

Rab1 and Ca²⁺ Are Required for the Fusion of Carrier Vesicles Mediating Endoplasmic Reticulum to Golgi Transport

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Abstract. Members of the *rab/YPTI/SEC4* gene family of small molecular weight GTPases play key roles in the regulation of vesicular traffic between compartments of the exocytic pathway. Using immunoelectron microscopy, we demonstrate that a dominant negative *rabla* mutant, *rabla(N124I)*, defective for guanine nucleotide binding in vitro, leads to the accumulation of vesicular stomatitis virus glycoprotein (VSV-G) in numerous pre-*cis*-Golgi vesicles and vesicular-tubular clusters containing *rab1* and β -COP, a subunit of the coatamer complex. Similar to previous observations (Balch et al. 1994. *Cell*. 76:841–852), VSV-G was

concentrated nearly 5–10-fold in vesicular carriers that accumulate in the presence of the *rabla(N124I)* mutant. VSV-G containing vesicles and vesicular-tubular clusters were also found to accumulate in the presence of a *rabla* effector domain peptide mimetic that inhibits endoplasmic reticulum to Golgi transport, as well as in the absence of Ca²⁺. These results suggest that the combined action of a Ca²⁺-dependent protein and conformational changes associated with the GTPase cycle of *rab1* are essential for a late targeting/fusion step controlling the delivery of vesicles to Golgi compartments.

THE biochemical mechanisms underlying vesicular transport between compartments of the exocytic and endocytic pathways are poorly understood, but are likely to involve the ordered assembly and disassembly of protein complexes that regulate vesicle budding, targeting, and fusion (reviewed in Pryer et al., 1992; Rothman and Orci, 1992). It is now evident that a collection of GTPases belonging to the *rab/YPT/SEC4*, *Arf*, *Sar1*, and heterotrimeric G_{αβγ} families serve as molecular switches to control diverse functions of the transport machinery (reviewed in Goud and McCaffery, 1991; Barr et al., 1992; Zerial and Stenmark, 1993; Nuoffer and Balch, 1994). In particular, transport from the ER to and between Golgi compartments in mammalian cells requires the small GTPase *rab1*. Neutralizing antibodies (Plutner et al., 1991; Schwaninger et al., 1992; Davidson and Balch, 1993; Peter et al., 1993) and *rab1*-specific peptides (Plutner et al., 1990; Balch et al., 1993) block transport in vitro. In addition, *trans*-dominant mutants with altered guanine nucleotide-binding properties inhibit transport in vivo (Tisdale et al., 1992) and in vitro (Nuoffer et al., 1994; Davidson and Balch, 1993).

In a previous study (Nuoffer et al., 1994), we established the biochemical and morphological properties of the *rabla*-(S25N) mutant, demonstrating that this mutant is likely to be restricted to the GDP-bound state, a condition that inhibits vesicle budding and the export of vesicular stomatitis virus glycoprotein (VSV-G)¹ from the ER. Given previous evidence that the *rabla(N124I)* mutant is also a dominant inhibitor of transport in vivo (Tisdale et al., 1992), we have analyzed the effects of this mutant on VSV-G transport in vitro using immunoelectron microscopy. We find that the N124I mutant is defective for guanine nucleotide binding in vitro, indicating that it is able to undergo rapid exchange independent of a guanine nucleotide exchange protein (GEP). This mutant does not inhibit vesicle budding from the ER. Instead, it prevents the fusion of VSV-G containing carrier vesicles to the Golgi stack. In the presence of the N124I mutant or in the absence of Ca²⁺ (by chelation with EGTA), VSV-G accumulates in 60-nm carrier vesicles, and clusters of vesicles and small tubules—pre-Golgi intermediates that populate the normal pathway in vitro (Balch et al., 1994). The combined results from this and our previous studies on the *rab1*(S25N) mutant suggest that while a conformational change as-

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1. **Abbreviations used in this paper:** GDI, guanine nucleotide dissociation inhibitor; GEP, guanine nucleotide exchange protein; ManII, α -1,2 mannosidase II; *rabla(N124I)*, dominant negative *rabla* mutant; tsO45, temperature-sensitive mutant of VSV-G; VSV-G, vesicular stomatitis virus glycoprotein; VTCs, vesicular tubular clusters.

sociated with the exchange of GDP for GTP may be necessary for the recruitment of rab1 during vesicle budding, GTP hydrolysis is likely to be critical for a late Ca²⁺-dependent vesicle targeting/fusion step.

Materials and Methods

Materials

A polyclonal reagent recognizing p58 was provided by J. Saraste (Saraste and Svensson, 1991). A monoclonal reagent recognizing p53 was generously provided by H.-P. Hauri (Biocenter, Basel, Switzerland). Secondary antibodies were obtained from the following sources: FITC-conjugated goat anti-rabbit F(ab)₂ and anti-mouse IgG from Zymed Laboratories, Inc. (South San Francisco, CA); gold-conjugated goat anti-rabbit from Amersham Corp. (Arlington Heights, IL). Rabbit antisera to β -COP were derived from an immunogenic peptide containing the unique EAEG sequence (Duden et al., 1991) as described (Wilson, B. S., et al., 1994).

Preparation of Recombinant rab1a Proteins

His₆-tagged rab1a(N124I) protein was prepared as described (Nuoffer et al., 1994).

Incubation In Vitro for Morphological Analysis

All materials, assays, and procedures used to analyze transport or the morphological distribution of VSV-G using indirect immunofluorescence were as described previously using permeabilized NRK cells (Plutner et al., 1992; Nuoffer et al., 1994). For electron microscopy, incubations were conducted in 35-mm tissue culture dishes (Costar Corp., Cambridge, MA) (Balch et al., 1994). The analysis of transport using perforated cells based on the processing of VSV-G to endoglycosidase D- (Beckers et al., 1987) or endoglycosidase H-resistant oligosaccharide forms (Davidson and Balch, 1993; Nuoffer et al., 1994) were performed as described.

Electron Microscopy and Immunolabeling

After transport in vitro, permeabilized NRK cells were fixed for 30 min with 3% paraformaldehyde and 0.1% glutaraldehyde, pH 7.4, in 0.1 M phosphate buffer, washed briefly in PBS, and processed for immunodiffusion or indirect immunogold labeling of ultrathin cryosections.

For immunodiffusion, cells were washed for 10 min in PBS containing 1% BSA, 0.01 M glycine, and 0.1% saponin, pH 7.4, and then incubated sequentially with a primary monoclonal antibody, P5D4, specific for the cytoplasmic tail of VSV-G (overnight), a rabbit anti-mouse bridging antibody (1 h), and 5 or 10 nm gold, goat anti-rabbit IgG conjugate (3 h). After washing, the cells were fixed (1 h) in 3% paraformaldehyde, 1.5% glutaraldehyde, and 7.5% sucrose in 0.1 M cacodylate buffer, pH 7.4. The cells were then pelleted, postfixed in 1% OsO₄ in veronal-acetate buffer, stained in block for 2 h in 0.5% uranyl acetate, pH 6.0, dehydrated, and embedded in epon. Where indicated, cells were counterstained with tannic acid as described (Orci et al., 1986).

For immunogold labeling of ultrathin cryosections, cells were harvested, pelleted, and cryoprotected by infiltration with a mixture of 2.3 M sucrose in 0.1 M phosphate buffer, pH 7.4, containing 20% polyvinyl pyrrolidone, mounted on aluminum nails, and frozen in liquid nitrogen. Ultrathin cryosections were cut on a Reichert Ultracut E, equipped with a Reichert FC-4 cryoattachment, collected on formvar/carbon-coated nickel grids, and quenched in 0.01 M glycine, pH 7.4. Sections were then incubated 2 h at room temperature with the anti-VSV-G monoclonal antibody in 10% FCS/PBS, a rabbit anti-mouse bridge (1 h), followed by a 5-nm gold, goat anti-rabbit IgG conjugate (2 h). The grids were then stained in 2% neutral uranyl acetate (10 min) and subsequently adsorption-stained with 0.2% uranyl acetate, 0.2% methyl cellulose, and 3.2% polyvinyl alcohol. Sections

were observed on an electron microscope (1200 EX; JEOL U.S.A. Inc., Peabody, MA, or CM-10; Philips Electronic Instruments Co., Mahwah, NJ).

Quantitation

Electron micrographs were enlarged to a magnification of 50,000–100,000-fold. The linear membrane density was determined by tracing of the membrane contour length of each compartment on photographic prints with aid of a measuring pen calibrated to give the total millimeter length for a particular magnification. For both vesicular-tubular clusters (VTCs) and Golgi stacks, only the external membrane length reflecting the distribution of gold particles was traced. The numbers reported are the number of gold particles per millimeter of membrane length of the compartment traced with the pen. In the case of 40–80-nm vesicles, the mean linear density is reported as that calculated for a 60-nm vesicle. The data presented for the distribution of VSV-G in the different intermediates and the Golgi for each time point in Fig. 2 are the combined averages of between two and three separate experiments. The error bar in each of these experiments reflects a number of variables that include the combined averages from different experiments, variation in the degree of infection of individual cells, and differences in rates of transport in vitro of VSV-G in different cells within each experiment. Statistical significance of data was determined by a Student's *t* test.

