated constitutively with a ras guanine nucleotide dissociation stimulator (GDS) (Feig and Cooper, 1988). This mutant is a potent inhibitor of mammalian cell proliferation and believed to antagonize normal ras function by competition for GDS. As shown in Fig. 2 (lane *f*), expression of rablb$_{22N}$ (~10-fold) above the endogenous pool of rablb (data not shown) potently inhibited transport resulting in <2% of VSV-G processed to the endo H_R forms. In contrast, overexpression of rablb$_{67L}$ (Table 2), which in ras (ras$_{61L}$) eliminates the intrinsic and GAP-stimulated GTPase activities (Haubruck and McCormick, 1991), did not inhibit transport (Fig. 2, lane *h*, 85% endo H_R) when expressed >10-fold over the endogenous wild type protein based on Western blotting (data not shown). The equivalent mutation in the SEC4p (SEC4$_{79L}$) does not inhibit transport from the





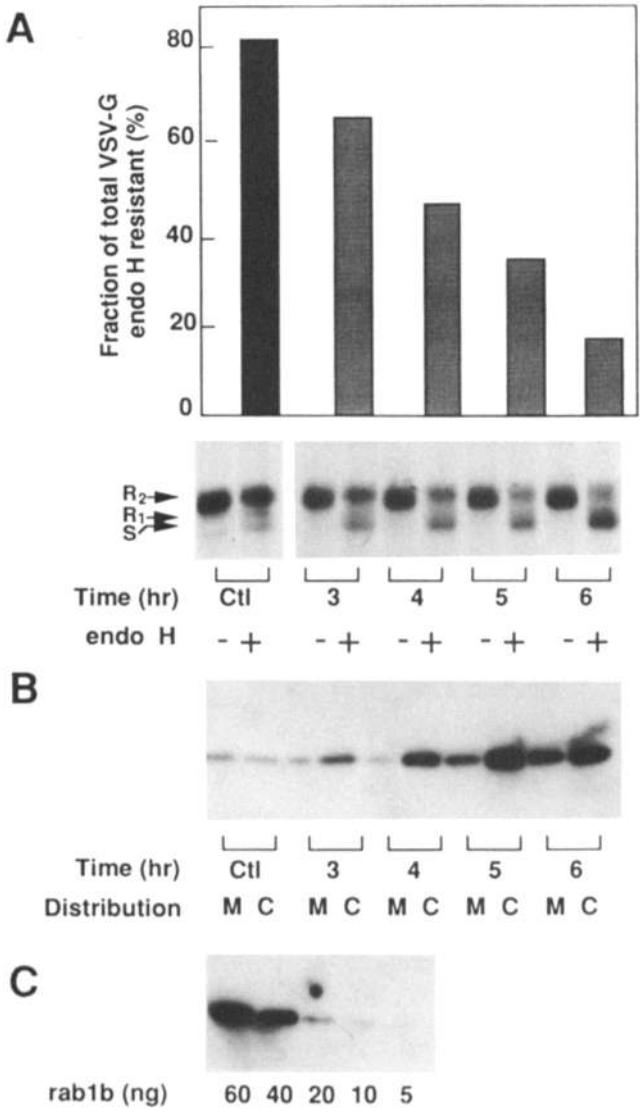


Figure 6. Relationship between rablb$_{12II}$ expression and inhibition of VSV-G transport from the ER to the Golgi complex. Infection/transfection conditions and analyses were as described in the legend of Fig. 3, and in Materials and Methods. (*A*) Extent of inhibition of VSV-G protein transport by rablb$_{12II}$ at 3, 4, 5, or 6 h post-transfection. (*B*) The level of expression of mutant rablb$_{12II}$ protein assessed by Western blotting after 3, 4, 5, or 6 h post-infection/transfection. Cytosolic fraction (*C*) and membrane fraction (*M*) were prepared as outlined in Materials and Methods. The control lane (*ctl*) shows the level of endogenous rablb. (*C*) The standard curve for rablb concentration was generated using purified recombinant rablb.

Figure 5. VSV-G protein is not delivered to the cell surface in HeLa cells expressing rablb$_{12II}$. Vaccinia infected cells (see Materials and Methods) were transfected with a plasmid expressing the temperature-sensitive mutant form of VSV-G protein (pAR-tsO45-G) in the absence (*A*) or presence (*B*) of the plasmid expressing rablb$_{12II}$ for 4 h at 39.5°C. After 4 h, cells were shifted to the permissive temperature (32°C) and incubated for 2 h. Cells were fixed and surface expression of tsO45 VSV-G protein determined by indirect immunofluorescence using an antibody specific for the extracellular domain of VSV-G as described in Materials and Methods.

Golgi to the cell surface in yeast (Walworth et al., 1989). As expected, rablb$_{22N}$ did not bind GTP after Western blotting whereas rablb$_{67L}$ GTP-binding was normal (Fig. 3, compare lanes *b* and *d*).

Rablb$_{121I}$ Lacking the Carboxyl-terminal CC Motif Is a Trans Dominant Inhibitor of Transport

Prenylation of the carboxyl-terminal cysteine residues of small GTP-binding proteins is required for membrane association and function (reviewed in Der and Cox, 1991) (Table 1). We have previously demonstrated that the carboxyl-terminal CC residues of rablb are post-translationally modified by a geranylgeranyl isoprenoid (Khosravi-Far et al., 1991). To assess the importance of this modification in rablb function, truncation (ΔCC) mutants were generated from the wild-type rablb and mutant rablb$_{121I}$ proteins. As shown in Fig. 7, deletion of the terminal CC residues from the wild-type protein had no effect on transport. VSV-G protein was efficiently processed to the endo H$_R$ form (Fig. 7, lane *d*, 65% endo H$_R$). Rablb$_{\Delta CC}$ bound GTP at levels comparable to the wild-type protein (not shown). In contrast, and to our surprise, the rablb$_{121I,\ \Delta CC}$ mutant inhibited ER to Golgi transport as efficiently as the nontruncated mutant (Fig. 6, lane *f*, <1% endo H$_R$) suggesting that a nonprenylated form of the mutant can effectively interfere with some aspect of the transport machinery governing rabl function.

