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 YPTI (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; Zahraouii et al., 1989; Chavrier et al., 1990a, b), has been proposed to play a key role in targeting of carrier vesicles between specific compartments (Bourne, 1988). Consistent with this model, the YPTI 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 (Zahraouii et al., 1989; Chavrier et al., 1990b). 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., 1990a), rab6 to the medial- and trans-Golgi compartments (Goud et al., 1990), rab5 and rab7 to early and late endosomes, respectively (Chavrier, 1990a), 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 YPTI, and are the likely mammalian homologues. Mouse rabla can replace YPTI 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 tsO45 strains of vesicular stomatitis virus (VSV), using the swelling method as described previously (Beckers et al., 1987). Tran[35S]methionine and [35S]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 rab, and human rab 2, 3A, and rab 6 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-rabphilin II were provided by D. Meyer (University of California, Los Angeles, CA). An affinity-purified polyclonal antibody specific for α1-6-sialyltransferease was obtained from J. Paulson (Cytel Corporation, La Jolla, CA). Two polyclonal sera for α1-2-mannosidase (Man I) and α1-2-mannosidase (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-rabB Fab fragments were prepared from mAb3c4 by using papain to couple agarose beads (Pierce Chemical Co., Rockford, IL) according to the manufacturer's directions.

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 pET3a) bacterial expression vector and induced rabbit proteins were purified by the procedures described previously (Tucker et al., 1986; Zahraoui et al., 1989; Touchot et al., 1987). Rabs were purified to >95% and 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-rab monoclonal antibodies, female Balb/C mice were immunized by an intraperitoneal injection of purified recombinant rabbl 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. Mice were boosted (intraperitoneal) twice every 2 weeks, 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 (Shulman et al., 1978) (ATCC CRL1381). 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 (Exceld; J. R. Scientific, Lenexa, KS) and the supernatant harvested and purified by ammonium sulfate precipitation. The ELISA assay was performed by binding of antibody (1 μg antigen/well in 50 μl of 50 mM Na2CO3 (pH 8.4)) to immunomells (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 phosphatase substrate (104-105; Sigma Chemical Co., St. Louis, MO) was added in 50 mM Na2CO3 and 1 mM MgOAc.

Results

Rab-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 rabl, 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., 1999a,b). Two of the monoclonals, mCl0a and m3D7C, recognized both the rabl and rabl proteins (92% identity; Touchot et al., 1987), but with two- to threefold lower af-
Table 1. Summary of Antibody Specificity

<table>
<thead>
<tr>
<th>Antibody (class)</th>
<th>Specificity</th>
<th>ER to Golgi compartment</th>
<th>Intra-Golgi compartment</th>
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<tbody>
<tr>
<td></td>
<td>rab1a</td>
<td>rab1b</td>
<td>rab2</td>
</tr>
<tr>
<td>p68</td>
<td>(+)§</td>
<td>+</td>
<td>(+)§</td>
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<tr>
<td>m5F2a (IgM)</td>
<td>–</td>
<td>+</td>
<td>–</td>
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<tr>
<td>m3E8a (IgM)</td>
<td>–</td>
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<td>–</td>
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<tr>
<td>m4D3c (IgG)</td>
<td>–</td>
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<tr>
<td>m5C6b (IgG)</td>
<td>–</td>
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<td>m1C10a (IgG)</td>
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<td>m3D7c (IgG)</td>
<td>+</td>
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<td>m4G2b (IgG)</td>
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* Transport inhibition reported on a relative scale in the presence of 5 μg monoclonal, or 10 μg polyclonal IgG: (−) no inhibition, (+) <25% inhibition, (+++) >80-90% inhibition.
† Cross-reactivity tested against Ha-ras, rho, rap, and rab1a, 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 rabi 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., 1990a; 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 SER 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 SER 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., 1990a), 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
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 re-
gion characteristic of the intermediate compartment. These
results suggest that the rablb protein is prevalent throughout
the early compartments of the secretory pathway, its mor-
phological distribution suggestive of its potential role in reg-
ulation of ER and Golgi protein transport.

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 pro-
teins, or bacterial extracts expressing recombinant fu-
sion 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 × 10⁵ cells) of the indicated cell line
(see Results) were probed with p68 or m5C6b.

