Rablb Regulates Vesicular Transport between the Endoplasmic Reticulum and Successive Golgi Compartments

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Abstract. We report an essential role for the *ras*-related small GTP-binding protein rablb in vesicular transport in mammalian cells. mAbs detect rablb in both the ER and Golgi compartments. Using an assay which reconstitutes transport between the ER and the *cis*-Golgi compartment, we find that rablb is required during an initial step in export of protein from the ER. In addition, it is also required for transport of protein between successive *cis*- and *medial*-Golgi compartments. We suggest that rablb may provide a common link between upstream and downstream components of the vesicular fission and fusion machinery functioning in early compartments of the secretory pathway.

TRANSPORT through the secretory pathway of eukaryotic cells involves the fission and fusion of carrier vesicles between the ER, Golgi cisternae, and the cell surface. Critical to our understanding of the basis for vesicular trafficking is providing a molecular explanation for the mechanisms which regulate vesicle fission and fusion, and ensure specificity to transport between distinct compartments. Genetic studies in yeast have identified a number of SEC gene products required for transport of protein between the ER and the Golgi complex, and between the Golgi complex and the cell surface (Novick et al., 1980; Kaiser and Schekman, 1990). One of the best studied of these proteins, NSF, which was first identified in mammalian cells and subsequently found to be homologous to the SEC18 gene product in yeast, is required at multiple steps in both the exocytic and endocytic pathways. It is therefore likely to be a component involved in the general fusion machinery (Wilson et al., 1989; Beckers et al., 1989; Diaz et al., 1989). In contrast, a new family of *ras*-related small GTP-binding proteins, which includes the YPT1 (Schmitt et al., 1988; Segev et al., 1988) and SEC 4 (Salminen and Novick, 1987) gene products in yeast, and the *rab* gene family in mammalian cells (Haubruck et al., 1987; Touchot et al., 1987; Zahraoui et al., 1989; Chavrier et al., 1990*a*,*b*), has been proposed to play a key role in targeting of carrier vesicles between specific compartments (Bourne, 1988). Consistent with this model, the YPT1 protein is required for transport between the ER and Golgi compartments (Schmitt et al., 1988; Segev et al., 1988; Bacon et al., 1989; Baker et al., 1990), whereas SEC4 is required for transport of protein from the *trans*-Golgi to the cell surface in yeast (Salminen et al., 1987; Walworth et al., 1989). In mammalian cells, at least 19 related members of the *rab* gene family have been identified (Zahraoui et al., 1989; Chavrier et al., 1990*b*). Of these, five have now been localized to the various compartments of the exocytic and endocytic pathways; rab2 to an intermediate compartment between the ER and the Golgi compartment (Chavrier et al., 1990*a*), rab6 to the *medial*- and *trans*-Golgi compartments (Goud et al., 1990), rab5 and rab7 to early and late endosomes, respectively (Chavrier, 1990*a*), and rab3a to synaptic vesicles (Fischer v. Mollard et al., 1990) (reviewed in Balch, 1990). In addition, we have shown that a synthetic peptide to the putative *rab* effector domain, analogous to the *ras* effector domain regulating GTP binding and hydrolysis (Pai et al., 1989, 1990; Milburn et al., 1990; Bourne et al., 1991), is a potent inhibitor of ER to Golgi and intra-Golgi transport in vitro (Plutner et al., 1990). These combined results reinforce the hypothesis that members of the *rab* family are key regulatory molecules involved in trafficking between compartments.

To further explore the functional role of small GTP-binding proteins in ER to Golgi trafficking we have generated both monoclonal and polyclonal antibodies to the rabl protein. Of the rab proteins, rabla and rablb have the most amino acid identity (66–75%) to YPT1, and are the likely mammalian homologues. Mouse rabla can replace YPT1 function in yeast (Haubruck et al., 1989). We now demonstrate that rablb is (*a*) morphologically distributed between both the ER and the Golgi compartments; (*b*) essential for an early step in vesicular transport between the ER and the Golgi compartments; and (*c*) required for transport between Golgi compartments. The surprising requirement for rablb activity in two different transport steps suggests that rablb may serve a key role in the assembly and disassembly of a common biochemical machinery involved in vesicle fission and fusion during early stages of the secretory pathway.

Materials and Methods

Materials

Semi-intact cells used for the analysis of ER to Golgi transport were prepared from wild-type or clone 15B CHO cells infected with either the wild-type or the tsO45 strains of vesicular stomatitis virus (VSV)[1] using the swelling method as described previously (Beckers et al., 1987). Tran[^{35}S]-label [^{35}S]methionine and [^{35}S]cysteine (>1,000 Ci/mmol) was purchased from ICN Biomedicals, Inc. (Irvine, CA). Cytosol used in transport assays was prepared from uninfected CHO wild-type cells as described previously (Beckers et al., 1987; Beckers and Balch, 1989). Endoglycosidase D (endo D) was obtained from Boehringer Mannheim Biochemicals (Indianapolis, IN) or prepared from the culture supernatant of *Diplococcus pneumoniae* (Glasgow et al., 1977). Clones for rat rablb, and human rab 2, rab 3A, and rab 6 were obtained from A. Tavitian (INSERM, Paris, France). The clones for canine rabla, and fusion proteins for canine rab4 and rab5 were obtained from M. Zerial (EMBL, Heidelberg, Germany). Rho protein was obtained from A. Hall, Chester Beatty Institute, London, England. Polyclonal anti-RER antibody and monoclonal anti-ribophorin II were provided by D. Meyer (University of California, Los Angeles, CA). An affinity-purified polyclonal antibody specific for α-1,6-sialyltransferase was obtained from J. Paulson (Cytel Corporation, La Jolla, CA). Two polyclonal serums for α-1,2-mannosidase II (Man II) were obtained from K. Moremen (Massachusetts Institute of Technology, Boston, MA). A polyclonal serum to protein disulfide isomerase (PDI) was provided by L. Gerace (Scripps Research Institute, La Jolla, CA). A polyclonal antibody to rab2 was obtained from I. Macara, University of Rochester, Rochester, NY. Anti-rablb Fab fragments were prepared from m4D3c by using papain coupled to agarose beads (Pierce Chemical Co., Rockford, IL) according to the manufacturer's directions with the modification that digestion was carried out in 25 mM HEPES (7.2), 125 mM KOAc, 10 mM cysteine-HCl, and 5 mM EDTA at a ratio of 5 μl packed beads to 125 μg antibody in a reaction volume of 100 μl for 2 h.

Expression of Recombinant Rab Proteins

The generation of bacterial expression constructs of each rab sequence, and the expression and purification of recombinant proteins are described in detail elsewhere (Khosravi-Far et al., 1991). Briefly, each rab cDNA was introduced into the pAR3040 (also designated pET-3a) bacterial expression vector and induced rab proteins were purified by the procedures described previously (Tucker et al., 1986; Zahraoui et al., 1989; Touchot et al., 1987). Rablb was purified to >95%. rab 4 and 5 induced cultures were lysed in SDS sample buffer and total bacterial extracts were resolved on SDS-PAGE.

Preparation of Antibodies

For the production of anti-rablb monoclonal antibodies, female Balb/C mice were immunized by an intraperitoneal injection of purified recombinant rablb protein in AluGel-S (Accurate Scientific, Westbury, NY). Typically, 100 μg of protein was diluted into PBS and combined with an equal volume of adjuvant just before use. Mice were boosted (intraperitoneal) after 2 wk, followed by a final intravenous injection at 3 wk with 50 μg of protein. Four days later, the animals were sacrificed and their spleens removed and dissociated to single cells for fusion using the SP-2/0-Ag14 mouse myeloma cell line (Schulman et al., 1978) (ATCC CRL1581). Hybridomas were plated in 96-well microtiter dishes, and positive cell lines (identified by an ELISA assay) were cloned in soft agar. Cells were adapted to serum-free medium (Excell; J. R. Scientific, Lenexa, KS) and the supernatant harvested and purified by ammonium sulfate precipitation. The ELISA assay was performed by binding of antigen (1 μg antigen/well in 50 μl of 50 mM Na_2CO_3 (pH 8.4)) to immunowells (Nunc, Naperville, IL) overnight. Wells were washed and blocked with 5% goat serum in PBS (PBS/Goat) for 60 min at 37°C. mAbs were added in PBS/Goat and incubated for 1–3 h at 37°C. Wells were washed three times with PBS/Goat followed by addition of alkaline phosphatase conjugated goat anti-rabbit or goat anti-mouse (Jackson Laboratory, Bar Harbor, ME) at 1:2,000 in PBS/Goat for 1 h, washed, and then alkaline phosphate substrate (104–105; Sigma Chemical Co., St. Louis, MO) was added in 50 mM Na_2CO_3 and 1 mM $MgCl_2$. Results were quantitated using a microplate reader (model 3550; Bio-Rad Laboratories, Cambridge, MA). Western blotting was performed by incubating the appropriate antibody in a buffer containing 25 mM Tris (8.0), 8 g/liter NaCl, 0.2 g/liter KCl, 0.05% Tween 20, and 5% nonfat milk, followed by washing and incubation in the presence of ^{125}I-protein A, and autoradiography.