To determine whether additional sequence information in the carboxyl-terminus of rablb was necessary to yield the trans dominant phenotype produced by the rablb$_{121I}$ mutant, we replaced the COOH-terminal 35 amino acids of rablb$_{121I}$ with the carboxyl-terminal 35 amino acids of rab5 to generate the hybrid construct rablb$_{(1-150)121I}$/rab5$_{(161-189)}$ (Table 2). The carboxyl-terminus of rab5 has been suggested to encode key information dictating the localization of the rab5 protein to an early endosome compartment (Chavrier et al., 1991). This mutant chimera binds GTP indicating that it folds properly to form a native structure (data not shown). In contrast to rablb$_{121I,\ \Delta CC}$, overexpression (~10-fold) of the hybrid (data not shown) had no effect on VSV-G transport (Fig. 7).

Rabl Effector Domain Mutations

Mutations in amino acids 32–40 of ras (the effector domain [Table 1]) do not, for the most part, alter the intrinsic properties of ras yet abolish transforming activity of the oncogenic forms (Hall, 1990; Haubruck and McCormick, 1991). Mutations in this region which destroy ras transforming activity typically eliminate the ability of GAP to bind efficiently to ras. Although the ras and rab effector domains are highly divergent, the 35T residue within the ras effector domain is invariant among all ras and ras-related proteins (Table 1) (Velencia et al., 1991; Wittinghofer and Pai, 1991). Substitution of 35T with A results in loss of ras transforming activity and prevents GAP stimulation of GTPase activity.

To determine whether mutations in the rablb effector domain would inhibit ER to Golgi transport, three different mutants were generated (Table 2): rablb$_{40A}$, rablb$_{41M}$ and rablb$_{44N}$. In the case of rablb$_{41M}$ GTP-binding is comparable to the wild-type protein (Fig. 3, lane *c*). Rablb$_{41M}$ and rablb$_{44N}$ are identical to YPTlp effector domain mutants recently reported to yield secretory defects in ER to Golgi transport in yeast (Becker et al., 1991). As shown in Fig. 8, we have detected weak, yet reproducible inhibition with rablb$_{41M}$ (Fig. 8, 50% endo H$_R$). This mutation in YPTlp yields the strongest *trans* dominant phenotype (Becker et al., 1991). In contrast, no inhibition was observed by overexpression of the rablb$_{40A}$ or rablb$_{44N}$ (Fig. 8). Because the 44N substitution in YPTl results in a temperature sensitive-phenotype (Becker et al., 1991) we tested whether a block in transport could be detected at an elevated temperature (39.5°C) in the presence of the rablb$_{44N}$ protein. As before, no inhibitory phenotype was detected (data not shown). In all cases, expression levels were ~10-fold that of the endogenous pool (data not shown). These results are compatible with the observation that effector domain mutations in mammalian ras are generally neither *trans* dominant nor growth inhibitory (Hall, 1990; Haubruck and McCormick, 1991).

Mutations in the GTP-Binding Domains of rab2 are Trans Dominant Inhibitors of ER to Golgi Transport

The rab2 protein, which is highly divergent from rablb (<40% identity), has been immunolocalized to the tubular-vesicular compartments believed to function in ER to Golgi

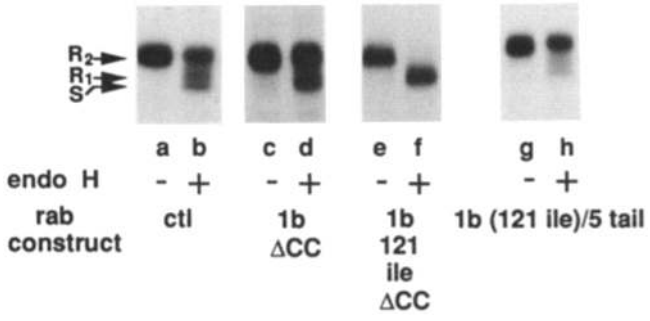


Figure 7. The effect of carboxyl-terminal mutations in rablb on VSV-G protein transport from the ER to the Golgi complex. Infection/transfection conditions and analyses were as described in Materials and Methods. Overexpression of the double mutant, rablB$_{121I,\ \Delta CC}$, resulted in a complete inhibition of VSV-G transport.

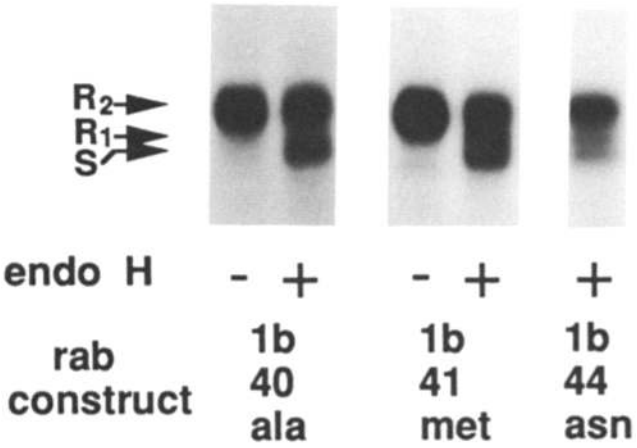


Figure 8. Effect of VSV-G transport in cells expressing mutations in the effector domain of rablb. Infection/transfection conditions and analyses were as described in Materials and Methods.