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 anti-
body inhibits 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.
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 (man9) asparagine-linked oligosaccharides acquired during nascent synthesis in the RER, to the man9 oligosaccharide form as a consequence of transport of VSVG to the cis-Golgi and processing by «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 VSVG protein (VSV strain ts045) (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 VSVG protein exclusively in the ER before initiation of transport (Beckers et al., 1987). Transport of VSVG 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 rabl 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 rabl (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 rabl 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
prior to incubation results in significantly reduced level of inhibition (Fig. 6A, lane d). In contrast, pretreatment of CHO cytosol had only a minor effect on the observed efficiency of transport (Fig. 6A, 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. 6A, lane d), supplementation of the assay with recombinant rablb did not stimulate transport in semi-intact cells which had been pretreated with antibody (Fig. 6B, 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-associ-

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.
As shown in Fig. 7 A, vesicular trafficking between the cis- and medial-Golgi compartments as measured by the coupled incorporation of \( ^3H \)-N-acetylglucosamine \( (^3H\text{-GlcNAc}) \) 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 VSVG 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 VSVG 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 \( G_{\text{man9}} \) form of VSVG containing one endo H-resistant oligosaccharide and occurs with a \( t_{1/2} \) of \( \sim 20 \) min (Fig. 7 B, ●). Transport to the medial-Golgi compartment results in the appearance of the \( G_{\text{man5}} \) form (\( t_{1/2} \) of \( \sim 75 \) min) containing two endo H-resistant oligosaccharides (Fig. 7 B, ●). When antibody was added before incubation, transport of wild-type VSVG from the ER through the cis-Golgi compartment (as indicated by the appearance of the total \( G_{\text{man9}} + G_{\text{man5}} \) forms (Fig. 7, \( \Delta t = 0 \) min, □) was only partially inhibited. This result contrasts with transport of tsO45 VSVG protein from the ER to the cis-Golgi which is completely sensitive to antibody (Fig. 6 A; data not shown). Since wild-type VSVG, unlike tsO45 VSVG, 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 \( G_{\text{man1}} \) intermediate (Fig. 7 B, \( \Delta t = 0 \) 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
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 VSVG 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 t1/2 of <10 min. In contrast, transport through a second antibody-sensitive step (cis- to medial-Golgi) occurred with a t1/2 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 VSVG 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 man₃-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 VSVG protein at a late step in transport which is now insensitive to the nonhydrolyzable analog of GTP, GTP₇S (Beckers and Balch, 1989). These results indicate that the step in which GTP (or a GTP₇S-sensitive protein) is recruited to newly forming vesicles can be completed in the absence of Ca²⁺. Transport from this step is readily reversible by the addition of Ca²⁺ (Beckers and Balch, 1989). To determine whether rablb was also recruited into a transport intermediate preceding the late Ca²⁺-sensi-
Figure 7. Transport between the cis- and medial-Golgi compartments is inhibited by anti-rab1. (A) Enriched Golgi fractions were assayed for transport of wild-type VSVG 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 VSVG 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 (\(\bullet\)), or incubated on ice for 60 min in the presence of m4D3c (\(\square\)). 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 VSVG as described in Materials and Methods. GH is the form of VSVG protein transported from the ER to the cis-Golgi compartment; GH2 is the form of VSVG protein transported from the ER to the medial-Golgi compartment; GH + GH2 is the total VSVG protein which has passed through the cis-Golgi compartment.

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.

Figure 8. Rab1b 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 (\(\circ\)), or incubated on ice for 60 min in the presence m4D3c (\(\bullet\)). 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 (\(\bigcirc\)), or presence of 5 mM EGTA (\(\bullet\), \(\bigcirc\)). 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 \(\mu\)M (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.
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 prepared against YPT1 which suggested that a cross-reactive

In contrast to previous studies using a polyclonal antibody

tion and function of the rablb protein in mammalian cells. In these studies we have provided evidence for the distribution and function of the rablb protein. Rablb function was found to be required at an early step in transport. We observed that ts045 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 GTPyS 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 SARI (Nakano et al., 1988; Nakano and Muramatsu, 1989) and rab2 (Chavrier et al., 1990a). 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-
cell 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, rab2 may also participate in a late, antibody insensitive, Ca2+-dependent fusion reaction. This interpretation was previously suggested by the ability of a synthetic peptide homologous to the putative rab2 effector domain to inhibit a late step in transport (Plutner et al., 1990). Whether this reflects the activity of ple smallGTP-binding proteins, the data also support a more additional role for rab2, has been recently localized to the putative recycling compartment between the ER and the Golgi (Chavrier et al., 1990a). 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 YPTI 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 an yet unknown rabb-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 fusion 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 fusion/ 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., 1990a) and the recent identification of at least 18 additional rab-related proteins in MDCK cells (Chavrier et al., 1990b) 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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