1. *Abbreviations used in this paper*: endo D, endoglycosidase D; endo H, endoglycosidase H; Man I, α-1,2-mannosidase; Man II, α-1,2-mannosidase II; PDI, protein disulfide isomerase; RER, rough ER; SER, smooth ER; VSV, vesicular stomatitis virus.

Immunofluorescence

NRK cells were plated on round glass coverslips, and grown for 2 d before use. Fixation was in 2% paraformaldehyde in PBS for 15 min at room temperature. Fixation was quenched with 10 mM ammonium chloride in PBS for 10 min, blocked for 10 min with 5% goat serum in PBS (PBS/Goat), and permeabilized in the presence of freshly prepared 0.1% saponin. Cells were stained with specific antibody for 20 min in PBS/Goat, washed, and stained with FITC or TRITC goat anti-mouse or goat anti-rabbit affinity-purified reagents (Calbiochem, La Jolla, CA) at 1:50 in PBS/Goat. Coverslips were mounted in Moviol (Calbiochem) and viewed under a confocal scanning laser microscope (MRC-600; Bio-Rad Laboratories).

Incubation Conditions and Analysis of Transport

The ER to Golgi transport assays using clone 15B semi-intact cells infected with tsO45 VSV were performed as described previously (Beckers et al., 1987; Beckers and Balch, 1989; Plutner et al., 1990). For assays using wild-type cells infected with wild-type VSV, cells were labeled as described previously (Beckers and Balch, 1987; Schwaninger et al., 1991) except that the pulse of [^{35}S]methionine (150 μCi) was reduced to 3 min at 37°C before transfer of cells to ice and preparation of semi-intact cells. Briefly, transport incubations contained in a final total volume of 40 μl (final concentration): 25 mM Hepes-KOH (pH 7.2), 27 mM KOAc (wild-type cells), or 88 mM KOAc (15B cells), 2.5 MgOAc, 5 mM EGTA, 1.8 mM $CaCl_2$, 1 mM ATP, 5 mM creatine phosphate, 0.2 IU of rabbit muscle creatine phosphokinase, 25 μg cytosol, and 5 μl (25–30 μg of protein; 1–2 × 10^5 cells) of semi-intact cells. UDP-GlcNAc was added to a final concentration of 1 mM where indicated in the Results to detect the appearance of the endoglycosidase H (endo H)-resistant forms of VSV-G protein. Assays were supplemented with antibody reagents as indicated in the Results. Transport was initiated by transfer to 32°C in the case of semi-intact cells containing tsO45 VSV-G, and 37°C for semi-intact cells containing wild-type VSV. After termination of transport by transfer to ice, the membranes were pelleted by a brief (15 s) centrifugation in a microfuge at top speed. For analysis of processing of VSV-G protein to the man_5 form, the pellet was subsequently solubilized in an endo D digestion buffer and digested with endo D as described previously (Beckers et al., 1987). For endo H digestion, samples were pelleted and the material solubilized by the addition of 50 μl of 0.1% SDS in 100 mM NaOAc (pH 5.6) and boiling for 5 min. Samples were digested overnight at 37°C in the presence of 1 mU of endo H. Endo D and endo H digestions were terminated by adding a 5× concentrated gel sample buffer (Laemmli, 1970) and boiling for 5 min. The samples were analyzed by SDS-PAGE using 7% acrylamide gels (Beckers et al., 1987), autoradiographed, and the fraction of VSV-G protein processed to the endo D-sensitive or endo H-resistant forms was determined by densitometry (Beckers et al., 1987; Beckers and Balch, 1989).

Results

Rablb-specific Antibodies

To elucidate the potential role of the *rab* gene family in the regulation of transport between the ER and the Golgi compartments, we have generated both monoclonal and polyclonal antibodies to rabl expressed in bacteria. As summarized in Table I, all of the mAbs strongly recognized rablb, but did not cross react with other representative members of the *rab* gene family including rab2, rab3a, rab4, rab5, rab6, or other members of the *ras*-related superfamily including Ha-ras, rhoA, and rap (Downward, 1990; Bourne et al., 1991*a*,*b*). Two of the monoclonals, m1C10a and m3D7C, recognized both the rabla and rablb proteins (92% identity; Touchot et al., 1987), but with two- to threefold lower af-

Table I. Summary of Antibody Specificity

Antibody (class)	Specificity				Inhibition of transport*	
	rab1a	rab1b	rab2	Other‡	ER to Golgi compartment	Intra-Golgi compartment
p68	(+)§	+	(+)‖	–	++	++
m5F2a (IgM)	–	+	–	–	+	+
m3E8a (IgM)	–	+	–	–	–	–
m4D3c (IgG)	–	+	–	–	+++	+++
m5C6b (IgG)	–	+	–	–	++	++
m1C10a (IgG)	+	+	–	–	+	nd
m3D7c (IgG)	+	+	–	–	+	nd
m4G2b (IgG)	–	+	–	–	+	nd

* Transport inhibition reported on a relative scale in the presence of 5 μg monoclonal, or 10 μg polyclonal IgG: (–) no inhibition, (+) <25% inhibition, (++) >50% inhibition, (+++) >80–90% inhibition.
‡ Cross-reactivity tested against Ha-ras, rho, rap, and rab3a, 4, 5, 6.
§ Detects recombinant rab1a with ~10-fold less sensitivity than recombinant rab1b.
‖ Detects recombinant rab2 with ~100-fold less sensitivity than recombinant rab1b.

finity for rabla compared to rablb. The polyclonal antibody p68 was also found to detect the rabla protein (~10-fold lower affinity) and the rab2 protein (~100-fold lower affinity) (Fig. 1 *A*). The mAb m4D3c did not recognize any of the rab proteins on Western blots, but was specific for the rablb protein based on ELISA results (data not shown). It is likely that this antibody recognizes a conformation specific epitope present on the native protein. A typical pattern of cross-reactivity for the mAb m5C6b and polyclonal antibody p68 with different rab proteins or with related families of the *ras*-superfamily of small GTP-binding proteins is shown in Fig. 1 *A*.

Western blot analysis showed that antibodies p68 and m5C6B react with a protein which co-migrates with rabl in a number of cell lines including mouse NIH-3T3 fibroblasts, NRK cells, and CHO cells (Fig. 1 *B*).

Rablb Is Found in Both the ER and Golgi Compartments

YPT1 (Segev et al., 1988) and SEC4 in yeast (Goud et al., 1988) and rab proteins in mammalian cells (Chavrier et al., 1990*a*; Goud et al., 1990; Fischer v. Mollard et al., 1990) have distinctive subcellular distributions likely to reflect their potential role in the regulation of vesicular trafficking between different compartments of the secretory pathway (Balch, 1990). We used indirect immunofluorescence and confocal microscopy on NRK cells to assess the distribution of the rablb protein in exocytic organelles. The distribution detected by the mAbs comprised two morphological classes. As shown in Fig. 2, *A* and *B*, antibodies of class I, which are represented by the distribution patterns observed with m5F2a and m3E8a, detected rablb exclusively in an extensive reticular network. The distribution colocalized with proteins recognized by a polyclonal reagent directed against total rough ER (RER) membranes (Fig. 2), and other ER specific markers including ribophorin I and PDI (data not shown). The Golgi region, as identified by the distribution of α-1,2-mannosidase II (Man II) (for example, see Fig. 2 *C*) lacked detectable rablb using class I antibodies. In contrast, class II antibodies (represented by m4D3c and m5C6b) detected rablb in both the ER and the Golgi compartments (Fig. 2, *C* and *D*), similar to that observed with the p68 antibody (data not shown). Preincubation of antibodies with recombinant rablb, but not with other rab proteins, inhibited the observed patterns of fluorescence. Similar distributions for each class of antibodies were observed with Hela, NIH-3T3 and BHK cell lines (data not shown).

Rablb Distributes with the ER and Golgi Compartments in Rat Liver Membrane Fractions

To provide additional evidence that the rablb protein is abundant in both the ER and Golgi compartments, rat liver was subfractionated into nuclear, RER, smooth ER (SER), Golgi compartments, and other subcellular fractions (Fleischer and Kervina, 1974). Using the p68 antibody (Fig. 3 *A*) or m5C6b (Fig. 3 *B*), a protein co-migrating with rablb was detected in both the Golgi and SER fractions. In addition, this protein was detected at low concentration in the cytosol, nuclear, and RER fractions (Fig. 3).