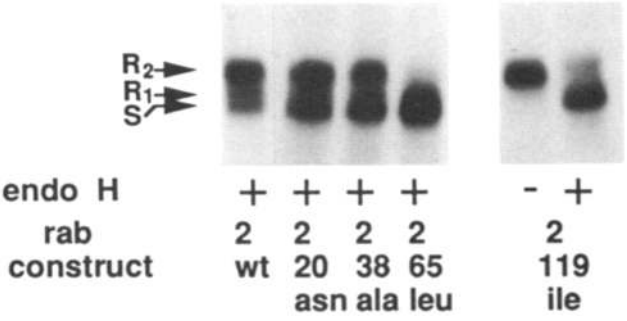


Figure 9. Evaluation of GTP-binding and effector domain mutants of rab2 on ER to Golgi transport of VSV-G protein. Infection/transfection conditions and analyses were as described in Materials and Methods.

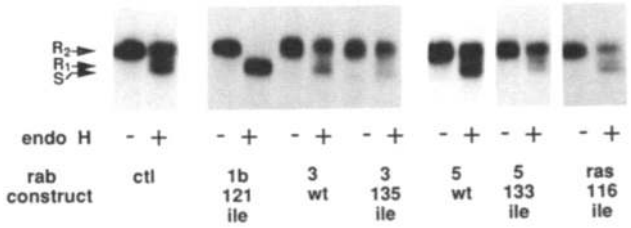


Figure 10. Substitution of 116I (ras numbering) in the GTP-binding domains of rab3a, rab5, and ras does not inhibit VSV-G protein transport from the ER to the Golgi complex when expressed in Hela cells. Infection/transfection conditions and analyses were as described in Materials and Methods.

transport (Chavrier et al., 1990*a*). However, no genetic or biochemical evidence is currently available which demonstrates its involvement in vesicular traffic.

To determine if rab2 functions in ER to Golgi transport, rab2 mutants were generated based on our observations for the rablb constructs (Table 2). We first tested $rab2_{119I}$ (equivalent to $rablb_{121I}$), a mutant which was likely to yield a *trans* dominant inhibitory phenotype. $Rab2_{119I}$ failed to bind GTP on Western blots (Fig. 3, lane *j*) and strongly inhibited protein transport from the ER to the Golgi complex (Fig. 9, <5% endo H resistant). In contrast, expression of rab2 wild-type protein neither stimulated nor inhibited the transport of VSV-G protein to the Golgi (Fig. 9, 65% endo H_R). Three additional mutants were generated (Table 2): 1) $rab2_{20N}$, analogous to the ras_{17N} mutation, 2) $rab2_{38A}$, an effector domain mutation, and 3) $rab2_{65L}$ corresponding to the oncogenic ras_{61L} mutation. We observed (Fig. 2, lane *f*) only a partial inhibition in membrane traffic by $rab2_{20N}$ (Fig. 9, 45% endo H_R), although it was expressed as efficiently as rabl proteins (~10-fold) and did not bind GTP after Western blotting (Fig. 3, lane *g*). Surprisingly, the $rab2_{65L}$ mutant was a potent inhibitor of transport (Fig. 9, <1% endo H_R) and bound GTP at levels comparable to wild-type (Fig. 3, lane *i*). The equivalent $rablb_{67L}$ had no effect on transport (Fig. 2, lane *h*). We have also consistently detected a weak, but reproducible inhibitory phenotype with the $rab2_{38A}$ effector domain mutation (Fig. 9, 40% endo H_R). As expected, GTP-binding by this mutant was comparable to wild type (Fig. 3, lanes *f* and *h*). The combined results suggest not only that rab2 is critical for ER to Golgi traffic, but the biochemical interactions facilitating rab2 function differ from those for rablb.

Overexpression of rab3a, rab5, rab6 Wild Type and GTP-Binding NKXD Mutant Proteins Have No Effect on Vesicular Traffic Between the ER and the Golgi Complex

To show that VSV-G protein transport inhibition between the ER and Golgi compartments is specific to those rab mutants described above, we expressed rab proteins which have been reported to participate in other steps of vesicular transport. To this end, we introduced the equivalent ras_{116I} mutation in rab3a, rab5, and rab6. Rab3 is involved in regulated exocytosis in endocrine, exocrine, and neural tissues and has been localized to exocytic vesicles (Darchen et al., 1990; Fisher von Mollard et al., 1990). Rab5 has been localized to endosomal compartments and is involved in early endosome fusion in vitro (Chavrier et al., 1991; Gorvel et al., 1991). The rab6 protein has been localized to the medial-*trans* compartments of the Golgi complex (Goud et al., 1990). As indicated in Fig. 10, overexpression (~10-fold) of the wild-type or mutant $rab3a_{135I}$, wild-type or mutant $rab5_{133I}$, or H-ras_{116I} had no statistically significant inhibitory or stimulatory effect on processing of VSV-G protein to the endo H_R form. In addition, we have been unable to detect any inhibitory effect of overexpression of $rab6_{126I}$ on processing of VSV-G to the terminally sialylated, complex form (Fig. 11). Given that sialyltransferase is a late-processing enzyme predominantly found in the *trans* Golgi compartment and trans Golgi network, these results suggest that rab6 is not essential for transport between *cis*, medial or *trans* Golgi compartments. Similarly, the inability of H-ras, rab3a, rab5 and rab6 NKXD mutant proteins to inhibit VSV-G transport between the ER and the Golgi complex indicates that these proteins do not interact with the biochemical machinery governing rabl or rab2 function.

Discussion

Disruption of ER to Golgi Transport by rabl and rab2 Mutations Affecting GTP Binding and Hydrolysis

Mutational analysis of ras and ras-related proteins has re-

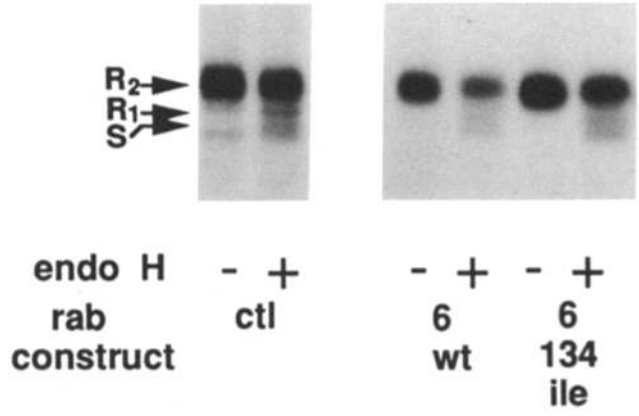


Figure 11. Expression of $rab6_{126I}$ does not inhibit VSV-G protein transport from the ER to the Golgi complex. Infection/transfection conditions and analyses were as in Materials and Methods.

vealed critical residues within conserved domains involved in GTP binding, exchange and hydrolysis required for function. We have used the structure/function data reported for the ras protein as our prototype to map functional domains in the rab proteins necessary for vesicular traffic from the ER to the Golgi complex.