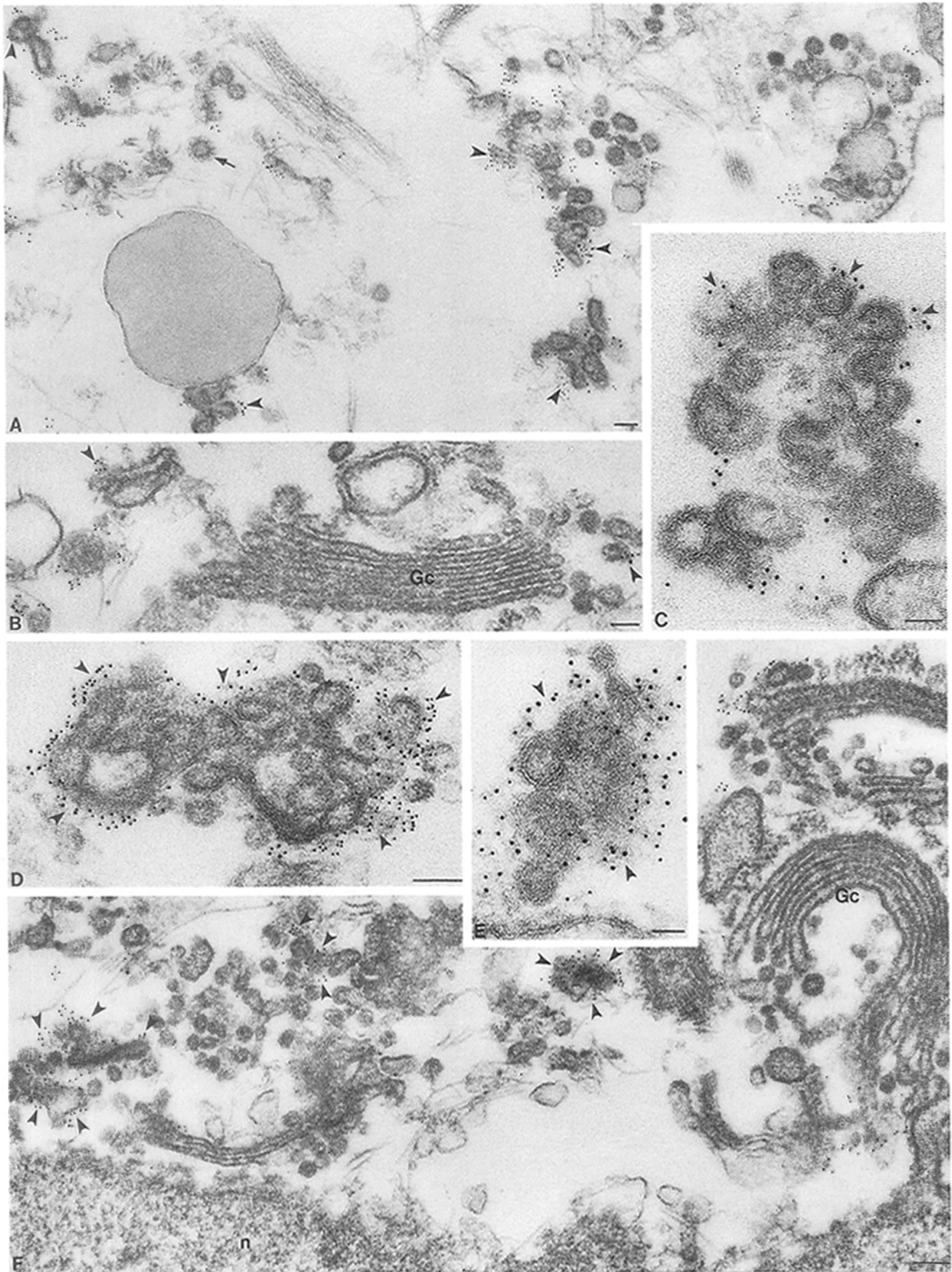
Results

VSV-G Protein Accumulates in Carrier Vesicles and VTCs in the Presence of GTP γ S

We have recently established that VSV-G is sorted and concentrated into 60-nm carrier vesicles upon export from the ER (Balch et al., 1994). To examine the role of GTP hydrolysis in protein concentration and vesicle budding from the ER, we first examined the effect of the nonhydrolyzable GTP analogue, GTP γ S, on the export of VSV-G using quantitative immunoelectron microscopy and an assay that efficiently reconstitutes this stage of the secretory pathway in vitro using permeabilized NRK cells (Plutner et al., 1992). For this purpose and to synchronize the export of VSV-G from the ER, we used a temperature-sensitive mutant of VSV-G (tsO45) which has a thermoreversible folding defect and is retained in the ER when cells are held at the restrictive temperature (Lafay, 1974). After shifting the cells to the permissive temperature (32°C), tsO45 VSV-G is efficiently transported to the Golgi stack in vivo (Tisdale et al., 1992) and in vitro (Plutner et al., 1992; Balch et al., 1994).

When permeabilized NRK cells containing tsO45 VSV-G in the ER were incubated for 45 min at the permissive temperature in the presence of GTP γ S, and the distribution of VSV-G was analyzed by immunoelectron microscopy, VSV-G was not detected in Golgi elements (Fig. 1B). Instead, VSV-G was found in both carrier vesicles and clusters of vesicles and small tubular elements (VTLS) (Fig. 1, A–C). Carrier vesicles and VTCs that accumulate in the presence of GTP γ S were identical to those previously shown to be pre-Golgi transport intermediates under normal incubation conditions in vitro (Balch et al., 1994). However, in the presence of GTP γ S, VTCs not only contained fewer tubular elements than intermediates observed at a similar time point in the ab-

Figure 1. GTP γ S, EGTA and a rab1 effector domain peptide cause accumulation of VSV-G protein in pre-Golgi intermediates. VSV-G is seen in transport vesicles and VTCs after incubation for 45 min at 32°C in the presence of 10 μ M GTP γ S (A–C), and in VTCs after incubation (45 min, 32°C) in the presence of 25 μ M rab3AL (E) or 10 mM EGTA (D and F) as described in the Materials and Methods. In the presence of GTP γ S (B) or EGTA (F), VSV-G is not transported to the Golgi. Methods for labeling and immunoelectron microscopy using the immunodiffusion protocol were as described in Materials and Methods. Bar, 0.1 μ m in A, B, D, and F; and 0.05 μ m in C and E.



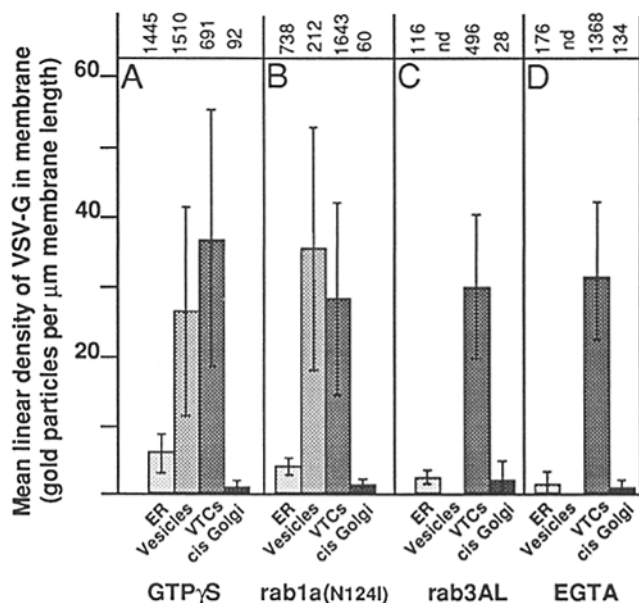


Figure 2. Quantitative analysis of the distribution of VSV-G during incubation in vitro in the presence of transport inhibitors. Permeabilized cells were incubated for 45 min in the presence of 10 μ M GTP γ S, 10 mM EGTA, 25 μ M rab3AL peptide (Plutner et al., 1990), or rab1a(N124I) (1 μ M), and the mean linear density of VSV-G (number of gold particles per micrometer of membrane contour length) in the ER, ER to Golgi carrier vesicles, VTCs, and the Golgi stack was determined as described in the Materials and Methods. ER to Golgi carrier vesicles were identified as 40–80-nm structures that contained >1 gold particles. The error bars define the SEM reflecting the distribution range of VSV-G in individual compartments used to generate the mean value as indicated in the Results. The total number of gold particles counted for each experimental condition are indicated at the top of the figure. The data presented for the distribution of VSV-G in the different intermediates and the Golgi for each time point are the combined averages of between two and five separate experiments. Within an individual experiment or within individual cells examined at high resolution, the fold change in density of VSV-G over that found in the ER ranged from 5.2 to 22 (nine experiments) in VTCs and 4- to 24-fold (four experiments) in the Golgi stack. *nd*, none detected.

sence of inhibitor (Balch et al., 1994), but showed a high content of vesicular profiles, suggesting that they were largely composed of clusters of 60-nm vesicles (Fig. 1, A and C). Vesicles and VTCs containing VSV-G formed in the absence (Balch et al., 1994) or presence of GTP γ S also contained smooth dense coats (Fig. 1 C). VTCs are the high resolution counterpart to the numerous punctate, pre-Golgi structures previously detected using indirect immunofluorescence in vitro in the presence of GTP γ S (Schwaninger et al., 1992).