As shown in Fig. 3 *B*, analysis of Western blots using antibodies specific for the Golgi marker proteins α-1,2-mannosidase II (Man II) and sialyltransferase (Sialyl-Tr) indicated that <1% of the total rablb in the SER fraction could be accounted for by Golgi contamination. Conversely, using antibodies to the resident ER proteins ribophorin I and PDI, shows that the Golgi fraction is minimally contaminated with ER membranes (Fig. 3 *B*). Quantitative analysis of these immunoblots indicates that while the Golgi fraction contains a two- to threefold higher specific concentration (per mg protein), the SER fraction contains nearly 70–80% of the total rablb protein in liver homogenates, with <5% localized to the cytosolic, nuclear, and RER fractions.

Rablb Colocalizes with rab2, a Marker for an Intermediate Compartment between the ER and the Golgi

Given the apparent distribution of rablb to both ER and Golgi compartments, we examined whether the rablb protein colocalizes with rab2, a small GTP-binding previously demonstrated to colocalize with p53 (Chavrier et al., 1990*a*), a marker protein for a tubular-vesicular compartment prevalent at the *cis* face of the Golgi compartment (Schweizer et al., 1988, 1990, 1991). A number of lines of evidence suggest that transport of protein from the ER to the Golgi compartment involves transfer of itinerant protein through this

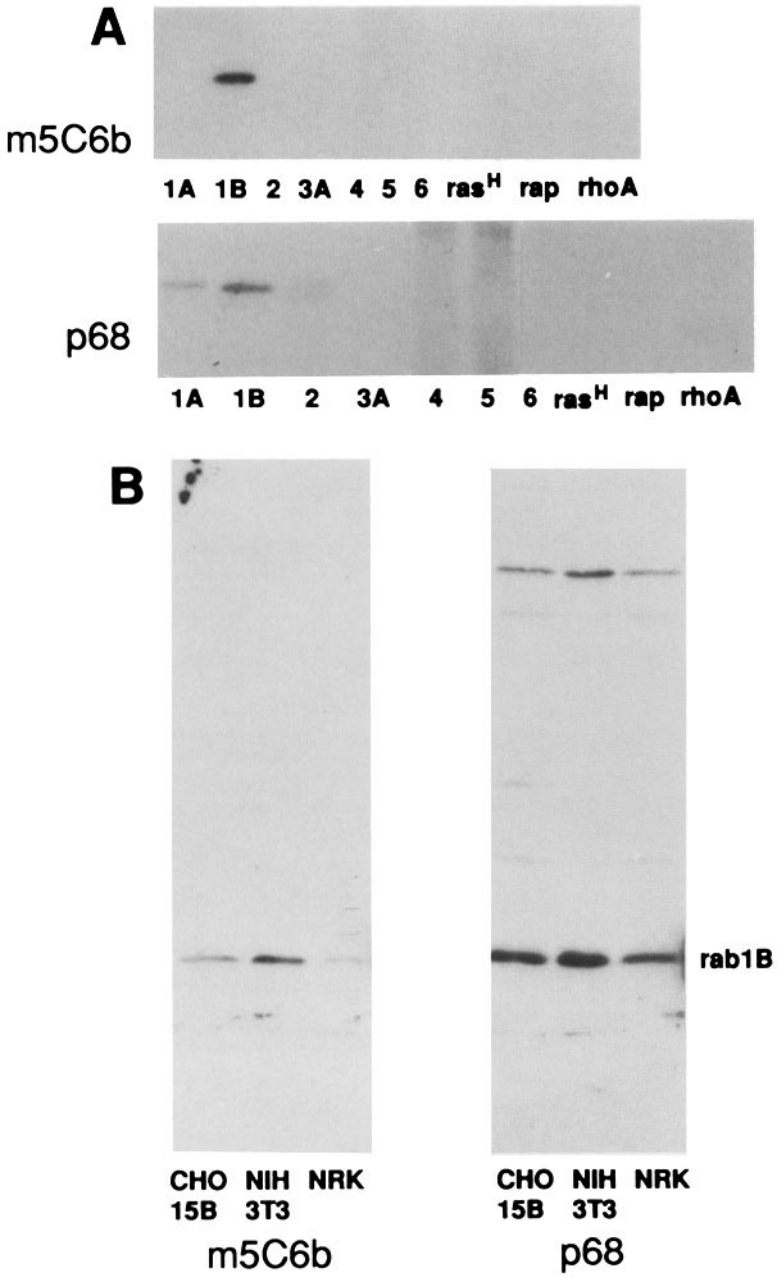


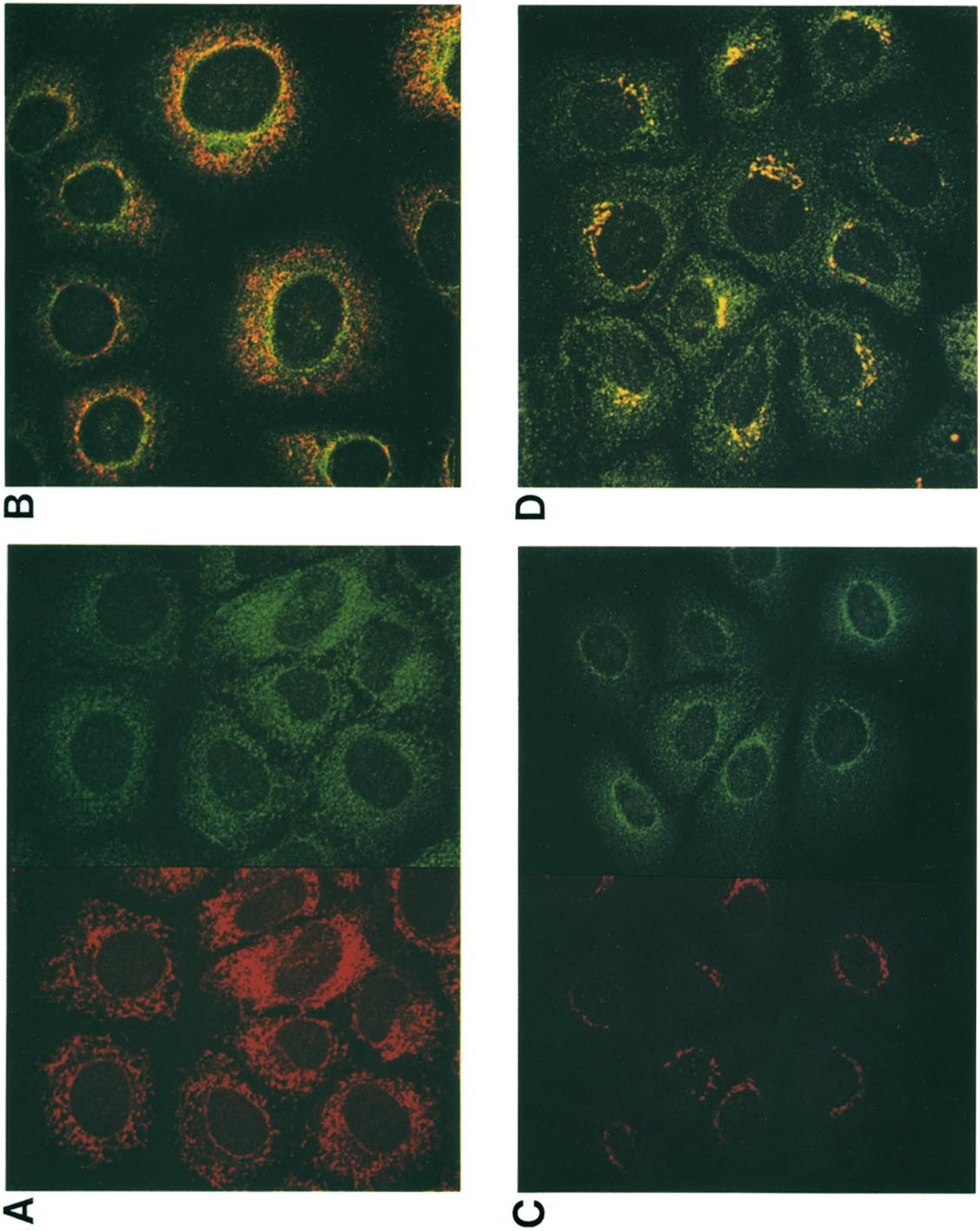
Figure 1. Specificity of p68 and m5C6b for members of the *rab* and *ras*-superfamily of small GTP-binding proteins. (*A*) 100 ng of the indicated recombinant proteins, or bacterial extracts expressing recombinant fusion proteins (rab 4 and 5), were probed on Western blots with the polyclonal antibody p68, or the mAb m5C6b as described in the Materials and Methods. (*B*) 25 μg (1–2 $\times$ 10^5 cells) of the indicated cell line (see Results) were probed with p68 or m5C6b.

pre-Golgi reticulum (Schweizer et al., 1990; Saraste and Kuismanen, 1984; Saraste et al., 1986). As shown in Fig. 4, using indirect immunofluorescence, rablb was found to colocalize with rab2 in a prominent reticular pre-Golgi region characteristic of the intermediate compartment. These results suggest that the rablb protein is prevalent throughout the early compartments of the secretory pathway, its morphological distribution suggestive of its potential role in regulation of ER and Golgi protein transport.