Since guanine nucleotide binding regulates the biological activity of ras, we generated the N to I substitution in the consensus NKXD (ras residues 116-119) GTP-binding domain present in all ras-related proteins. While this substitution activates ras transforming potential (Der et al., 1988), analogous mutants of YPT1 (Schmitt et al., 1986; Schmitt et al., 1988), SEC4 (Walworth et al., 1989), and rab5 (Gorvel et al., 1991) exhibit *trans* dominant inhibitory phenotypes. To identify *trans* dominant inhibitors of ER to Golgi transport we made use of the T7 RNA polymerase produced by recombinant vaccinia virus to direct high level transient expression of rab proteins and VSV-G protein. In this system, $rablb_{121I}$ was a potent inhibitor of VSV-G protein transport from the ER to the Golgi complex based on the observation that the two *N*-linked oligosaccharides of VSV-G protein failed to be processed to early post-ER, *cis* Golgi forms (Schwaninger et al., 1991). Indirect immunofluorescence studies confirmed the biochemical results: VSV-G failed to enter the Golgi stack and was not detected on the cell surface. Thus, it is unlikely that the inability to trim VSV-G protein oligosaccharides to endo H_R forms is due to a processing defect per se or an "end-run" around the Golgi complex. The punctate intermediates in which VSV-G accumulated in the presence of the $rablb_{121I}$ mutant was strikingly similar to the distribution of a putative pre-Golgi intermediate compartment postulated to be involved in ER to Golgi transport (Saraste and Kuismanen, 1984; Saraste and Svensson, 1991; Schweizer et al., 1988). Although the data presented here does not directly distinguish between accumulation of VSV-G protein in a terminal export site (budding vesicle) associated with the ER network or accumulation in a downstream post-ER, pre-Golgi transport intermediate, our preliminary analysis using immunoelectron microscopy strongly suggests the latter interpretation (W. E. Balch and M. G. Farquhar, unpublished data). The identification of the intracellular compartment where transport is blocked by other *trans* dominant negative mutations is currently under investigation.

Overexpression of $rabla_{121I}$ yielded an identical biochemical phenotype to that observed for $rablb_{121I}$. Rabla and rablb are 95% identical, with the major differences being found in both conservative and nonconservative substitutions in the carboxyl-terminus (Zahraoui et al., 1989). Given the ability of mouse rabla to substitute for YPT1p in yeast (Haubruck et al., 1989), our results suggest that rabla and rablb proteins may be functionally interchangeable in mammalian cells. Redundancy in expression of highly-related isotypes is not unusual among members of the ras-superfamily of small GTP-binding proteins (Valencia et al., 1991).

Biochemical and mutational analyses of rab-related proteins found in the exocytic pathway of yeast (Schmitt et al., 1988; Bacon et al., 1989; Walworth et al., 1989; Baker et al., 1990; Kaiser and Schekman, 1990; Becker et al., 1991; Rexach and Schekman, 1991) supports our interpretation that rab1 is essential for ER to Golgi transport. Specifically, $YPT1_{121I}$ and $SEC4_{133I}$ do not bind GTP and are dominant-lethal mutants (Salminen and Novick, 1987; Walworth et al., 1989). Although the morphological and biochemical phenotype of $YPT1_{121I}$ in the secretory pathway of yeast has not been examined, temperature-sensitive mutants of YPT1 accumulate invertase and carboxypeptidase Y in the core-glycosylated pre-Golgi oligosaccharide forms, and accumulate ER and pre-Golgi transport vesicles in vivo and in vitro (Schmitt et al., 1988; Segev et al., 1988; Bacon et al., 1989; Walworth et al., 1989; Baker et al., 1990; Kaiser and Schekman, 1990; Becker et al., 1991). Likewise, $SEC4_{133I}$ leads to the accumulation of transport vesicles between the *trans* Golgi compartment and the cell surface (Walworth et al., 1989). These data support our biochemical observations and morphological studies suggesting that $rablb_{121I}$ expression results in the accumulation of VSV-G protein in pre-Golgi compartments. However, unlike the reversal of the dominant lethal phenotype observed by overexpression of wild-type protein on the negative effects of $SEC4_{122I}$ and $YPT1_{121I}$ in yeast (Schmitt et al., 1988; Walworth et al., 1989), overexpression of rablb wild type was unable to compete with $rablb_{121I}$. We suggest that $rablb_{121I}$ may inhibit transport by an irreversible binding to an effector molecule(s) required for rablb function.

The 116I (ras numbering) mutation in the NKXD domain of rab3a, rab5, rab6, and H-ras did not inhibit transport between the ER and Golgi membranes, consistent with their lack of activity in the early compartments of the secretory pathway. Although rab6 has been localized to medial/*trans* compartments of the Golgi complex (Goud and McCaffrey, 1991), overexpression of $rab6_{126I}$ failed to inhibit processing of VSV-G to the complex sialic acid containing structure. Transport between the medial and *trans* Golgi compartments is likely to involve vesicular carriers (Rothman, 1987). The inability of $rab6_{121I}$ to inhibit VSV-G transport suggests that rab6 does not regulate this step in the pathway. The combined results strengthen the general model that specific rab proteins participate in unique molecular interactions which regulate the function of different membrane-bound compartments in the endocytic and exocytic pathways. The analogous ras_{116I} substitution in different rab proteins will likely serve as a general and sensitive tool to define the role of uncharacterized rab proteins in endomembrane function.