To ascertain whether VSV-G was concentrated in vesicles and VTCs in the presence of GTP γ S to a similar degree as that observed under normal incubation conditions (Balch et al., 1994), the mean linear membrane density of VSV-G was determined using immunoelectron microscopy. As shown in Fig. 2 A, the mean linear density of VSV-G in isolated carrier vesicles formed in the presence of GTP γ S was 25.8 ± 15.8 gold particles/ μ m (assuming a 60-nm vesicle). These vesicles had an average of 5.0 ± 3.1 gold particles/vesicle (298

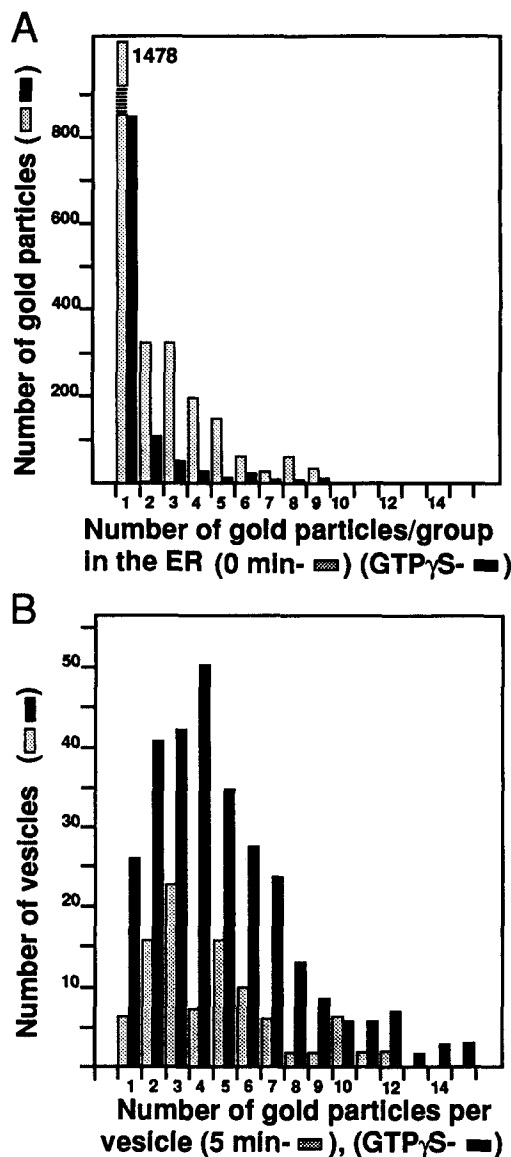


Figure 3. Quantitative analysis of the distribution of VSV-G along the membrane contour length of the ER or in carrier vesicles. (A) The distribution of VSV-G in the ER membrane before incubation in vitro (*open bars*) (five experiments, 78 fields) or after 45 min in the presence of GTP γ S (*solid bars*) (three experiments, 48 fields) as described in the Materials and Methods is reported as the number of gold particles detected as individual particles or in groups of two or more particles (see Results). (B) The number of gold particles in individual vesicles after 5 min incubation in vitro (*open bars*) (two experiments, 29 fields) or after 45-min incubation in the presence of GTP γ S (*solid bars*) (three experiments, 48 fields).

vesicles counted) (Fig. 3 B, *solid bars*). These numbers are comparable to those obtained for vesicles formed after only 5 min of incubation in the absence of inhibitor (Fig. 3 B, *open bars vs solid bars*) (Balch et al., 1994). The mean linear density of VSV-G in these VTCs was 37.1 ± 20.6 gold particles/ μ m (with an average of 30.8 ± 11.8 gold particles/VTC, 44 VTCs counted) (Fig. 2 A). Even after a 45-min incubation at 32°C in the presence of GTP γ S, the concentration of VSV-G in the ER (6.1 ± 3.4 gold particles/ μ m) (Fig. 2 A) was not significantly different ($P < 0.001$) from that ob-

served before incubation (5.2 ± 2.1 gold particles/ μm) (Balch et al., 1994). These results suggest that while vesicle budding occurs in the presence of GTP γ S, transport components present in the assay may be unable to efficiently recycle in the absence of GTP hydrolysis, thereby reducing the overall extent of export from the ER compared to normal incubation conditions in the absence of the inhibitor.

To assure ourselves that the increased linear density of VSV-G in vesicles or VTCs formed in the presence of GTP γ S was not a consequence of an unusual change in its linear distribution throughout the ER membrane before vesicle budding, we assessed the distribution of gold particles as individual particles or groups of particles on the ER membrane in the presence or absence of the inhibitor (Fig. 3 A). Even after a 45-min incubation in the presence of GTP γ S, gold particles remaining in the ER membrane were found to be distributed predominantly as individual particles. 819 out of 1,048 particles or groups of particles detected were scored as individual particles (Fig. 3 A). In this experiment, the mean distribution of gold particles retained in the ER in the presence of GTP γ S was found to be 1.5 ± 1.1 gold particles/group (Fig. 3 A, *solid bars*). This result is not significantly different ($P < 0.01$) from our previous results, which documented the distribution of gold particles in the ER before incubation *in vitro* in the absence of GTP γ S (Fig. 3 A, *open bars*) (1.5 ± 1.2 gold particles/group) (Balch et al., 1994). Thus, incubation in the presence of GTP γ S does not lead to a general aggregation of VSV-G along the surface of the ER membrane. Concentration is closely coupled to vesicle budding. Moreover, the 5- to 10-fold increase in density of VSV-G in vesicles and VTCs in the presence of inhibitor is comparable to the increased concentration of VSV-G in identical structures formed in the absence of inhibitor (Balch et al., 1994).

The Rabla(N124I) Mutant is Defective in Guanine Nucleotide Binding and Biochemically Distinct from the Rabla(S25N) Mutant

To analyze specifically the role of rab1 in vesicle targeting and fusion, we explored the properties of the rabla mutant, in which the asparagine in position 124 of the third guanine nucleotide binding motif (NKXD) is substituted by isoleucine. The N124I mutant has a number of properties that distinguish it from those of the rabla(S25N) mutant, which has a preferential affinity for GDP (Nuoffer et al., 1994). First, the rabla(N124I) mutant fails to bind both GDP or GTP at detectable levels *in vitro* (Table I). The equivalent mutation in H-ras(N116I) is similarly defective for GDP/GTP binding and is transforming, suggesting that it causes constitutive activation a consequence of a GEP-independent, high exchange rate (Walter et al., 1986). Second, the N124I mutant is a potent inhibitor of ER to Golgi transport when transiently expressed *in vivo* (Fig. 4, lane c), even in the absence of the carboxyl-terminal CC motif (Fig. 4, lane d), suggesting that isoprenylation is not essential for function. However, further truncation of the carboxyl terminus leads to loss of inhibitory activity (Fig. 4, lane f), reflecting either the requirement for the hypervariable domain for membrane localization or, alternatively, misfolding and instability *in vivo*. Third, the dominant negative effects of the rabla(N124I) mutant were not sensitive to coexpression with wild type rabla *in vivo* (Fig. 4, lane e), suggesting that it functions in an irre-

Table I. Guanine Nucleotide Binding to Purified Wild Type and Mutant Rabla

His ₆ -rabla	Bound nucleotide (mol/mol)	
	α [³² P]GTP	[³ H]GDP
Wild type	0.85	0.27
N124I	<0.01	<0.01

Guanine nucleotide binding to purified wild type and mutant rabla. His₆-tagged wild type and mutant rabla were purified as described (Nuoffer et al., 1994), and 31-pmol (0.7- μg) aliquots were incubated for 45 min at 30°C in an assay buffer comprising 50 mM Hepes-potassium hydroxide, pH 8, 5 mM EDTA, 2 mM MgCl₂, 1 mM DTT, 0.1 $\mu\text{g/ml}$ BSA, and either 1.4 μM [³H]GDP (specific activity = 3 Ci/mmol) or 1.1 μM α [³²P]GTP (specific activity = 6 Ci/mmol) (final vol = 100 μl). Nucleotide exchange was terminated by the addition of excess MgCl₂, and bound nucleotide measured by liquid scintillation counting after capture of the protein on 0.45 μm nitrocellulose membranes (BA85; Schleicher & Schuell, Inc., Keene, NH).

versible fashion. Finally, the intact rabla(N124I) mutant failed to incorporate [³H]geranylgeranylpyrophosphate when incubated in the presence of cytosol prepared from rat liver homogenates (data not shown). Given the previous observation that additional secondary or tertiary structure is essential for the geranylgeranylation of CC- and CXC-containing proteins (Khosravi-Far et al., 1991, 1992), it is apparent that the conformation of the N124I mutant is different from that of either wild type rab1 or the rab1(S25N) mutant.