Transport between the ER and the Golgi Compartments Is Inhibited by Anti-rablb Antibodies

While the distribution of rablb in both the ER and Golgi compartments is consistent with its potential role in vesicular trafficking between these compartments, a strong test of this hypothesis would be to demonstrate that rablb specific antibodies inhibit ER to Golgi transport. We have developed an

Figure 2. Rablb is found in both the ER and Golgi compartments. Rablb distribution was detected using indirect immunofluorescence and confocal microscopy as described in Materials and Methods. (*A*) Codistribution of the anti-RER antibody (rhodamine, left panel) with the class I antibody m5F2a (fluorescein, right panel), or Panel B: A merge of confocal data sets showing co-localization of anti-RER and m5F2a antibodies. Co-localization is indicated by the yellow to orange color. Panel C: Co-distribution of α-1,2-mannosidase II (Man II) a *cis/medial* Golgi marker enzyme (rhodamine, left panel) with the class II antibody m5C6b (fluorescein, right panel). (Panel D) A merge of confocal data sets showing co-localization of Man II and m5C6b in the *cis/medial* Golgi compartments.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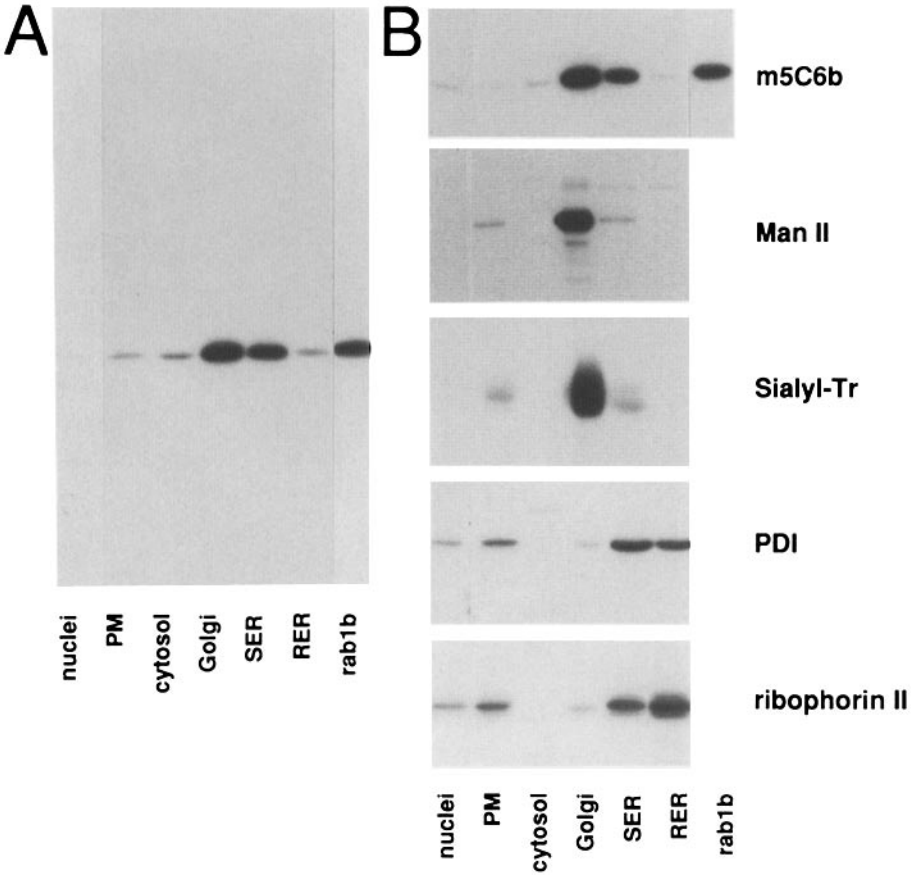

Figure 3. Distribution of rablb to rat liver fractions. Rat liver fractions were prepared as described in Materials and Methods. Western blots using 50 μg of total protein from each fraction were probed with the indicated antibody as described in Materials and Methods. (*A*) Rat liver fractions probed with p68. (*B*) Rat liver fractions probed with m5C6b, Man II, sialyltransferase (*Sialyl-Tr*), protein disulfide isomerase (*PDI*), or ribophorin II.

in vitro assay which reconstitutes the specific transport of protein from the ER to the *cis*-Golgi compartment using semi-intact cells, a population of cells in which the plasma membrane has been perforated to expose intact ER and Golgi structures (Beckers et al., 1987, 1990; Beckers and Balch, 1989). In this assay, the transport of the surface glycoprotein of VSV (VSV-G protein) is measured by following the trimming of the two high mannose (man_9) asparagine-linked oligosaccharides acquired during nascent synthesis in the RER, to the man_5 oligosaccharide form as a consequence of transport of VSV-G to the *cis*-Golgi and processing by α-1,2-mannosidase I (Man I) (Beckers et al., 1987). To focus exclusively on ER to *cis*-Golgi transport, our assay uses a thermoreversible mutant form of VSV-G protein (VSV strain tsO45) (Lafay, 1974) which is defective in transport at the restrictive temperature (39.5°C), but highly efficient at the permissive temperature (32°C), allowing us to prepare semi-intact cells with VSV-G protein exclusively in the ER before initiation of transport (Beckers et al., 1987). Transport of VSV-G in these semi-intact cells requires energy in the form of ATP and soluble cytosolic factors released during preparation of semi-intact cells. Transport is potently inhibited by GTPγS (Beckers and Balch, 1989) and by a synthetic peptide homologous to the putative rab protein effector domain, a key switch region likely to regulate GTP binding and hydrolysis through interaction with upstream or downstream effectors (Plutner et al., 1990).

To test for rablb function in ER to Golgi transport, a complete assay containing semi-intact cells, cytosol, and ATP was incubated for 60 min on ice in the presence of the monoclonal or polyclonal antibodies before initiation of transport by incubation at 32°C for 90 min. As shown in Fig. 5 *A*, strong inhibition (>90%) of transport was observed for p68, m4D3c, and m5C6b; less inhibition (<50%) was observed for m5F2a, m4G2b, m1C10a, and m3D7a; while no inhibition of transport was observed for m3E8a. The strongest neutralizing antibody, m4D3c, inhibited transport by 50% at concentrations of <0.5 μg (Fig. 5 *B*). To insure that inhibition by m4D3c was not because of membrane aggregation in the presence of divalent antibodies, the effect of monovalent Fab fragments was tested. Addition of increasing concentrations of Fab fragments mirrored the inhibition observed with intact divalent antibody. However, proportional inhibition required a 10-fold molar increase in Fab fragment concentration over that observed for the divalent antibody (data not shown). Inhibition of transport by m4D3c was blocked by preincubation with recombinant rablb (Fig. 6 *A*, lane *e*), but not rabla or other rab and *ras*-related proteins (data not shown).