In addition to the requirement for rab1, we have provided the first evidence that rab2 is essential for transport from the ER to the Golgi complex. Our data supports the observation that rab2 is localized to pre-Golgi tubular-vesicular elements believed to be involved in ER to Golgi transport (Chavrier et al., 1990a; Saraste and Kuismanen, 1984; Saraste and Svensson, 1991; Schweizer et al., 1988). The evolutionary divergence of rab2 from rab1 (<40% identity [Valencia et al., 1991]) suggests that a distinct collection of upstream and downstream effector proteins regulating guanine nucleotide-binding, hydrolysis and/or membrane-association exists for the rab2 protein. To date, a rab2 homologue has not been detected in yeast. Whether the mammalian and yeast pathways differ in this respect is not known.

Mutations in a second domain involved in GTP-binding (GXXXXGKS/T) were examined (Table 1, ras numbering 10–17). The 17N substitution in ras is thought to result in formation of a GDP-complexed form (Feig and Cooper, 1988) due to the tight-binding of a ras specific GDS to this mutant protein. Because this protein is essential for normal ras func-

tion, cells expressing this mutant are growth inhibited. While the rablb$_{22N}$ was a potent inhibitor of transport, the identical substitution in rab2 had a markedly reduced effect. We would propose that GDP/GTP exchange is critical for regulation of carrier vesicle function by rab 1. Although GDI and GDS proteins have now been identified for a number of rab and rab-related small GTP-binding proteins (reviewed in Takai et al., 1992) their role in transport and other cellular functions remains to be elucidated.

We also examined the function of the highly conserved DTAGZEX domain (Table 1, ras numbering 57–63) which interacts with the phosphoryl group (Table 1). In the case of ras, this domain is critical for GTPase activity (Hall, 1990) and insures specificity to ras-GAP (Maruta et al., 1991). Ras$_{61L}$ mutants have impaired intrinsic and GAP-stimulated GTPase activities which favor formation of the active, GTP-complexed form and results in transformation (Der et al., 1986). Whereas rablb$_{67L}$ did not inhibit transport, rab2$_{65L}$ was a potent *trans* dominant inhibitor of VSV-G protein transport. The failure of rablb$_{67L}$ to arrest transport mirrors the observation that a similar mutation in SEC4 (SEC4$_{79L}$) has no effect on vesicular transport from the *trans* Golgi to the cell surface in yeast (Walworth et al., 1989). In contrast, the inhibitory phenotype observed for rab2$_{65L}$ agrees with results reported for ras, suggesting that the inability to hydrolyze GTP or enhanced GAP-binding contributes significantly to transport inhibition. Why rab1 and rab2 differ in this respect is unknown. It may simply reflect differing roles of the GDP versus GTP bound forms in protein traffic. In this regard, the models that have been proposed to provide a general interpretation for rab protein function are likely to be oversimplified (Bourne, 1988). Clarification of these differences will require purification of the relevant proteins and characterization of their activities in vitro in the context of vesicular transport.

Mutations in the rab1 and rab2 effector domains (Table 1, residues 32–40 [ras numbering]) were generated to disrupt the interaction with putative downstream effector molecules. Although effector domain mutations in YPT1 show a strong *trans* dominant phenotype (Becker et al., 1991) and a marked reduction in GTPase stimulation by YPT1-GAP (Becker et al., 1991), identical mutations in rab1 or rab2 only partially inhibited transport. Mouse rabla can substitute for YPT1p function in yeast only when the protein is strongly overexpressed (Haubruck et al., 1989). The combined results suggest that high levels of expression of rabla in null mutants lacking YPT1 may be necessary to compensate for reduced affinity of rabla for yeast components rather than intrinsic stability.

Mutations in the Carboxyl-terminal Domain

Post-translational modifications of the carboxyl-terminal cysteine residues of ras and related small GTP-binding proteins are critical for function in vivo (reviewed in Maltese, 1990; Der and Cox, 1991). Due to the importance of prenylation for function of ras and ras-related proteins, we anticipated that the CC truncation would eliminate the *trans* dominant inhibition produced by rablb$_{121I}$ expression. Both the unprocessed (ΔCC) mutants of YPT$_{133I}$ and SEC4$_{133I}$ in yeast (Schmitt et al., 1984; Walworth et al., 1989), or unprocessed oncogenic RAS/ras mutants in yeast and mammalian cells lack *trans* dominant function (Der and Cox, 1991). To our surprise the double mutant, rablb$_{121I,\Delta CC}$ remained a potent inhibitor of transport. A reasonable explanation is that lack of prenylation may lead to phenotypically distinct results for rab and ras-related proteins which reflect differences in their biochemical interactions. In the case of rablb$_{121I}$, the unprenylated form may still participate in a subset of biochemical reactions characteristic of the wild-type protein. In support of this interpretation, at least 50% of the unprocessed form of rablb$_{121I,\Delta CC}$ sediments with membranes (W. E. Balch, unpublished observations). In this case, the rablb$_{121I}$ mutant may irreversibly bind a critical component(s) essential for transport leading to inhibition.

To gain insight into the role of additional residues in the carboxyl terminus for rab1 function, we generated the rablb$_{121I}$/rab5 chimera. At least 35 amino acids of the COOH-terminal tail of rab5 have recently been reported to be critical for localization of rab5 to the early endosome (Chavrier et al., 1991). A rab2 chimera containing the carboxyl-terminus of rab5 is found in the early endosome (Chavrier et al., 1991). Because the rablb$_{121I}$/rab5 chimera had no inhibitory effect on transport, our results indicate that the carboxyl-terminal tail of the rablb protein either contains essential sequence information facilitating its interaction with the biochemical machinery governing transport, or that mislocalization (i.e., the endosomal pathway) efficiently segregates the mutant from proteins involved in ER to Golgi trafficking.