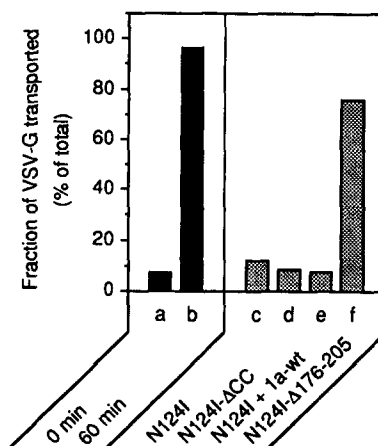


Figure 4. The N124I mutant inhibits transport irreversibly and does not require prenylation for dominant negative function *in vivo*. HeLa cells were infected with the T7 RNA polymerase-recombinant vaccinia virus vTF7-3 and cotransfected with plasmids encoding VSV-G and the indicated rabla constructs as described previously (Nuoffer et al., 1994). After 5-h transfection, cells were pulse-labeled with *trans*-³⁵S label for 10 min, not chased (lane a) or chased for 60 min (lanes b-f). VSV-G was immunoprecipitated, digested with endo H, and the endo H-sensitive, the endo H-resistant intermediate, and the terminally glycosylated forms were separated by SDS-PAGE. Autoradiographs were quantitated by scanning densitometry as described previously (Nuoffer et al., 1994). Transport is expressed as the percentage of total VSV-G converted to endo H-resistant forms. Lanes a and b show cells transfected with VSV-G only; in lanes c-f, cells were cotransfected with VSV-G and the indicated plasmid(s). In lane e, cells were transfected with both rabla(N124I) and rabla-wt. In all cases, expression levels of wild type and mutant proteins (about two- to eightfold) were confirmed using Western blotting (data not shown).

Presence of the Rabla(N124I) Mutant Leads to the Accumulation of VSV-G in Pre-Golgi Carrier Vesicles and VTCs In Vitro

To assess directly the consequences of the rabla(N124I) mutant on ER to Golgi transport, the effects of the mutant on the processing of VSV-G oligosaccharides to the *cis*-Golgi, endoglycosidase D-sensitive form was analyzed in vitro. In this case, perforated 15B CHO cells were incubated in the presence of cytosol at the permissive temperature in the presence of recombinant rabla(N124I) prepared from *Escherichia coli* (Nuoffer et al., 1994). As shown in Fig. 5 A, rabla(N124I) strongly inhibited transport in vitro in a dose-dependent fashion with half-maximal inhibition occurring at ~400 ng (final concentration = 0.5 μ M). Inhibition was abolished if the protein was denatured by boiling before addition to the assay. Preincubation of the mutant in the presence of cytosol reduced the amount of mutant protein required for half-maximal inhibition by ~50%, suggesting that cytosolic factor(s) may participate in establishing the inhibitory phenotype of the rabla(N124I) mutant (data not shown).

Analysis of the temporal sensitivity of the transport reaction to the rabla(N124I) mutant revealed that transport could be inhibited only at early, but not late times of incubation (Fig. 5 B). Cells incubated in the absence of the inhibitor rapidly became insensitive to the subsequent addition of the mutant protein. For example, >50% of maximal transport activity became resistant to the addition of the rabla(N124I) mutant during the first 15 min of incubation (Fig. 5 B, closed squares), a time point at which <10% of the VSV-G had reached the *cis*-Golgi compartment and processed to the endoglycosidase D-sensitive form (Fig. 5 B, open squares). Given that the first 20 min of incubation in vitro is the time period during which VSV-G is sorted and concentrated via 60-nm carrier vesicles (Balch et al., 1994), these results raised two possibilities. Either the N124I mutant inhibits vesicle budding per se, or the mutant protein (or a rab1-regulated transport factor) needs to be recruited during vesicle formation to function at a later vesicle targeting/fusion step.

To distinguish between these two possibilities, the transport of tsO45 VSV-G was examined in vitro using indirect immunofluorescence after incubating permeabilized NRK cells at 32°C in the presence or absence of mutant protein. As expected, before the start of the incubation, tsO45 VSV-G was restricted to the ER (Fig. 6 A) and could not be detected in the Golgi stack, based on the distribution of the *cis*/medial Golgi marker α -1,2 mannosidase II (Man II) (Velasco et al., 1993) (Fig. 6 B). Incubation in the absence (Fig. 6, C and D) or presence of excess wild-type rabla (Fig. 6, E-J) led to the redistribution of VSV-G from the ER to pre-Golgi punctate structures that overlapped with p58 (Fig. 6, E [VSV-G] and F [p58]), a marker for pre-Golgi intermediates (Saraste et al., 1987; Saraste and Svensson, 1991) and to the Golgi compartments containing Man II (Fig. 6, G and I [VSV-G] and H and J [Man II]). In contrast, in the presence of the rabla(N124I) mutant, while the Golgi stack remained intact, overlap of VSV-G with Man II was largely abolished (Fig. 6, M [VSV-G] and N [Man II]; arrows, Golgi compartments), a result consistent with the biochemical data (Fig. 5). However, the N124I mutant, unlike the S25N mutant (Nuoffer et al., 1994), did not affect the export of VSV-G from the ER to p58-containing pre-Golgi intermediates (Fig. 6, K [VSV-G] and L [p58], arrowheads). Using confocal

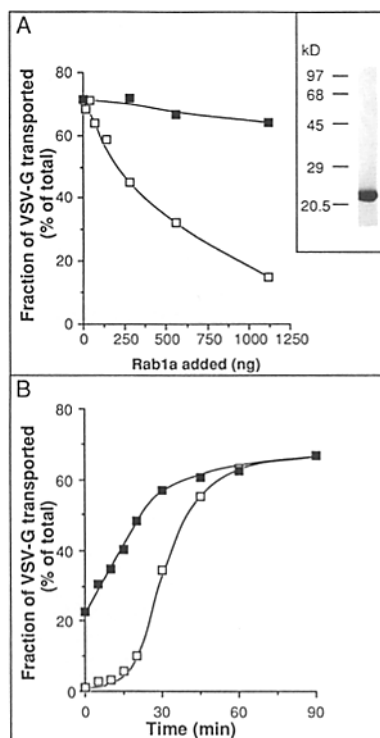


Figure 5. Inhibition of vesicular transport in vitro by rabla(N124I). (A) Vesicular transport between the ER and *cis*-Golgi in perforated 15B CHO cells was measured as described previously (Beckers et al., 1987). Assay mixtures were supplemented with the indicated amounts of rabla wild type (■) or rabla(N124I) (□). The inset shows SDS-PAGE analysis of 2 μ g of the purified mutant protein stained with Coomassie blue. (B) Temporal sensitivity of VSV-G transport to rabla(N124I). At the times indicated, the assays were shifted to ice, and the cells collected by centrifugation (3 min at 500 g). The membranes were resuspended in fresh reaction mixtures supplemented with 1.25 μ g rabla(N124I) and either maintained on ice (□) or incubated at 32°C for the remainder of a total of 90 min (■).

microscopy, the extent of overlap of VSV-G with p58-containing structures was ~75%, with <5–10% overlap with Man II-containing Golgi compartments.

Using immunoelectron microscopy, we next identified the pre-Golgi structures accumulating VSV-G in the presence of the rabla(N124I) mutant after a 45-min incubation at the permissive temperature in vitro. VSV-G was exported from the ER and accumulated in vesicles and numerous VTCs with a high proportion of vesicular profiles (Fig. 7, A and C–D). As shown in Fig. 2 B, the mean linear densities of VSV-G in vesicles (34.5 ± 18.8 gold particles/ μ m, 36 vesicles counted) and VTCs (29.7 ± 11.8 gold particles/ μ m, 37.1 ± 17.0 particles/VTC; 44 VTCs counted) in the presence of rabla(N124I) were similar to the density observed in vesicles in the absence of the mutant protein after 15 min incubation at the permissive temperature (29 ± 17 gold particles/ μ m; Balch et al., 1994). However, the N124I mutant completely blocked transport of VSV-G to Golgi stacks (Fig. 7 A, large arrows). Quantitation revealed that the linear membrane density of VSV-G in Golgi stacks was only 1.2 ± 1.3 gold particles/ μ m (two gold particles per stack, 28 Golgi stacks counted) (Fig. 2 B). No inhibition of transport to the *cis* face of the Golgi stack was observed in the presence of excess wild type rabla (Fig. 7 B), a result consistent with the