To determine whether the functional pool of rablb protein is present in the membrane containing semi-intact cells or the soluble cytosolic pool (cytosol) provided by a high-speed supernatant prepared from CHO cells, semi-intact cells and cytosol were pretreated separately with m4D3c for 60 min on ice before the addition of the untreated fraction (cytosol or semi-intact cells), ATP, and incubation at 32°C for 90 min. As shown in Fig. 6 *A* (lane *f*), pretreatment of the semi-intact cells alone was sufficient to cause inhibition of transport. Pretreatment of membranes on ice was found to be essential for inhibition as the addition of antibody immediately

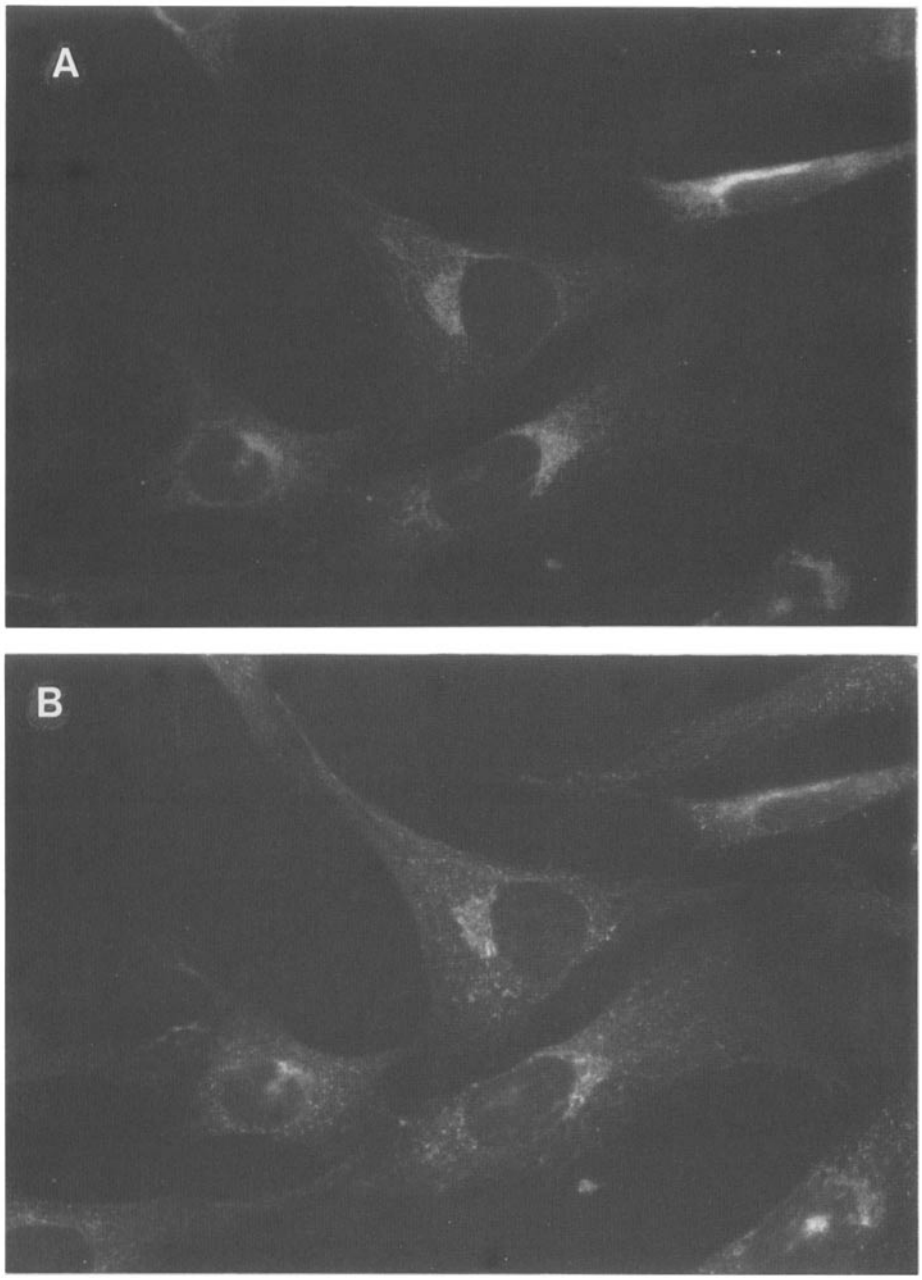

Figure 4. Colocalization of rablb and rab2. Rablb and rab2 distribution was determined using indirect immunofluorescence as described in Materials and Methods. Panel A shows the distribution of rablb, panel B the distribution of rab2.

prior to incubation results in significantly reduced level of inhibition (Fig. 6 *A*, lane *d*). In contrast, pretreatment of CHO cytosol had only a minor effect on the observed efficiency of transport (Fig. 6 *A*, lane *h*), suggesting that the functional pool of rablb required for ER to Golgi transport is at least initially associated with the membrane fraction present in semi-intact cells.

Although pretreatment of m4D3c with *Escherichia coli* recombinant rablb efficiently neutralized the ability of m4D3c to inhibit transport (Fig. 6 *A*, lane *d*), supplementation of the assay with recombinant rablb did not stimulate transport in semi-intact cells which had been pretreated with antibody (Fig. 6 *B*, lane *a*). Since rablb expressed in bacteria lacks posttranslational modifications including carboxyl-terminal prenylation and carboxy-methylation (Khosravi-Far et al., 1991) likely to be essential for membrane association and function, we tested whether a native, membrane-associated form of rablb protein present in CHO cells could stimulate transport in antibody inhibited cells. As shown in Fig. 6 *B* (lane *b*), addition of untreated CHO membranes to semi-intact cells pretreated with m4D3c for 60 min on ice efficiently stimulated transport. This *trans* complementation was abolished by pretreating these membranes with m4D3C (Fig. 6 *B*, lane *c*), suggesting that a functional, recycling pool of rablb was provided by the addition of untreated membranes.

While the inhibition of ER to Golgi trafficking suggests that rablb plays a key role in regulation of transport at this stage of the secretory pathway, localization of rablb to the Golgi apparatus led us to test whether intra-Golgi transport also required the function of the rablb protein. For this purpose, Golgi fractions which efficiently reconstitute transport between the *cis*- and *medial*-Golgi compartments (Balch et al., 1984) were incubated in vitro in the presence or absence

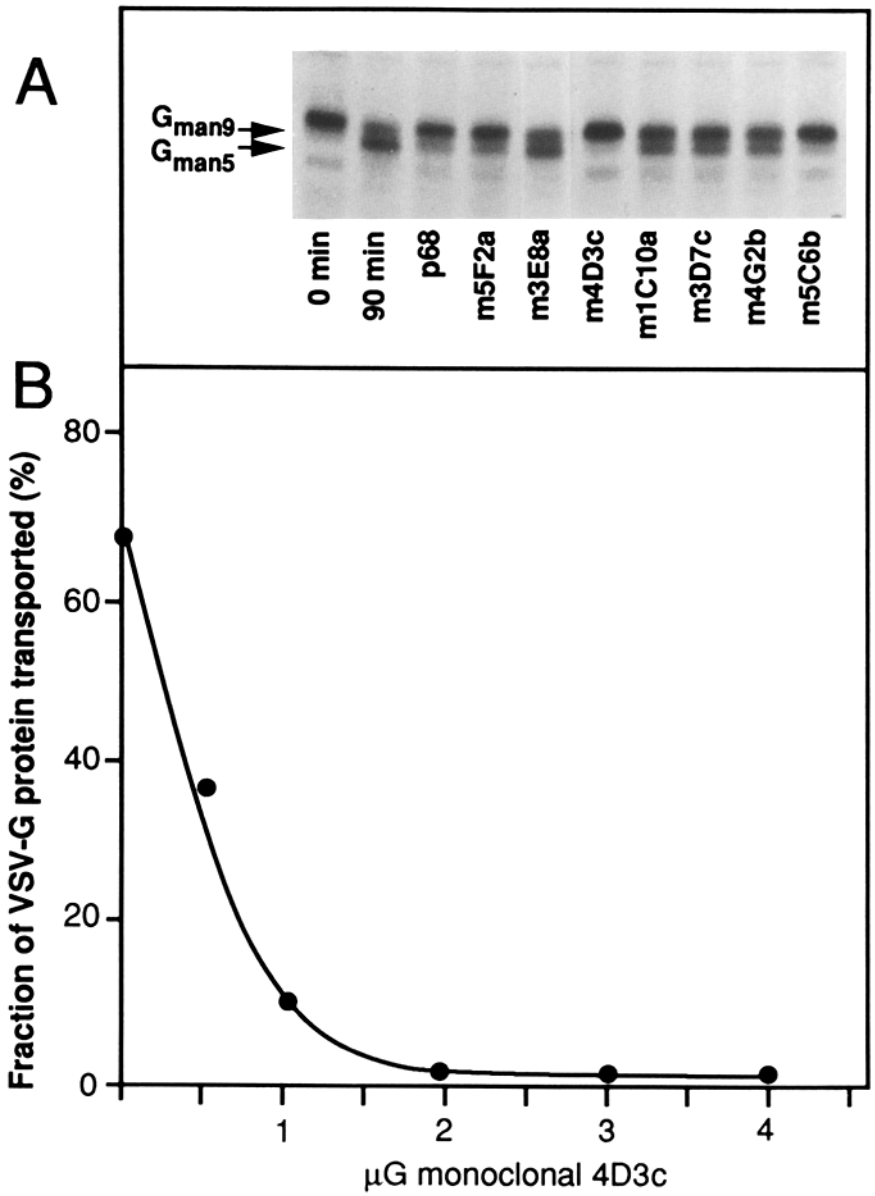

Figure 5. Inhibition of ER to Golgi transport in semi-intact cells. (*A*) Semi-intact cells (*SIC*) and cytosol were pretreated for 60 min on ice with 5 μg of the indicated antibody before incubation for 90 min at 32°C in the presence of ATP as described in Materials and Methods. The upper band (G_{man9}) is the oligosaccharide-containing form of VSV-G protein found in the ER; the lower band (G_{man5}) is the form of VSV-G protein which has been processed by the *cis*-Golgi enzyme man I, indicating transport in vitro to the Golgi compartments. (*B*) As indicated in *A*, except semi-intact cells and cytosol were pretreated for 60 min on ice with the indicated concentration of m4D3c before incubation for 90 min at 32°C.

of m4D3c. As shown in Fig. 7 *A*, vesicular trafficking between the *cis*- and *medial*-Golgi compartments as measured by the coupled incorporation of ^{3}H-*N*-acetylglucosamine (^{3}H-GlcNAc) was inhibited when Golgi membranes were pretreated with m4D3c for 60 min on ice, but not inhibited when the antibody was neutralized by preincubation with rablb.