Role of rab Proteins in Vesicular Trafficking Between the ER and Golgi Compartments

The mutational analysis of rab protein function is consistent with a number of biochemical probes we have developed to explore rab1 protein function in vitro. Recently we demonstrated that transport between the ER and the Golgi complex in vitro can be inhibited by a monoclonal antibody specific for rablb (Plutner et al., 1991). In the absence of rablb, VSV-G protein fails to exit the ER suggesting that rab1 or rablb-associated proteins are essential for vesicle formation (Plutner et al., 1992). Upon initiation of transport in vitro, VSV-G protein becomes rapidly transported to pre-Golgi intermediates enriched in rab1 (Plutner et al., 1992; Schwaninger et al., 1992). This rapid recruitment of rab1 coupled to the observation that the GDP-bound form of the SEC4 protein is required for membrane association (Kabcenell et al., 1990; Saski et al., 1991) suggests that the rablb$_{22N}$ mutant may inhibit transport by blocking the function of components involved in GDP/GTP exchange during an early step in vesicle formation. While the consequences of overexpression of the GDP-bound mutant form on cell morphology remain to be elucidated, an activated form, such as the rablb$_{121I}$ mutant may inhibit a later targeting or fusion step. This interpretation is supported by the observation that VSV-G protein accumulates in punctate vesicular structures which are reminiscent of pre-Golgi intermediates (Saraste and Svensson, 1991) in the presence of mutant protein in vivo, and in the presence of the rab3AL peptide in vitro (Schwaninger et al., 1992). Moreover, it is consistent with the observation that similar activating mutations in SEC4 (Walworth et al., 1989) or YPT1 (Segev et al., 1988; Bacon et al., 1989; Baker et al., 1990; Kaiser and Schekman, 1990; Rexach and Schekman, 1991) result in the accumulation of intermediate

transport vesicles in vivo and in vitro. The role of rab2 in transport is unknown. However, the observation that endocytosis involves at least two rab proteins, rab4 and rab5 (Gorvel et al., 1991; van der Sluijs et al., 1991) suggests that multiple rab proteins may regulate sequential or competing biochemical interactions between intracellular compartments.

Through examination of the biochemical and morphological consequences of expression of these and additional mutants on protein transport in vivo and in vitro we hope to gain insight into the role of rab1 and rab2 in ER to Golgi traffic.

We thank A. Tavitian, M. Zerial, J. Rose, C. Machamer and K. Nielson (Stratagene Corp.) for providing reagents used in these studies. TSRI manuscript number 7457-CB.

This research was supported by grants from the National Institutes of Health to W. E. Balch (GM33301, GM42336, and CA27489) and C. J. Der (CA42978, CA52072, CA55008). E. J. Tisdale is a recipient of a National Institutes of Health postdoctoral fellowship (CA 09270).

Received for publication 30 June 1992 and in revised form 28 August 1992.

References

Bacon, R. A., 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.

Baker, D., L. Wuestehube, R. Schekman, D. Botstein, and N. Segev. 1990. GTP-binding Ypt1 protein and Ca^{2+} function independently in a cell-free protein transport reaction. *Proc. Natl. Acad. Sci. USA.* 87:355–359.

Balch, W. E., M. M. Elliott, and D. S. Keller. 1986. ATP-coupled transport of vesicular stomatitis virus G protein between the endoplasmic reticulum and the Golgi. *J. Biol. Chem.* 261:14681–14689.

Balch, W. E. 1990. Small GTP-binding proteins in vesicular transport. *Trends Biochem. Sci.* 15:473–477.

Balch, W. E., R. A. Kahn, and R. Schwaninger. 1992. ADP-ribosylation factor (ARF) is required for vesicular trafficking between the endoplasmic reticulum (ER) and the *cis* Golgi compartment. *J. Biol. Chem.* 267:13053–13061.

Barbacid, M. 1987. ras genes. *Annu. Rev. Biochem.* 56:779–827.

Becker, J., T. J. Tan, H. Trepte, and D. Gallwitz. 1991. Mutational analysis of the putative effector domain of the GTP-binding Ypt1 protein in yeast suggests specific regulation by a novel GAP activity. *EMBO (Eur. Mol. Biol. Organ.) J.* 10:785–792.

Beckers, C. J. M., and W. E. Balch. 1989. Calcium and GTP: essential components in vesicular trafficking between the endoplasmic reticulum and Golgi apparatus. *J. Cell Biol.* 108:1245–1256.

Beckers, C. J. M., D. S. Keller, and W. E. Balch. 1987. Semi-intact cells permeable to macromolecules: use in reconstitution of protein transport from the endoplasmic reticulum to the Golgi complex. *Cell.* 50:523–534.

Bourne, H. R. 1988. Do GTPases direct membrane traffic in secretion? *Cell.* 53:59–61.

Bourne, H. R., D. A. Sanders, and F. McCormick. 1990. The GTPase superfamily: a conserved switch for diverse cell functions. *Nature (Lond.).* 348: 125–131.

Bourne, H. R., D. A. Sanders, and F. McCormick. 1991. The GTPase superfamily: conserved structure and molecular mechanism. *Nature (Lond.).* 349: 117–127.

Chardin, P. 1991. Small GTP-binding proteins of the ras family: a conserved functional mechanism? *Cancer Cells.* 3:117–126.

Chavrier, P., J.-P. Gorvel, E. Steizer, K. Simons, J. Gruenberg, and M. Zerial. 1991. Hypervariable C-terminal domain of rab proteins acts as a targeting signal. *Nature (Lond.).* 353:769–772.

Chavrier, P., R. G. Parton, H. P. Hauri, K. Simons, and M. Zerial. 1990*a*. 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. 1990*b*. Molecular cloning of YPT1/SEC4-related cDNAs from an epithelial cell line. *Mol. Cell. Biol.* 10:6578–6585.

d'Enfert, C., L. J. Wuestehube, T. Lila, and R. Schekman. 1991. Sec12p-dependent membrane binding of the small GTP-binding protein Sar1p promotes formation of transport vesicles from the ER. *J. Cell Biol.* 114:663–670.