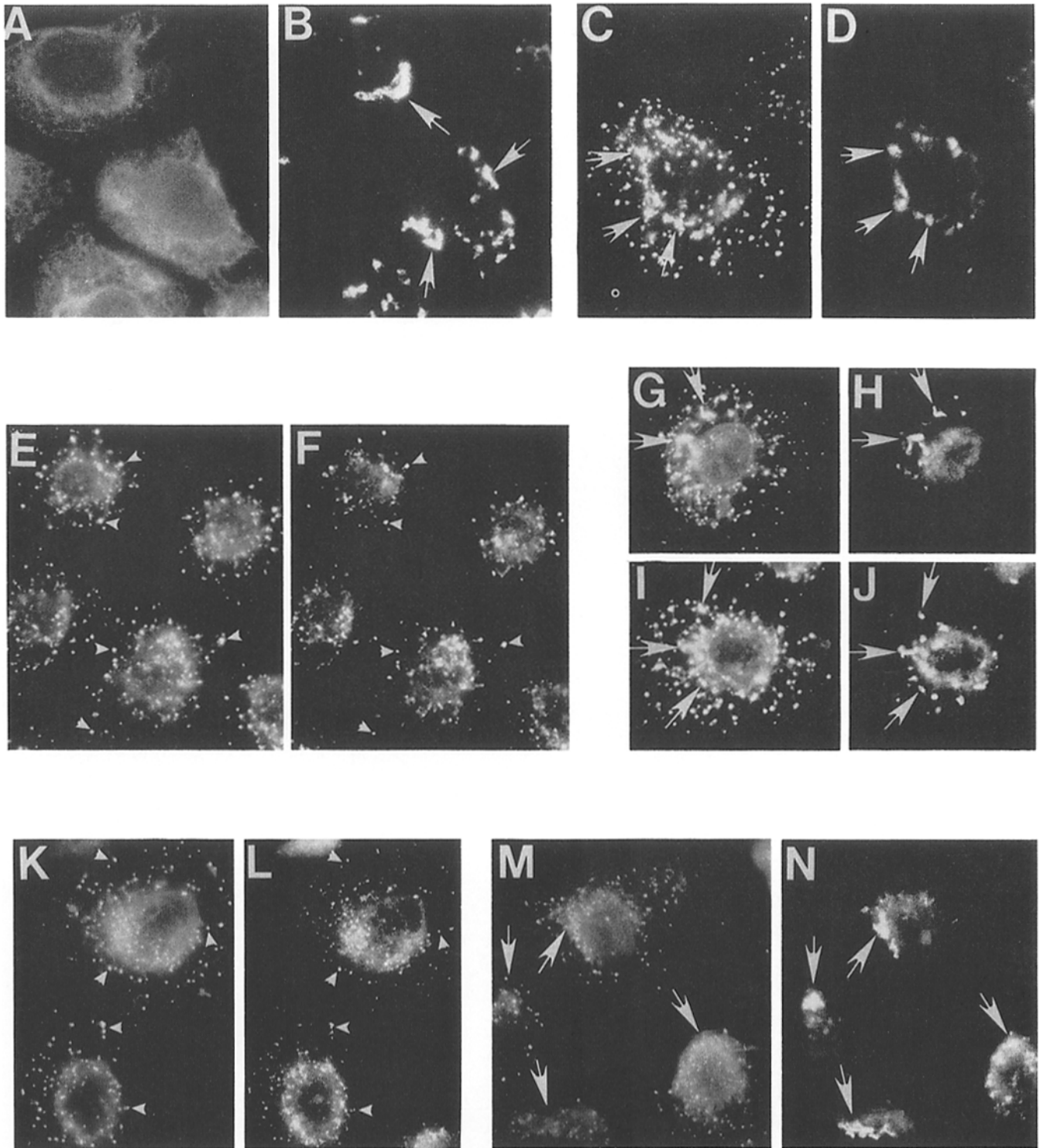
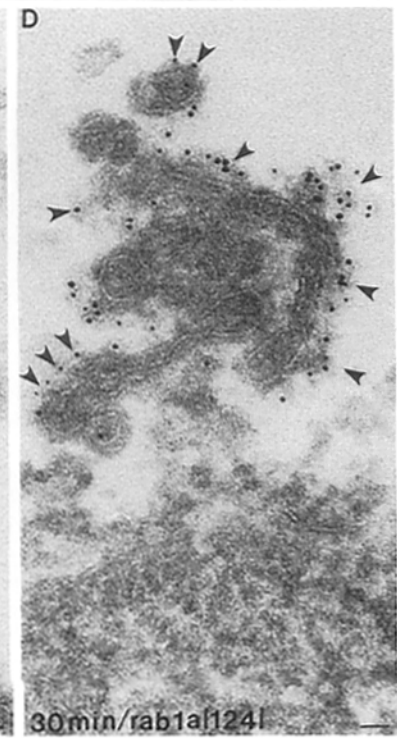
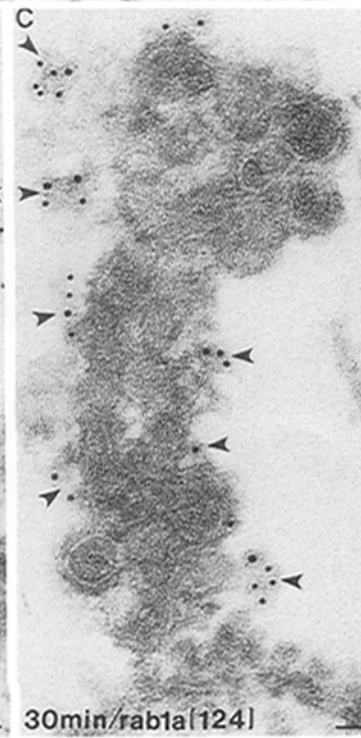
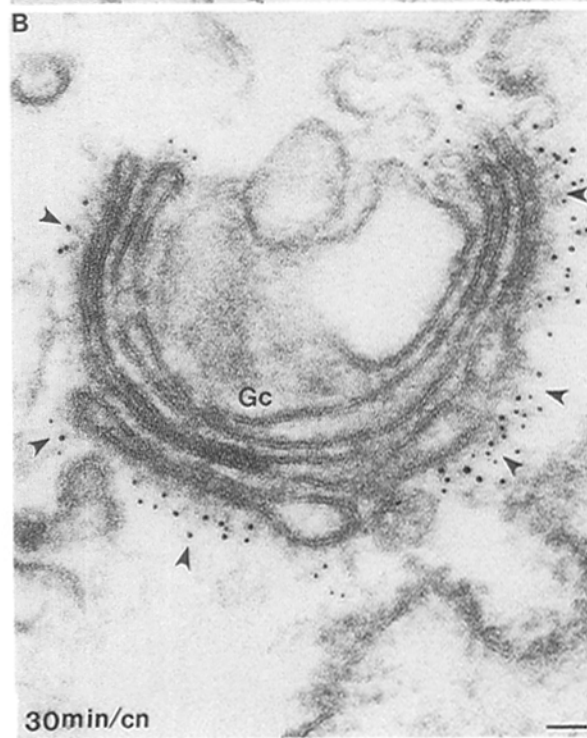
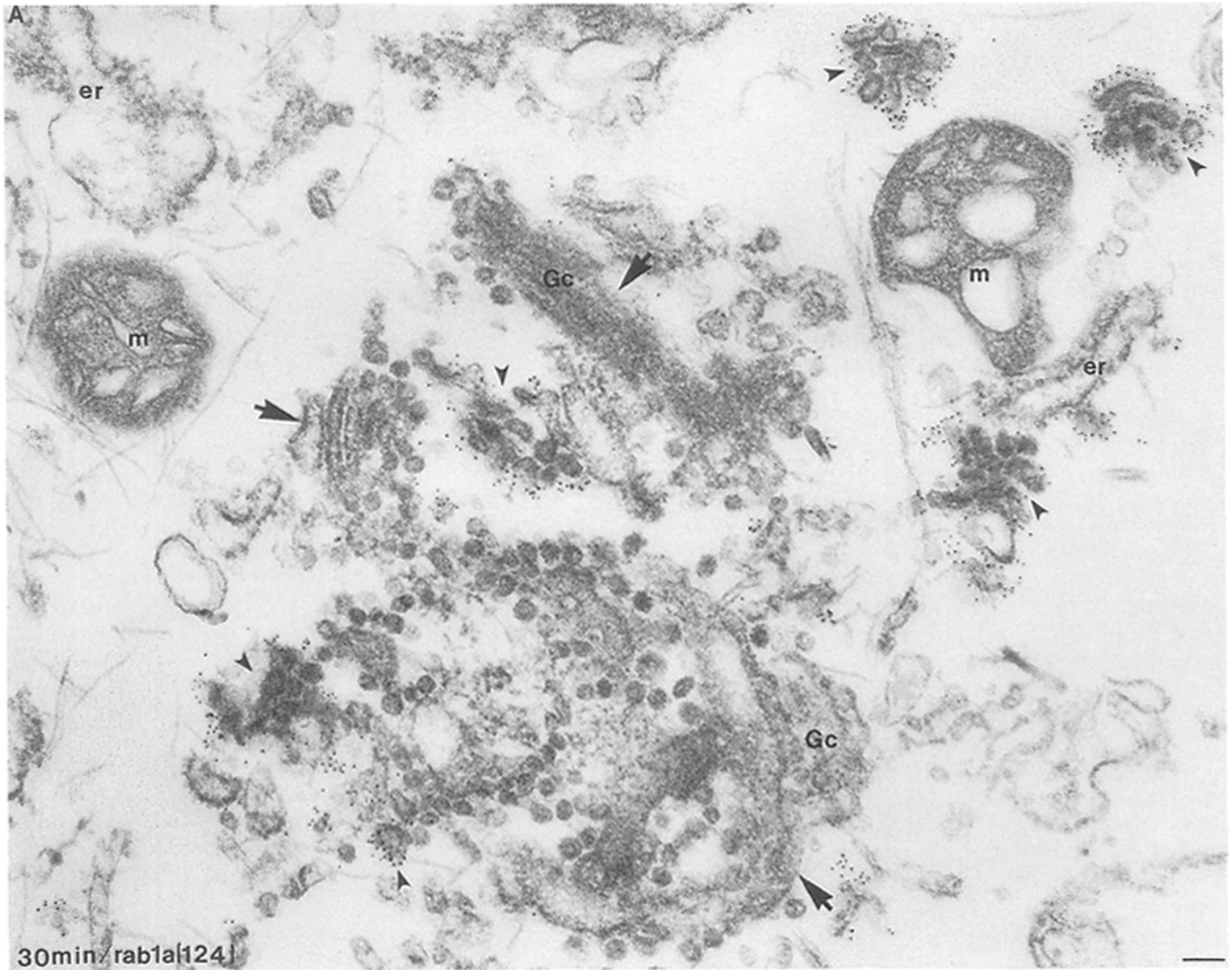