Sequential transport of wild-type VSV-G from the ER through the *cis*- and *medial*-Golgi compartments in semi-intact cells was also found to be sensitive to antibody. We can readily detect intra-Golgi transport in semi-intact cells by quantitating the sequential processing of the two VSV-G protein oligosaccharides to the endo H-resistant structures (Schwaninger et al., 1991). Transport from the ER to the *cis*-Golgi compartment results in the appearance of the G_{H1} form of VSV-G containing one endo H-resistant oligosaccharide and occurs with a $t_{1/2}$ of ~20 min (Fig. 7 *B*, ■). Transport to the *medial*-Golgi compartment results in the appearance of the G_{H2} form ($t_{1/2}$ of ~75 min) containing two endo H-resistant oligosaccharides (Fig. 7 *B*, ●). When antibody was added before incubation, transport of wild-type VSV-G from the ER through the *cis*-Golgi compartment (as indicated by the appearance of the total G_{H1} + G_{H2} forms (Fig. 7, Δt = 0 min, □) was only partially inhibited. This result contrasts with transport of tsO45 VSV-G protein from the ER to the *cis*-Golgi which is completely sensitive to antibody (Fig. 6 *A*; data not shown). Since wild-type VSV-G, unlike tsO45 VSV-G, rapidly migrates to export sites during the brief pulse-chase period in vivo before preparation of semi-intact cells (W. E. Balch, unpublished results), the rablb protein may be required during a very early step in vesicular transport. In contrast, transport from the ER to the *medial*-Golgi compartment (as indicated by the appearance of the G_{H2} intermediate (Fig. 7 *B*, Δt = 0 min, ○) was completely sensitive to antibody when added before initiation of transport, providing direct evidence that a temporally distinct intra-Golgi transport step requires rablb.

To determine the rate at which VSV-G protein matures

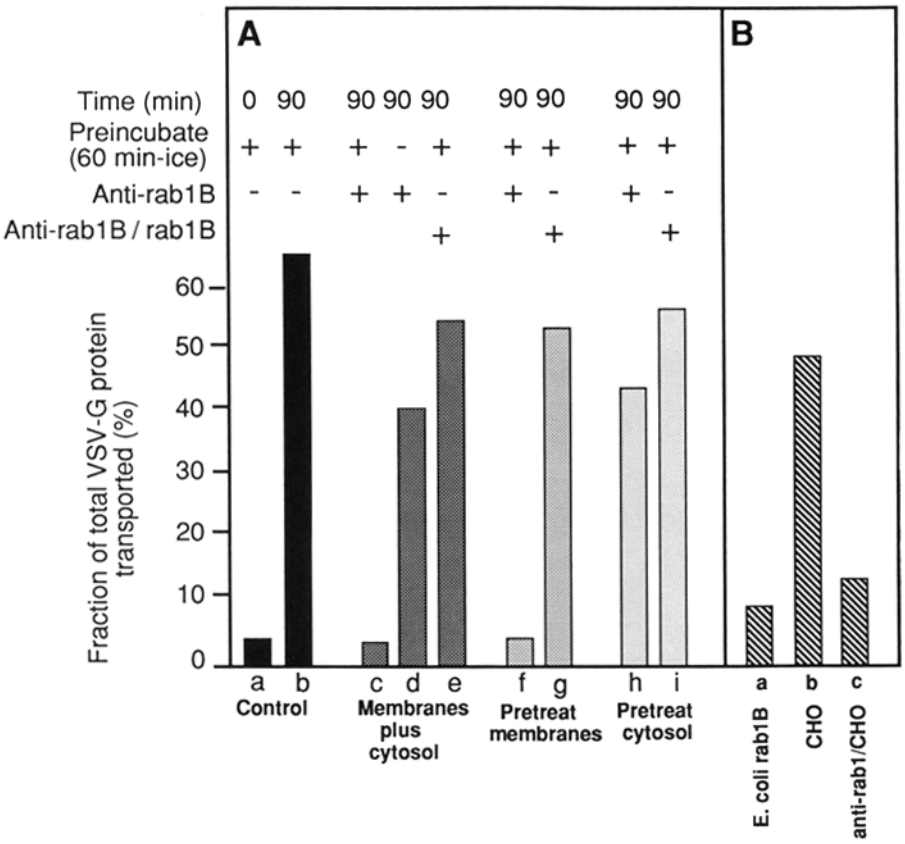

Figure 6. The membrane fraction contains the pool of rablb sensitive to inhibition by anti-rablb antibody. (*A*) Semi-intact cells (*SIC*) and/or cytosol were pretreated with m4D3c for 60 min on ice before incubation for 90 min at 32°C with the untreated fraction as indicated (lanes *c*, *f*, and *h*). In lanes *d*, *g*, and *i* m4D3c was preincubated for 60 min on ice in the presence of 5 μg (2.5-fold molar excess) of recombinant rablb protein before incubation with cells and/or cytosol as indicated. In lane *d*, m4D3c was added immediately before initiation of transport. (*B*) Recombinant *E. coli* rablb (lane *a*), or CHO membranes (lane *b*) were added to semi-intact cells (pretreated for 60 min on ice with m4D3c) and incubated for 90 min at 32°C. In lane *c*, the CHO cell membranes were pretreated with m4D3c for 60 min on ice before addition to the assay.

through steps sensitive to antibody during transport from the ER to the *cis*- and *medial*-Golgi compartments, we incubated semi-intact cells for increasing time at 37°C in vitro before addition of antibody. Using this protocol, any VSV-G protein which is mobilized past the rablb requiring step would be expected to be efficiently delivered to the subsequent Golgi compartment in an antibody insensitive fashion. As shown in Fig. 7 *B* (□), transport through the first antibody-sensitive step (ER to *cis*-Golgi) occurred with a $t_{1/2}$ of <10 min. In contrast, transport through a second antibody-sensitive step (*cis*- to *medial*-Golgi) occurred with a $t_{1/2}$ of 25 min. These results are consistent with our previous results in which we have demonstrated that transport between the ER and the *medial*-Golgi compartment in vitro requires two sequential vesicular transport steps (Schwaninger et al., 1991).

Rablb Is Required at an Early Step in ER to Golgi Transport

To extend the above results and provide additional support for the role of rablb in export of protein from the ER, we examined the temporal requirement for tsO45 VSV-G transport in vitro. We have previously established that transport in vitro between the ER and the Golgi occurs in two kinetically distinguishable steps, the first being characterized by a lag period in which VSV-G protein is exported from the ER and transferred to the *cis*-Golgi compartment (vesicle fission and targeting) and a late step in which vesicles fuse with the *cis*-Golgi compartment, resulting in rapid processing by Man I (Beckers et al., 1987, 1990; Beckers and Balch, 1989). To determine whether rablb function is required at either an early or late step (or both), semi-intact cells, cytosol, and ATP were preincubated for increasing times at 32°C. At each time point indicated in Fig. 8 cells were transferred to ice, incubated with m4D3c for 60 min, and subsequently returned to 32°C where they were incubated for a total time of 90 min. As suggested above, we found that VSV-G protein was rapidly mobilized into a transport intermediate which is insensitive to antibody inhibition. After a 20–30-min incubation at 37°C, a time-point in which only 10–20% of the total VSV-G protein was delivered to the *cis*-Golgi compartment as measured by the appearance of the man_5-processed form (Fig. 8 *A*, ○), >80% of the VSV-G protein transported had reached a step which was insensitive to antibody (Fig. 8 *A*, ●).