Darchen, F., A. Zahraoui, F. Hammel, M.-P. Monteils, A. Tavitian, and D. Scherman. 1990. Association of the GTP-binding protein rab3a with bovine adrenal chromaffin granules. *Proc. Natl. Acad. Sci. USA.* 87:5692–5696.

Der, C. J., and A. D. Cox. 1991. Isoprenoid modification and plasma membrane association: critical factors for ras oncogenicity. *Cancer Cells.* 3:331–340.

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

Der, C. J., B. Weissman, and M. J. MacDonald. 1988. Altered guanine nucleotide binding and H-ras transforming and differentiating activities. *Oncogene.* 3:105–112.

Feig, L. A., and G. M. Cooper. 1988. Inhibition of NIH3T3 cell proliferation by mutated ras proteins with preferential affinity for GDP. *Mol. Cell. Biol.* 8:3235–3243.

Fisher von Mollard, G., G. Mignery, M. Baumert, M. Perin, T. Hanson, P. Burger, and J. R. Sudhöf. 1990. rab3 is a small GTP-binding protein exclusively localized to synaptic vesicles. *Proc. Natl. Acad. Sci. USA.* 87:1988–1992.

Fuerst, T. T., E. G. Niles, F. W. 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.

Gorvel, J. P., 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. *Cur. 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.

Hall, A. 1990. ras and GAP—Who's controlling whom? *Cell.* 61:921–923.

Haubruck, H., and F. McCormick. 1991. Ras p21: effects and regulation. *Biochim. Biophys. Acta.* 1072:215–229.

Haubruck, H., R. Prange, C. Vorgias, and D. Gallwitz. 1989. The ras-related mouse ypt1 protein can functionally replace the YPT1 gene product in yeast. *EMBO (Eur. Mol. Biol. Organ.) J.* 8:1427–1432.

Hicke, L., and R. Schekman. 1990. Molecular machinery required for protein transport from the endoplasmic reticulum to the Golgi complex. *BioEssays.* 12:253–258.

Horiuchi, H., M. Kawata, M. Katayama, Y. Hoshida, T. Musha, S. Ando, and Y. Takai. 1991. A novel prenyltransferase for a small GTP-binding protein having a C-terminal Cys-Ala-Cys structure. *J. Biol. Chem.* 266:16981–16984.

Kabcenell, A. K., B. Goud, J. K. Northrup, and P. J. Novick. 1990. Binding and hydrolysis of guanine nucleotides by Sec4p, a yeast protein involved in the regulation of vesicular traffic. *J. Biol. Chem.* 265:9366–9372.

Kahn, R. A., C. Goddard, and M. Newkirk. 1988. Chemical and immunological characterization of the 21-kDa ADP-ribosylation factor of adenylate cyclase. *J. Biol. Chem.* 263:8282–8287.

Kaiser, C. A., and R. Schekman. 1990. Distinct sets of SEC genes govern transport vesicle formation and fusion early in the secretory pathway. *Cell.* 61:723–733.

Khosravi-Far, R., R. Lutz, A. D. Cox, R. Clark, K. Kato, J. R. Bourne, P. J. Casey, M. Sinensky, W. E. Balch, J. E. Buss, and C. J. Der. 1991. Isoprenoid modification of rab proteins terminating in CC or CXC. *Proc. Natl. Acad. Sci. USA.* 88:6264–6268.

Kinsella, B. T., and W. A. Maltese. 1991. rab-GTP binding proteins implicated in vesicular transport are isoprenylated in vitro at cysteines within a novel carboxyl-terminal motif. *J. Biol. Chem.* 266:8540–8544.

Kohl, N. E., R. E. Diehl, M. D. Schaber, E. Rands, D. D. Soderman, B. He, S. L. Moores, D. L. Pompliano, S. Ferro-Novick, S. Powers, K. A. Thomas, and J. B. Gibbs. 1991. Structural homology among mammalian and Saccharomyces cerevisiae isoprenyl-protein transferase. *J. Biol. Chem.* 266:18884–18888.

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

Lafay, F. 1974. Envelope viruses of vesicular stomatitis virus: effect of temperature-sensitive mutations in complementation groups III and V. *J. Virol.* 14:1220–1228.

Maruta, H., J. Holden, A. Sizeland, and G. D'Abaco. 1991. The residues of ras and rap proteins that determine their GAP specificities. *J. Biol. Chem.* 266:11661–11668.

Melancon, P., B. S. Glick, V. Malhotra, P. J. Weidman, T. Serafini, M. L. Gleason, L. Orci, and J. E. Rothman. 1987. Involvement of GTP-binding "G" proteins in transport through the Golgi stack. *Cell.* 51:1053–1062.

Moores, S. L., M. D. Schaber, S. D. Mosser, E. Rands, M. B. O'Hara, V. M. Garsky, M. S. Marshall, D. L. Pompliano, and J. B. Gibbs. 1991. Sequence dependence of protein isoprenylation. *J. Biol. Chem.* 266:14603–14610.

Moss, B. 1989. Vaccinia virus vectors. *Ann. NY Acad. Sci.* 569:86–103.

Moss, B., B.-Y. Ahn, B. Amedgadzie, P. D. Gershon, and J. G. Keck. 1991. Cytoplasmic transcription system encoded by vaccinia virus. *J. Biol. Chem.* 266:1355–1358.

Nakano, A., and M. Muramatsu. 1989. A novel GTP-binding protein, Sar1p, is involved in transport from the endoplasmic reticulum to the Golgi apparatus. *J. Cell Biol.* 109:2677–2691.

Oka, T., S.-I. Nishikawa, and A. Nakano. 1991. Reconstitution of GTP-binding Sar1 protein function in ER to Golgi transport. *J. Cell Biol.* 114:671–679.