Figure 6. VSV-G accumulates in p58-containing intermediates in the presence of rab1a(N124I). Permeabilized NRK cells were maintained on ice (*A* and *B*) or incubated at 32°C for 80 min in the absence of recombinant rab protein (*C* and *D*), the presence of rab1b wild type (2 μM) (*E–J*), or rab1a(N124I) (2 μM) (*K–N*) as described in the Materials and Methods. The distribution of VSV-G, Man II (a marker for the medial Golgi), or p58 (a marker for pre-Golgi intermediates in NRK cells) was determined by indirect immunofluorescence. *A*, *C*, *E*, *G*, *I*, *K*, and *M* show the distribution of VSV-G; *B*, *D*, *H*, *J*, and *N* show the distribution of Man II; *F* and *L* show the distribution of p58.



efficient processing of VSV-G to Golgi forms in the presence of wild type rabla (Fig. 5). Thus, in contrast to the S25N mutant, N124I mutant inhibits exclusively a late vesicle targeting/fusion step.

A Peptide Identical to the Effector Domain of Rabla Inhibits Transport and Accumulates VSV-G in VTCs

Consistent with a role for rabl in vesicle targeting/fusion, we previously observed a late block in transport using a synthetic peptide identical to the effector domain of rabl (Plutner et al., 1990; Balch et al., 1993). When examined using indirect immunofluorescence (Plutner et al., 1990; Schwaninger et al., 1992), incubation of perforated cells with this peptide mimetic leads to the accumulation of VSV-G in punctate pre-Golgi structures. Using immunoelectron microscopy, we found that incubation in the presence of the effector domain peptide led to the accumulation of VSV-G in VTCs morphologically similar to those observed in the presence of the rabla(N124I) mutant (Fig. 1 E). The mean linear density of VSV-G in these VTCs was 30.5 ± 11 gold particles/ μm with an average of 26.1 ± 10.4 gold particles/VTC (19 VTCs examined) (Fig. 2 C). Consistent with the efficient inhibition of transport by the peptide, no significant transport to the Golgi stack could be detected (Fig. 2 C). Moreover, little VSV-G could be detected in independent carrier vesicles (Fig. 2 C), and the profiles of most VTCs were vesicular rather than tubular in shape (Fig. 1 E). These results are consistent with the possibility that the peptide may sequester the function of an effector protein required for a late vesicle targeting or fusion step.

Rabl and β -COP Colocalize with VSV-G in VTCs

We have recently provided biochemical evidence that the nonclathrin coat protein β -COP (Duden et al., 1991; Serafini et al., 1991; Waters et al., 1991; Orci et al., 1993) is essential for export of VSV-G from the ER and may be recruited coordinately with rabl as part of an 18 to 19S precoat particle (Peter et al., 1993). These results predict that the coat structure present on vesicles and VTCs should contain both rabl and β -COP. Using an antibody specific for β -COP, both vesicles and budding profiles at the tips of cisternae and along the *cis* face of the Golgi stack labeled strongly for β -COP in perforated cells incubated for 30 min at 32°C in vitro (Fig. 8 A). In addition, VTCs containing VSV-G found either adjacent to the nucleus (Fig. 8 B) or in more peripheral locations (not shown) contained β -COP. Similarly, rabl colocalized with VSV-G on the external surface of VTCs (Fig. 8 C) and on the *cis*/medial compartments of Golgi stacks (data not shown). This degree of colocalization is consistent with our previous observations that rabl and β -COP are abundant on pre-Golgi punctate intermediates and Golgi stacks based on

indirect immunofluorescence (Plutner et al., 1992; Schwaninger et al., 1992).

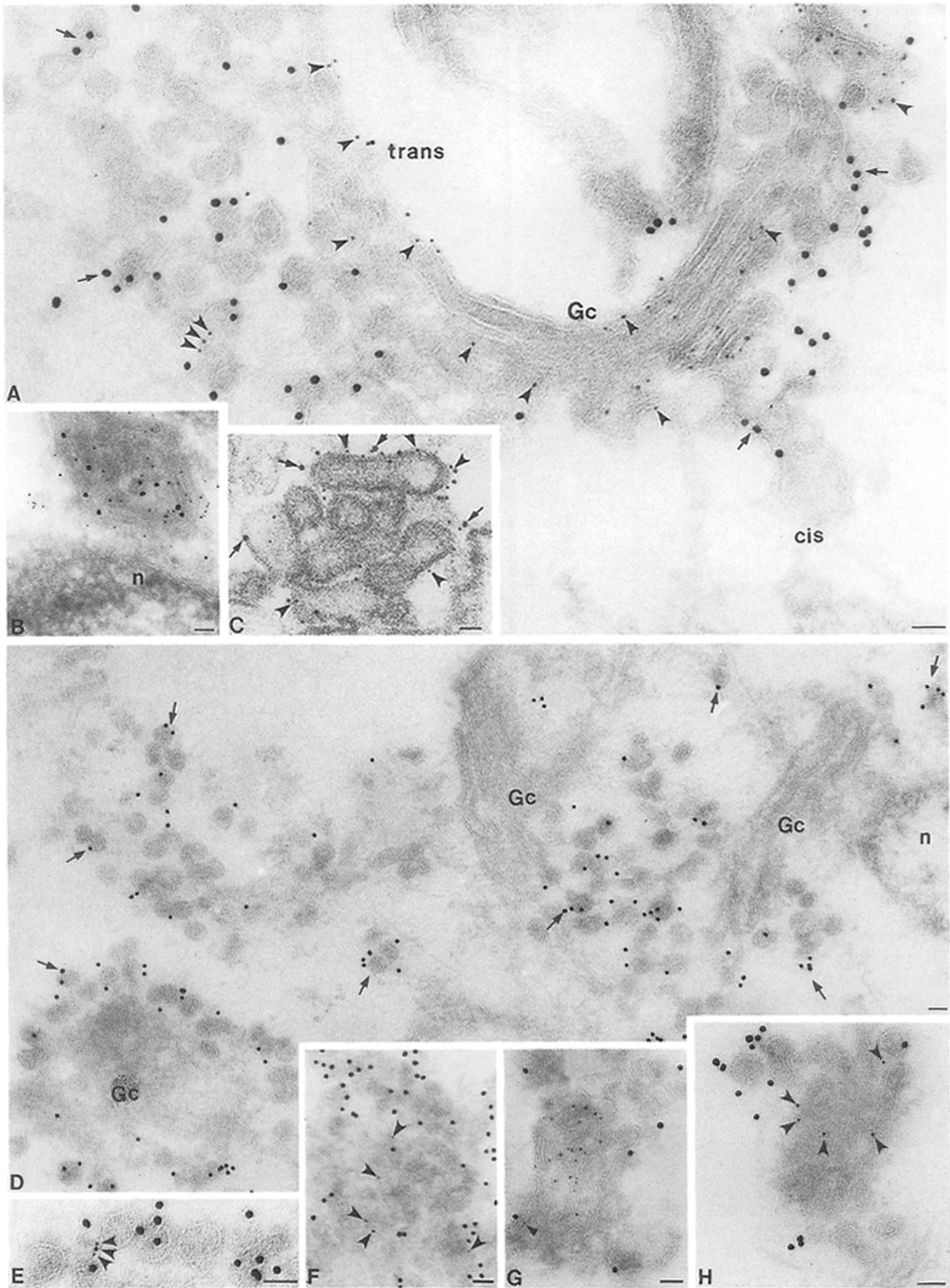
To insure that the VSV-G- and β -COP-containing vesicles observed in Fig. 8 A were not derived from vesicles budding from early Golgi compartments, the distribution of β -COP was examined in cells incubated in the presence of GTP γ S. As expected, under these conditions, the Golgi stack was devoid of VSV-G (compare Fig. 8 D to Fig. 8 A), although β -COP containing budding profiles at the tips of Golgi cisternae, as well as numerous β -COP containing vesicles lacking VSV-G, could be detected along the proximal face. Despite the clear transport block, VSV-G containing vesicles and VTCs derived from the ER also labeled strongly for β -COP (Fig. 8, E-H). The combined results establish that the p58-containing vesicular carriers mediating ER to Golgi traffic in vitro (Balch et al., 1994) contain both rabl and β -COP.