Two additional lines of evidence support the above conclusion that rablb was required at an early step in ER to Golgi transport. First, semi-intact cells pretreated with antibody can be efficiently complemented in *trans* with rablb-containing CHO membranes only during the first 20–30 min of incubation, a time period in which transfer to the Golgi, but not fusion, is complete (Beckers and Balch, 1989; Beckers et al., 1990; data not shown). Second, we have previously demonstrated that preincubation in the presence of EGTA results in accumulation of VSV-G protein at a late step in transport which is now insensitive to the nonhydrolyzable analog of GTP, GTPγS (Beckers and Balch, 1989). These results indicate that the step in which GTP (or a GTPγS-sensitive protein) is recruited to newly forming vesicles can be completed in the absence of Ca^{2+}. Transport from this step is readily reversible by the addition of Ca^{2+} (Beckers and Balch, 1989). To determine whether rablb was also recruited into a transport intermediate preceding the late Ca^{2+}-sensi-

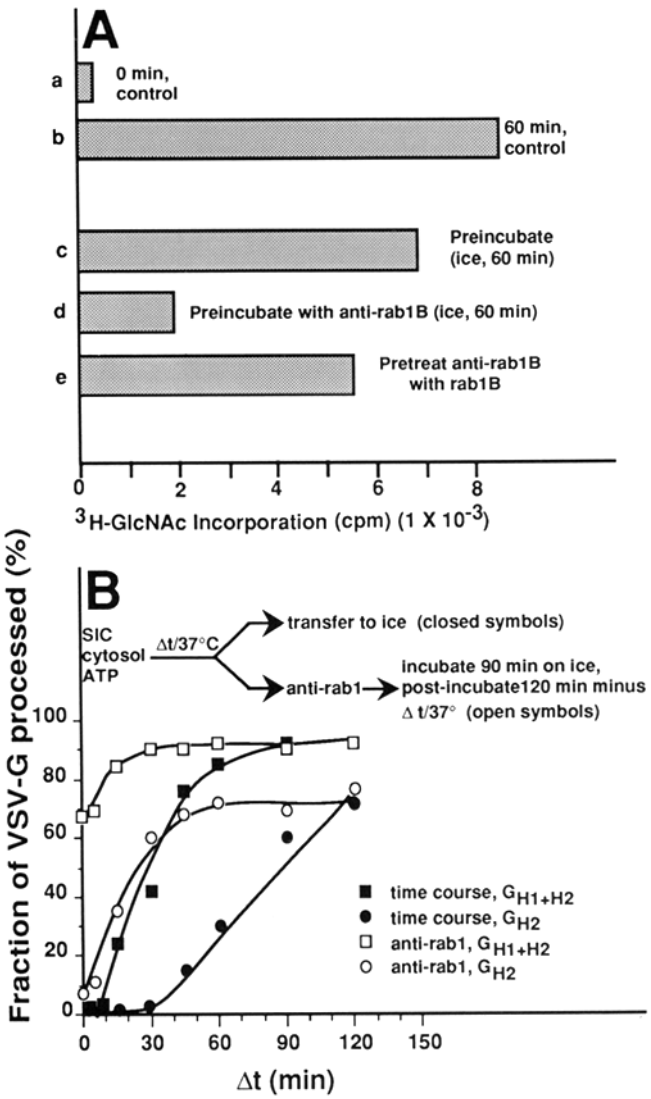

Figure 7. Transport between the *cis*- and *medial*-Golgi compartments is inhibited by anti-rablb. (*A*) Enriched Golgi fractions were assayed for transport of wild-type VSV-G protein between the *cis*- and *medial*-Golgi compartments by the coupled incorporation of ^{3}H-GlcNAc as described previously (Balch et al., 1984). (*B*) Sequential transport of wild-type VSV-G protein from the ER to the *cis*- and *medial*-Golgi compartments was measured by preincubating wild-type semi-intact cells (*SIC*), cytosol and ATP for increasing time at 37°C (as described in Materials and Methods). At the indicated time, cells were transferred to ice (●, ■), or incubated on ice for 60 min in the presence of m4D3c (○, □). Subsequently, cells treated with antibody were postincubated in the presence of antibody for a total time of 90 min at 37°C, and quantitated for the appearance of the endo H–resistant forms of VSV-G as described in Materials and Methods. G_{H1} is the form of VSV-G protein transported from the ER to the *cis*-Golgi compartment; G_{H2} is the form of VSV-G protein transported from the ER to the *medial*-Golgi compartment; G_{H1} + G_{H2} is the total VSV-G protein which has passed through the *cis*-Golgi compartment.

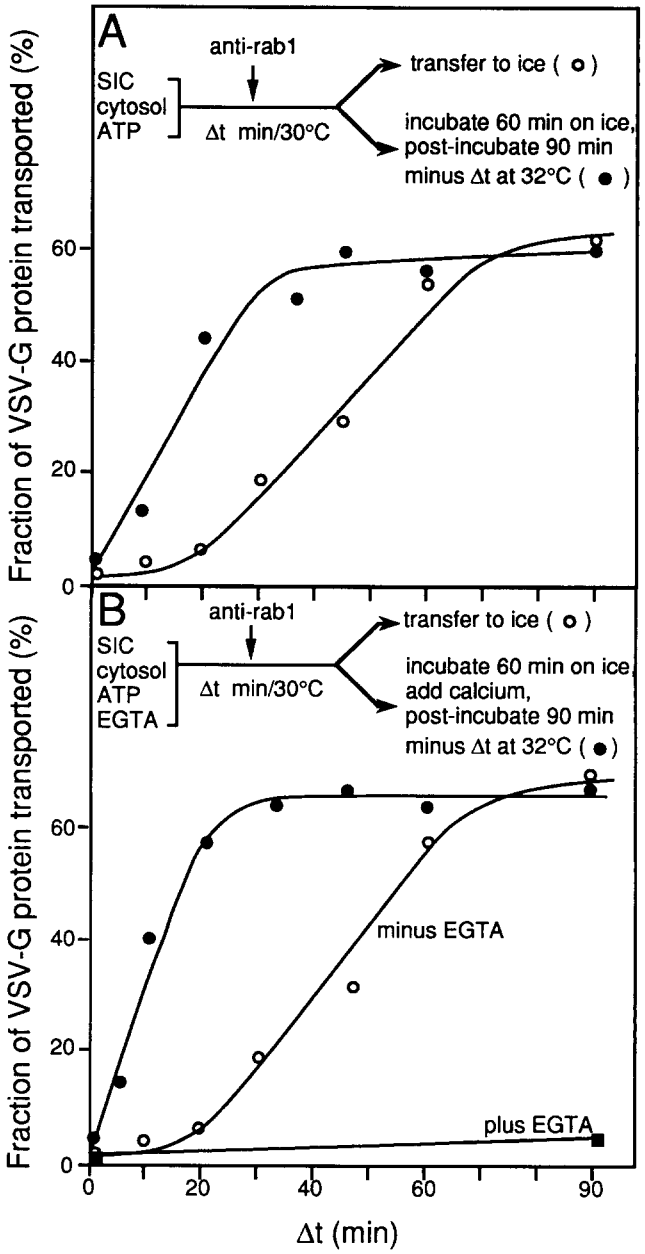

Figure 8. Rablb is required for an early step in vesicular transport. (*A*) Semi-intact cells (*SIC*), cytosol and ATP were incubated for increasing time at 32°C (as described in Materials and Methods). At the indicated time cells were transferred to ice (○), or incubated on ice for 60 min in the presence m4D3c (●). Subsequently, cells treated with antibody were postincubated in the presence of antibody for a combined total time of 90 min at 32°C. (*B*) Semi-intact cells, cytosol, and ATP were incubated for increasing time at 32°C in the absence (○), or presence of 5 mM EGTA (●, ■). At the indicated time cells were transferred to ice and incubated for 60 min in the presence of m4D3c. Subsequently, treated cells were supplemented with Ca^{2+} to bring the final free Ca^{2+} concentration to 0.1 μM (Beckers and Balch, 1989) and postincubated in the presence of antibody for a total time of 90 min at 32°C. A continuous incubation for 90 min in the presence of EGTA is illustrated by the closed squares.

tive fusion step, semi-intact cells were preincubated in the presence of EGTA for increasing time. As shown in Fig. 8 *B*, sensitivity to m4D3c was rapidly lost. After 30 min in the presence of EGTA, further transport in the presence of Ca^{2+} was resistant to antibody.