Patel, G., M. J. MacDonald, R. Khosravi-Far, M. M. Hisaka, and C. J. Der. 1992. Alternate mechanisms of ras activation are complementary and favor formation of ras-GTP. *Oncogene.* 7:283–288.

Plutner, H., H. Davidson, J. Saraste, and W. Balch. 1992. Morphological analysis of protein transport from the endoplasmic reticulum to Golgi membranes

in digotonin permeabilized cells: Role of the p58 containing compartment. *J. Cell Biol.* In press.

Plutner, H., A. D. Cox, S. Pind, R. Hosravi-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.

Plutner, H., R. Schwaninger, S. Pind, and W. E. Balch. 1990. Synthetic peptides of the Rab effector domain inhibit vesicular transport through the secretory pathway. *EMBO (Eur. Mol. Biol. Organ.) J.* 9:2375–2383.

Rexach, M. F., and R. W. Schekman. 1991. Distinct biochemical requirements for the budding, targeting, and fusion of ER-derived transport vesicles. *J. Cell Biol.* 114:219–229.

Rose, J. K., L. Buonocore, and M. A. Whitt. 1991. A new cationic liposome reagent mediating nearly quantitative transfection of animal cells. *Biotechniques.* 10:520–525.

Rothman, J. E. 1987. Transport of the vesicular stomatitis glycoprotein to the *trans* Golgi membranes in a cell-free system. *J. Biol. Chem.* 262:12502–12510.

Rothman, J. E., and L. Orci. 1992. Molecular dissection of the secretory pathway. *Nature (Lond.).* 355:409–415.

Salminen, A., and P. J. Novick. 1987. A ras-like protein is required for a post-Golgi event in yeast secretion. *Cell.* 49:527–538.

Saski, T., K. Kaibuchi, A. K. Kabcenell, and P. J. Novick. 1991. A mammalian inhibitory GDP/GTP exchange protein (GDP dissociation inhibitor) for smg p25A is active on the yeast SEC4 protein. *Mol. Cell. Biol.* 11:2909–2912.

Saraste, J., and E. Kuismanen. 1984. Pre- and post-Golgi vacuoles operate in the transport of Semliki Forest virus membrane glycoproteins to the cell surface. *Cell.* 38:535–549.

Saraste, J., and K. Svensson. 1991. Distribution of the intermediate elements operating in ER to Golgi transport. *J. Cell Sci.* 100:415–430.

Schmitt, H. D., M. Puzicha, and D. Gallwitz. 1988. Study of a temperature-sensitive mutant of the ras-related YPT1 gene product in yeast suggests a role in the regulation of intracellular calcium. *Cell.* 53:635–647.

Schmitt, H. D., P. Wagner, E. Pfaff, and D. Gallwitz. 1986. The ras-related YPT1 gene product in yeast: a GTP-binding protein that might be involved in microtubule organization. *Cell.* 47:401–412.

Schmitt, R. A., C. J. Schneider, and J. A. Glomset. 1984. Evidence for post-translational incorporation of a product of mevalonic acid into Swiss 3T3 cell proteins. *J. Biol. Chem.* 259:10175–10180.

Schwaninger, R., H. Plutner, G. Bokoch, and W. Balch. 1992. Multiple GTP-binding proteins regulate vesicular transport from the endoplasmic reticulum to Golgi membranes. *J. Cell Biol.* In press.

Schwaninger, R., C. J. M. Beckers, and W. E. Balch. 1991. Sequential transport of protein between the endoplasmic reticulum and successive Golgi compartments in semi-intact cells. *J. Biol. Chem.* 266:13055–13063.

Schweizer, A., T. Fransen, T. Baechi, L. Ginsel, and H.-P. Hauri. 1988. Identification, by a monoclonal antibody of 53-kD protein associated with a tubulovesicular compartment at the *cis*-side of the Golgi apparatus. *J. Cell Biol.* 107:1643–1653.

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.

Serafini, T., M. Amherdt, M. Brunner, R. A. Kahn, and J. E. Rothman. 1991. ADP-ribosylation factor is a subunit of the coat of Golgi-derived COP-coated vesicles: a novel role for a GTP-binding protein. *Cell.* 18:239–253.

Schmitt, H. D., P. Wagner, E. Pfaff, and D. Gallwitz. 1986. The ras-related YPT1 gene product in yeast: a GTP binding protein that might be involved in microtubule organization. *Cell.* 47:401–412.

Studier, F. W., A. H. Rosenberg, J. J. Dunn, and J. W. Dubendorff. 1990. Use of T7 RNA polymerase to direct expression of cloned genes. *Methods Enzymol.* 185:60–89.

Takai, Y., K. Kaibuchi, A. Kikuchi, and M. Kawata. 1992. Small GTP-binding proteins. *Int. Rev. Cyt.* 133:187–230.

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.* 30:4638–4648.

van der Sluijs, P., M. Hull, A. Zahraoui, A. Tavitian, and B. Goud. 1991. The small GTP-binding protein rab4 is associated with early endosomes. *Proc. Natl. Acad. Sci. USA.* 88:6313–6317.

Vos, J. C., and H. G. Stunnenberg. 1988. Derepression of a novel class of vaccinia virus genes upon DNA replication. *EMBO (Eur. Mol. Biol. Organ.) J.* 7:3487–3492.

Walworth, N. C., B. Goud, A. K. Kabcenell, and P. J. Novick. 1989. Mutational analysis of SEC4 suggests a cyclical mechanism for the regulation of vesicular traffic. *EMBO (Eur. Mol. Biol. Organ.) J.* 8:1685–1693.

Waters, M. G., I. C. Griff, and J. E. Rothman. 1991. Proteins involved in vesicular transport and membrane fusion. *Curr. Opin. Cell Biol.* 3:615–620.

Wittinghofer, A., and E. F. Pai. 1991. The structure of Ras protein: a model for a universal molecular switch. *TIBS.* 16:382–387.

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.