VSV-G Accumulates in Carrier Vesicles and VTCs in the Absence of Ca^{2+}

In yeast, the function of the rabl homologue Ypt1p has been reported to be sensitive to cytosolic Ca^{2+} (Schmitt et al., 1988). Moreover, mutant alleles of PRM1, a Ca^{2+} ATPase present in the Golgi, are strong suppressors of ypt1-1 defects in ER to Golgi transport (Rudolph et al., 1989). Consistent with a potential role for Ca^{2+} in ER to Golgi transport, we have previously demonstrated that EGTA is a potent inhibitor of transport in vitro (Beckers and Balch, 1989; Beckers et al., 1990).

The effect of incubation of perforated cells at the permissive temperature in the absence of Ca^{2+} is shown in Fig. 1. VSV-G was efficiently exported from the ER, but was found to accumulate in tightly packed VTCs (Fig. 1, D and F). As was the case with GTP γ S (Fig. 1, A-C), VTCs formed in the presence of EGTA show a high proportion of vesicular profiles compared to tubular profiles with a mean linear membrane density of 31.7 ± 9.4 gold particles/ μm (Fig. 2 D). However, VTCs accumulated in the presence of EGTA had an average value of 75 ± 26.4 gold particles/VTC (17 VTCs counted). The latter number is indicative of the larger size of the VTCs formed in the presence of EGTA compared to those observed under normal transport conditions at the same time point (21 ± 19 gold particles/VTC; Balch et al., 1994) or in the presence of GTP γ S (30.8 ± 11.8 gold particles/VTC). Moreover, there was a statistically significant ($P = 0.01$) decrease in the linear density of VSV-G in the ER (1.5 ± 1.62 gold particles/ μm membrane length) (Fig. 2 D), compared to that observed before incubation (5.2 ± 2.1 ; Balch et al., 1994) or in the presence of GTP γ S (Fig. 2 A), indicating that VSV-G was efficiently exported from the ER in the absence of cytosolic Ca^{2+} . The combined results suggest that Ca^{2+} is

Figure 7. VSV-G accumulates in pre-Golgi VTCs in the presence of rabla(N124I). Digitonin-permeabilized NRK cells were incubated at 32°C for 45 min in the presence of rabla(N124I) ($2 \mu\text{M}$) (A, C, and D) or wild-type rabla ($2 \mu\text{M}$) (B) as described in the Materials and Methods. In cells incubated with the rabla mutant VSV-G accumulates in VTCs (arrowheads) and does not reach the Golgi stacks (large arrows). C and D show enlargements of VTCs that accumulate in the presence of mutant protein. Methods for labeling and immunoelectron microscopy using the immunodiffusion protocol were as described in Materials and Methods. Bar, $0.1 \mu\text{m}$ in A; $0.05 \mu\text{m}$ in B; and $0.02 \mu\text{m}$ in C and D.



required at a late targeting or fusion step, which also requires rabl function.

Discussion

The Rabla(N124I) Mutant Inhibits Vesicle Targeting/Fusion

Incubation of perforated NRK cells in the presence of the rabla(N124I) mutant led to the accumulation of VSV-G in pre-Golgi vesicles and VTCs, efficiently inhibiting delivery to the Golgi stack. Similarly, transient expression of rablb(N121I) (a functionally equivalent isoform with 92% identity to rabla) in HeLa cells led to the accumulation of VSV-G in punctate, pre-Golgi structures that overlap with the distribution of the pre-Golgi intermediate marker protein p53 (Tisdale et al., 1992) (Plutner, H., and W. E. Balch, unpublished observation) (Schweizer et al., 1988). Consistent with these results, the yeast counterpart of rabl, Ypt1p, has also been suggested to be required for a late vesicle targeting/fusion step in vivo and in vitro (Segev et al., 1988; Bacon et al., 1989; Kaiser and Schekman, 1990; Rexach and Schekman, 1990; Segev, 1991). Moreover, Ypt1p has recently been shown to be a component of 50-nm carrier vesicles involved in ER to Golgi transport (Lian and Ferro-Novick, 1993). Thus, it is apparent that the presence of the N124I mutant blocks transport in a dominant fashion by preventing interaction of the endogenous wild type rabl with a transport component(s) required for the targeting or fusion of transport vesicles with the *cis*-Golgi compartment.

What is the defect in rabla(N124I) function responsible for this phenotype? The equivalent H-ras (N116I) mutant is transforming, suggesting that this mutation leads to constitutive activation (Walter et al., 1986). Based on the inability of both mutants to bind GDP or GTP in vitro and the structural conservation of ras-like GTPases, the N124I mutation may have a similar effect on rabla. If so, the ability of carrier vesicles to form, but not fuse, may reflect an alteration in the conformation of the rabl mutant which prevents its normal cycling from the GTP-bound to the GDP-bound form. In the absence of such a conformational switch, late events regulating vesicle targeting/fusion fail to be initiated. Since we have not directly demonstrated that the mutant is actively recruited during vesicle budding, two sites of action are currently possible. Either the mutant is recruited during vesicle budding in place of endogenous wild type rabl, or alternatively, the mutant binds directly to a downstream component required for vesicle targeting/fusion, thereby preventing completion of the transport cycle by vesicles containing wild type rabl. Interestingly, the rabl effector domain peptide inhibits transport at an identical step. Whether the component inhibited

by rabla(N124I) mutant and that sensitive to the peptide are identical remains to be determined.

Sorting and Concentration Is Complete upon Vesicle Budding from the ER

We previously demonstrated (Balch et al., 1994) that the only concentration step detectable in the transport of VSV-G to the *trans* Golgi network occurs during vesicle budding from the ER. In the present study, we detected no significant increase in the concentration of VSV-G in vesicles or VTCs accumulating in the presence of the rabla(N124I) mutant or inhibitors that block the fusion of carrier vesicles or VTCs to the Golgi stack. These results are consistent with the interpretation that sorting and concentration of VSV-G is completed upon budding from the ER (Balch et al., 1994). They argue against the possibility that concentration may occur as a result of, for example, recycling of membrane and lipid via additional rounds of budding from tubular elements present in VTCs. The results also provide evidence that the rabla(N124I) mutant, the effector domain peptide and the absence of Ca²⁺ lead to the accumulation of a common pre-Golgi intermediate by blocking the normal transport process. Since rabl is also required for transport between Golgi compartments (Nuoffer et al., 1994; Davidson and Balch, 1993), steps in which further concentration cannot be detected (Orci et al., 1986; Balch et al., 1994), it is presently unclear whether rabl per se is directly involved in protein sorting or concentration. However, a number of other small GTPases including Arf (Balch et al., 1992; Dascher and Balch, 1994) and Sar1 (Nakano and Muramatsu, 1989; Oka et al., 1991; d'Enfert et al., 1991; Barlowe et al., 1993; Kuge et al., 1994), as well as an unknown G_{αβγ} (s) protein (Schwaninger et al., 1992) are essential for export from the ER in mammalian cells. These proteins and/or their associated machineries are currently reasonable candidates for the regulation of steps related to protein sorting and concentration.

Role of VTCs in ER to Golgi Transport

Carrier vesicles budding from the ER of NRK cells rapidly (within 5–10 min) organize into VTCs in vitro (Balch et al., 1994), which are similar in structure to putative pre-Golgi elements containing p58 in intact cells (Saraste and Svensson, 1991). A characteristic of both p53- and p58-containing pre-Golgi intermediates in vivo (Schweizer et al., 1991; Saraste and Svensson, 1991), or those observed here in vitro, is the presence of short tubular elements. More extensively tubulated or vacuolated pre-Golgi elements have been observed to accumulate nascent membrane and secretory proteins upon incubation at reduced temperature (Saraste and Kuismanen, 1984; Saraste et al., 1986; Saraste and Svens-

Figure 8. Rabla and β-COP colocalize with VSV-G in VTCs. (A–C) Permeabilized NRK cells were incubated for 30 min at 32°C as described in Materials and Methods. (A) Distribution of β-COP (arrows, large gold) and VSV-G (arrowheads, small gold) in the Golgi stack. β-COP is prominent on the *cis* face of the Golgi and on peripheral vesicles. (B) Enlargement of β-COP- (large gold) and VSV-G- (small gold) containing VTCs showing colocalization of β-COP and VSV-G. (C) Colocalization of rablb (arrows, large gold) and VSV-G (arrowheads, small gold) in VTCs. (D–F) Permeabilized NRK cells were incubated for 45 min at 32°C in the presence of GTPγS. (D) A Golgi region lacking VSV-G. (E–F) ER-derived vesicles and VTCs contain both VSV-G (arrowheads, small gold) and β-COP (large gold). Methods for labeling and immunoelectron microscopy using the immunodiffusion protocol (C) or ultrathin cryosections (A–B and D–H) were as described in Materials and Methods. Bar, 0.05 μm in A–H.

son, 1991; Schweizer et al., 1988, 1990; Lotti et al., 1992) or during virus infection, particularly using viruses which mature in pre-Golgi intermediates (Tooze et al., 1984; Soedeik et al., 1993; Krijnse-Locker et al., 1994). We suggest that the morphology of pre-Golgi intermediates observed in different cell lines, in the presence of selected viruses and under different incubation conditions, is likely to vary considerably reflecting rate-limiting step(s) in either vesicle budding from the ER or in the targeting and fusion of peripherally derived 60-nm carrier vesicles to the perinuclear *cis*-Golgi compartment. Our results are most consistent with the interpretation that transport between the ER and the *cis*-Golgi compartment involves a single round of vesicle budding/fusion. Since VTCs lack continuity with the ER, lack the abundant ER marker protein CaBP3, and appear subsequent to the budding of individual vesicles, we consider it likely that they represent collection of vesicles en route to the *cis*-Golgi compartment.