Discussion

In these studies we have provided evidence for the distribution and function of the rablb protein in mammalian cells. In contrast to previous studies using a polyclonal antibody prepared against YPT1 which suggested that a cross-reactive protein to the YTP1 antibody is present only in the Golgi (Segev et al., 1988), we have shown using both morphological and biochemical criteria that most of the rablb protein (70–80%) is present in a smooth ER distribution. Less than 5% was associated with the nuclear envelope, RER or cytosolic fractions, while the remaining 20–30% was associated with the Golgi stack. Since the smooth ER fraction prepared from rat liver is likely to contain both transitional elements believed to be involved in protein export (Palade, 1975) and putative intermediate compartments in which transported proteins accumulate at reduced temperature (15–16°C) (Saraste and Kuismanen, 1984; Saraste et al., 1986; Schweizer et al., 1990), we provided evidence that rablb colocalizes with rab2, a newly identified small GTP-binding protein found in the intermediate compartment (Chavrier et al., 1990*a*). We are currently examining the role of rablb in the function of this potential intermediate compartment in ER to Golgi transport.

We were surprised to find that a subset of the mAbs only detected rablb in the ER. One explanation for this result is that this class (class I) recognizes an epitope on rablb which is accessible only when the protein is present in the ER. In the Golgi stack this epitope may either be masked directly by association with additional accessory (effector) proteins or indirectly through the presence of abundant coat proteins on Golgi cisternae and vesicles (Orci et al., 1986, 1989; Serafini et al., 1991). This interpretation is supported by the observation that class I antibodies are low affinity, pentameric IgM immunoglobulins. Since antibodies which detect both rabla and rablb show similar morphological distributions (data not shown), we presently have no evidence to suggest that these highly related proteins (92% identity) (Touchot et al., 1987; Zahraoui et al., 1989) are found in distinct subcellular distributions.

Consistent with the morphological distribution of rablb and yeast YPT1 (Molenaar et al., 1988; Baker et al., 1990; Bacon et al., 1989) or SEC4 (Salminen and Novick, 1987; Walworth et al., 1989), transport between the ER and the *cis*-Golgi compartment required a membrane-associated form of rablb. The activity of this membrane-associated form was potently neutralized by the mAb m4D3c, which we found recognized only the native protein. In the case of *ras*, only one strongly neutralizing antibody, Y13-259, has been identified to date (Barbacid, 1987). It recognizes an exposed hydrophilic domain comprising the switch two domain (residues 63–73) (Milburn et al., 1990; Pai et al., 1989, 1990) and blocks guanine nucleotide exchange and GAP binding (Barbacid, 1987). By analogy, while some of the mAbs which also recognized rablb strongly on ELISA assay had little or no effect on transport, m4D3c may recognize a native epitope essential for rablb function, an interpretation we are currently exploring through epitope mapping.

The mechanism through which m4D3c neutralizes the rablb function in vesicle formation is currently unknown. The necessity to preincubate semi-intact cells with antibody is consistent with the interpretation that it is necessary to titrate the endogenous pool of rablb into antigen–antibody complexes. Since Fab fragments efficiently inhibit transport (although required at a higher concentration that the divalent form), it is unlikely that membrane aggregation leads to the observed result. The loss of apparent binding affinity during preparation of Fab fragments from monoclonal antibodies is a well-recognized problem. The ability to reverse inhibition in antibody-treated cells by the addition of rablb-containing CHO membranes in *trans* at the beginning of the incubation demonstrated that the antibody complexed endogenous rablb pool present on ER membranes does not irreversibly block export. These results suggest that either rablb or a protein complex containing rablb may recycle between membranes. Two lines of evidence suggest that a rablb-containing protein complex is required. First, we demonstrated that we were unable to reverse inhibition by the addition of recombinant rablb. Since there is now strong evidence that rab proteins undergo posttranslational processing steps which promote their membrane association (Molenaar et al., 1988; Schafer et al., 1989; Leonard et al., 1990; Chavrier et al., 1990*a*; Fischer v. Mollard et al., 1990) these posttranslational modifications are likely to be essential for interaction of rablb with upstream or downstream effectors. Second, the *trans*-acting factors provided by CHO membranes can be prepared in a soluble form. Fractionation of the soluble pool suggests that rablb functions in the context of a molecular complex which contains at least two additional unknown factors (W. E. Balch, unpublished results). We are currently exploring the importance of both isoprenylation and carboxy-methylation, and the role of these accessory (effector) proteins for rablb function in vitro. Inhibition of rablb function was sufficient to block vesicular trafficking. Since rabla is also abundant in a broad range of tissues and cell types (Touchot et al., 1987; Zahraoui et al., 1989) its role in transport remains to be determined.

Rablb function was found to be required at an early step in transport. We observed that tsO45 VSV-G protein was rapidly mobilized in vitro into a transport intermediate which was insensitive to antibody. In this case, we found that rablb function was required during the lag period, a period of time (~20 min) which precedes fusion of the first wave of transport vesicles with the *cis*-Golgi compartment (Beckers et al., 1990). Similarly, wild-type VSV-G protein was rapidly mobilized into a transport intermediate in vivo during pulse–chase labeling of cells. Experiments directed at the study of protein transport using "wild-type" markers will have to take into account the possibility that recruitment of newly synthesized proteins into transport intermediates may in some cases be extremely rapid. These results suggest that the rablb protein is required for functional export of VSV-G protein from the ER. This is consistent with the morphological distribution of the rablb protein and our previous observation that GTPγS can inhibit transport only during the first 20–30 min (Beckers and Balch, 1989), indicating that incorporation of GTP into the transport machinery occurs early. The latter result may also reflect the requirement for other GTP-binding proteins including ARF (Stearns et al., 1990), a mammalian homologue to yeast SAR1 (Nakano et al., 1988; Nakano and Muramatsu, 1989) and rab2 (Chavrier et al., 1990*a*). However, these data are presently inconsistent with biochemical and genetic studies in yeast which suggest that YPT1 is not required for vesicle formation, rather vesi-

cle fusion (Baker et al., 1990; Kaiser and Schekman, 1990). These results raise the important issue that these small GTP-binding proteins are likely to be central regulators in a cascade of upstream and downstream effectors. Depending on the mutation and/or inhibitory reagent used to potentiate function, either an early (recruitment) or a late (downstream) event may be disrupted. For example, in addition to its requirement during vesicle formation as suggested by ability of m4D3c to inhibit an early step, rablb may also participate in a late, antibody insensitive, Ca^{2+}-dependent fusion reaction. This interpretation was previously suggested by the ability of a synthetic peptide homologous to the putative rablb effector domain to inhibit a late step in transport (Plutner et al., 1990). Whether this reflects the activity of an effector protein similar in function to *ras*-GAP in promoting GTP hydrolysis or a different effector function associated with fusion at the *cis*-Golgi face, is presently unknown. In addition to rabl, rab2 has been recently localized to the putative recycling compartment between the ER and the Golgi (Chavrier et al., 1990*a*). We are currently exploring its potential role in ER to Golgi transport.

We found that the rablb protein is also required for transport of VSV-G protein between successive Golgi compartments. These results suggest that rablb is involved in at least two early stages of vesicular trafficking: ER to *cis*-Golgi and *cis*- to *medial*-Golgi transport. These results are consistent with the potential role of YPT1 in trafficking between the ER and subsequent Golgi compartments in yeast (Baker et al., 1990; Bacon et al., 1989). What, then, is the function of rablb protein in regulation of transport? A model consistent with the striking morphological distribution of rab proteins in distinct compartments of the exocytic and endocytic pathways (Balch, 1990) proposes that small GTP-binding proteins are critical for specificity of vesicular trafficking (Bourne, 1988). In this interpretation, each rab protein encodes a targeting determinant which ensures that newly formed vesicles are delivered to a specific, downstream compartment. While our data cannot rule out the possibility that m4D3c cross reacts with as yet unknown rablb-related proteins which differentiate ER to Golgi and intra-Golgi trafficking or indirectly inhibit an effector protein common to multiple small GTP-binding proteins, the data also support a more general interpretation of rab function. In this interpretation, rablb regulates sequential steps in a common vesicle fission and fusion machinery operating between early compartments. In this case, rablb does not serve as the targeting determinant per se, but perhaps biochemically links common upstream and downstream components in the vesicle fission/fusion cycle. This interpretation, of course, does not rule out the possibility that other rab proteins may play key roles in vesicle targeting. Indeed, as suggested above, the presence of both rablb and rab2 protein in an early intermediate compartment between the ER and Golgi compartments (Chavrier et al., 1990*a*) and the recent identification of at least 18 additional rab-related proteins in MDCK cells (Chavrier et al., 1990*b*) indicates that members of the *rab* gene family may play multiple roles in regulation of the complex machinery involved in vesicular transport or organelle structure during different stages of the cell cycle.

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