Role of Ca^{2+} in Transport

Both ER to Golgi (Beckers et al., 1990) and intra-Golgi transport (Schwaninger et al., 1991; Davidson and Balch, 1993) require Ca^{2+} . In this paper we have shown that the fusion of vesicles or VTCs with the acceptor compartment is the site of Ca^{2+} dependence. Accumulation of VTCs was particularly striking in the absence of Ca^{2+} and, like those accumulated in the presence of the rab1 effector domain peptide, these VTCs frequently appeared to have a higher content of vesicles as opposed to the short, tubular elements observed in VTCs formed under normal incubation conditions, suggesting that Ca^{2+} was also required for the fusion of nascent vesicles to generate these tubular elements. Thus, VTCs principally represent a collection of vesicles en route to the perinuclear Golgi. The ability of ER-derived carrier vesicles to form short tubules in VTCs may reflect the well-documented ability of "like" compartments of the exocytic and endocytic pathways to fuse with each other.

It is now apparent that rab proteins and a family of Ca^{2+} -dependent proteins may specifically regulate the fusion of carrier vesicles mediating traffic between many different compartments of the exocytic pathway (Bennett and Scheller, 1993; Söllner et al., 1993). For example, rabphilin is a Ca^{2+} -binding protein possibly involved in rab3 function (Shiratakis et al., 1993). Moreover, several lines of evidence have suggested a link between the requirement for Ca^{2+} and rab1 function (Schmitt et al., 1988). The PMR1 gene in yeast encodes a Ca^{2+} -adenosine triphosphatase (ATPase) that colocalizes to early Golgi compartments (Antebi and Fink, 1992). Pmr1 mutants are strong suppressors of ypt1-1 defects in ER to Golgi transport (Rudolph et al., 1989), suggesting that these two components function in the context of a protein complex that participates in vesicle targeting and/or fusion. In some respects, the fusion of VTCs with the *cis*-Golgi compartment is reminiscent of the rab3/ Ca^{2+} -dependent fusion of vesicles to the cell surface during regulated secretion (reviewed in Holz et al., 1991; see Oberhauser et al., 1992; Padfield et al., 1992; Senyshyn et al., 1992). However, unlike regulated events at the cell surface, which require a transient increase in Ca^{2+} as part of an upstream signaling pathway, ER to Golgi vesicles and VTCs function optimally at normal intracellular (~ 100 nM) Ca^{2+} concentrations (Beckers and Balch, 1989; Rexach and Schekman, 1990). At this time, we prefer

the model in which both rab1 and a Ca^{2+} -binding protein facilitate the fusion of pre-Golgi carriers to the *cis*-Golgi compartment.

A Model for Rab1 Function in Vesicle Formation and Fusion

The cumulative evidence from this and previous studies (Tisdale et al., 1992; Davidson and Balch, 1993; Nuoffer et al., 1994) allows us to propose a tentative working model for the function of rab1 in ER to Golgi transport (Fig. 9). This model, in principle, is also applicable to vesicular traffic between early Golgi compartments. We propose that rab1 is recruited in the GDP-bound conformation, either as part of a 18-19S precoat particle containing β -COP or in the form of a smaller guanine nucleotide dissociation inhibitor (GDI)-rab1 complex (Fig. 9). The requirement for β -COP is derived from our recent observations that a functional pool of rab1 involved in vesicle budding is associated with a high molecular weight protein complex containing β -COP (Peter et al., 1993). Consistent with these results, we demonstrated in the present study that β -COP is associated with vesicles and VTCs containing VSV-G en route to the *cis* face of the Golgi stack in vitro. A similar distribution of β -COP to pre-Golgi carriers has been detected in vivo (Hendricks et al., 1993; Oprins et al., 1993; Pepperkok et al., 1993). GDI is now believed to be associated with the GDP-bound, cytosolic form of rab proteins and to assist in their delivery to the membranes (Soldati et al., 1993; Ullrich et al., 1993). The possibility that GDI is involved in an early recruitment step is consistent with our observations that a second, low molecular weight pool of rab1, which is likely to represent a GDI-rab1 complex (Balch, W. E., and F. Peter, unpublished observations), can also serve as a source for rab1 during vesicle budding from the ER (Peter et al., 1993). The necessity for recruitment of rab1 in the GDP-bound form is also consistent with our previous observation that overexpression of rab1b(Q67L), a mutant that is likely to be restricted to the GTP-bound form because of defective GTP hydrolysis, is not a dominant inhibitor of transport in vivo (Tisdale et al., 1992), suggesting that it is excluded from the

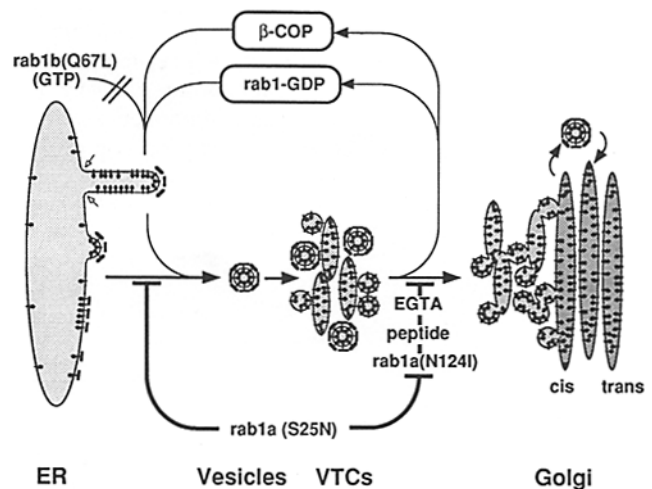


Figure 9. A summary of the role of rab1 in the regulation of vesicles and VTCs mediating vesicular traffic between the ER and the Golgi. See text for details.

transport machinery (Fig. 9). Finally, the model is consistent with the observation that recruitment is likely to involve a protein (GEP), given the inhibitory properties of the S25N mutant which markedly attenuates export from the ER by possibly inhibiting rab1-GEP (Nuoffer et al., 1994).

GTP hydrolysis, presumably mediated by a rab1-specific GTPase-activating protein, is likely to be involved in a late vesicle targeting/fusion step. This interpretation is consistent with the observation that rab1 is found on ER to Golgi vesicular carriers. As suggested above, the N124I substitution may restrict rab1 to a conformation which allows it to interact in an irreversible fashion with an effector involved in vesicle targeting or fusion (Fig. 9). While the putative target for rab1 involved in targeting/fusion is unknown, recent studies have found that H-ras(N116I) can form a complex with a purified ras-specific GEP (Hwang et al., 1993). These results raise the possibility that the N124I mutant can at least interact and be recruited by a rab1-GEP during vesicle budding. However, the mode of interaction of the S25N and N124I mutants with GEP and/or other transport components are clearly different in that the two mutants diverge with respect to their requirements for posttranslational isoprenylation, competition with wild type rab1, and morphological phenotypes. One possibility to account for these results is that while the S25N mutant reduces the overall activity or efficiency of rab1-GEP in promoting wild type rab1 recruitment, the N124I mutant adopts a conformation that supports its efficient recruitment during coat assembly and vesicle budding, but renders it unable to undergo a critical conformational change required to initiate vesicle targeting/fusion (Fig. 9). Since both mutants lead to inhibition of intra-Golgi transport and loss of the Golgi stack in a fashion distinct from that observed for brefeldin A or microtubule depolymerizing agents such as nocodazole (Nuoffer et al., 1994; Balch, W. E., unpublished results), it is apparent that the integrity of the Golgi stack is exceptionally sensitive to the disruption of vesicular traffic between compartments (see Wilson et al., 1994).

Given the complexities of events involved in vesicle budding and fusion, further experiments are currently in progress to identify rab1-GEP and the targeting/fusion machinery regulated by rab1 and rab1-specific GTPase-activating protein. With these components in hand, we should be able to refine the above working model, which is largely derived from the use of selected mutants defective in guanine nucleotide exchange and hydrolysis.

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