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# COPI Recruitment Is Modulated by a Rab1b-dependent Mechanism

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The small GTPase Rab1b is essential for endoplasmic reticulum (ER) to Golgi transport, but its exact function remains unclear. We have examined the effects of wild-type and three mutant forms of Rab1b in vivo. We show that the inactive form of Rab1b (the N121I mutant with impaired guanine nucleotide binding) blocks forward transport of cargo and induces Golgi disruption. The phenotype is analogous to that induced by brefeldin A (BFA): it causes resident Golgi proteins to relocate to the ER and induces redistribution of ER-Golgi intermediate compartment proteins to punctate structures. The COPII exit machinery seems to be functional in cells expressing the N121I mutant, but COPI is compromised, as shown by the release of β-COP into the cytosol. Our results suggest that Rab1b function influences COPI recruitment. In support of this, we show that the disruptive effects of N121I can be reversed by expressing known mediators of COPI recruitment, the GTPase ARF1 and its guanine nucleotide exchange factor GBF1. Further evidence is provided by the finding that cells expressing the active form of Rab1b (the Q67L mutant with impaired GTPase activity) are resistant to BFA. Our data suggest a novel role for Rab1b in ARF1- and GBF1-mediated COPI recruitment pathway.

## INTRODUCTION

The secretory pathway in mammalian cells consists of a linear assembly of dynamic compartments. Transport and recycling between these compartments occur through generation of transport intermediates from the donor compartment and the delivery of such intermediates to the appropriate acceptor compartment. A family of Rab proteins belonging to the Ras superfamily of small GTPases has emerged as essential regulators of all stages of membrane traffic (Pfeffer, 2001; Segev, 2001b; Zerial and McBride, 2001). Rabs have been proposed to act in diverse aspects of vesicular transport, including vesicle formation, motility, docking, and fusion. However, despite considerable advances, the exact mechanism of Rab function remains unclear.

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Abbreviations used: ER, endoplasmic reticulum; VTC, vesicular tubular clusters; BFA, brefeldin A; GFP, green fluorescent protein, GDI, GDP-dissociation inhibitor; ERGIC, ER-Golgi intermediate compartment; VSV-G, vesicular stomatitis virus glycoprotein.

In the early secretory pathway, two isoforms of Rab1, Rab1a and Rab1b, have been shown to be required for protein transport from the endoplasmic reticulum (ER) to the *cis*-Golgi (Plutner *et al.*, 1991; Tisdale *et al.*, 1992; Nuoffer *et al.*, 1994; Pind *et al.*, 1994). A yeast homologue of Rab1, YPT1, is also essential for ER-to-Golgi transport (Segev *et al.*, 1988). The cellular function of Rab1 is likely to involve temporally and spatially distinct interactions with its effectors. Two Rab1 effectors have been identified as the tethering factors p115 and GM130 (Allan *et al.*, 2000; Moyer *et al.*, 2001; Weide *et al.*, 2001). Similarly, in yeast, YPT1 has been shown to interact genetically with the yeast homologue of p115, USO1 (Sapperstein *et al.*, 1996).

Rabs cycle continuously between a GDP- and a GTP-bound form (Schimmoller *et al.*, 1998). The GTP-bound conformation is regarded as "active" and can interact with downstream effector proteins (Segev, 2001a; Zerial and McBride, 2001). Conversion of the GTP- to the GDP-bound form is caused by GTP hydrolysis, facilitated by a GTPase-activating protein. After GTP hydrolysis and the corresponding conformational shift, Rabs interact with a GDP dissociation inhibitor (GDI) that can extract them from the membrane and support their transient existence in the cytosol. The Rab-GDI complex is then specifically recognized by a membrane Rab receptor (the identity of which is un-

known) that displaces the GDI and subsequently allows the Rab to bind a new GTP in a reaction mediated by a guanine nucleotide exchange factor. Because the continuous cycling of Rabs between these states is necessary for their function, mutations that alter nucleotide loading, exchange, or hydrolysis can be used to explore the cellular role of $\alpha$ Rab.

To explore the function of Rab1b in vivo, we used a "dominant negative" approach shown to be useful in elucidating the functions of distinct Rabs and their effectors (Segev, 2001a; Zerial and McBride, 2001). We generated a Q67L mutant with low GTPase activity, an S22N mutant with low affinity for GTP but normal affinity for GDP (Nuoffer *et al.*, 1994), and an N121I mutant with low affinity for both GDP and GTP (Pind *et al.*, 1994). Although some mutants of Rab1b or the equivalent mutants of Rab1a (Q65L, S25N, N124I) have been partially characterized (Tisdale *et al.*, 1992; Nuoffer *et al.*, 1994; Pind *et al.*, 1994), previous experiments examined limited number of parameters and used different experimental systems. We used the same methodology to examine the effects of all the Rab1b mutants on (1) ER-Golgi traffic, by monitoring the transport of a cargo protein; (2) Golgi and ER-Golgi intermediate compartment (ERGIC) structure, by analyzing the localization of resident Golgi and ERGIC proteins; (3) the response of Rab1b effectors, by analyzing the behavior of p115 and GM130; (4) the status of the COPII machinery, by exploring the behavior of Sec13 and Sec31; and (5) the status of the COPI machinery, by exploring the behavior of $\beta$-COP.

Our data indicate that expression of the wild-type Rab1b and the constitutively active Q67L mutant has limited effect on ER-Golgi trafficking and Golgi structure. In contrast, the inactive S22N mutant causes partial Golgi disruption, whereas the inactive N121I mutant completely disrupts Golgi structure. The N121I mutant causes brefeldin A (BFA)-like phenotype and induces the relocation of resident Golgi proteins to the ER and the redistribution of ERGIC53, GM130, and p115 to punctate structures shown in BFA-treated cells to represent ER exit sites and arrested vesicular tubular clusters (VTCs) (Ward *et al.*, 2001). In addition, N121I causes the dissociation of $\beta$-COP from membranes, implicating Rab1b in a pathway leading to COPI recruitment. Supportive evidence for the role of Rab1b in COPI dynamics was provided by the rescue of the N121I phenotype by expression of ARF1 and GBF1, known mediators of COPI recruitment (Lippincott-Schwartz *et al.*, 1998; Kawamoto *et al.*, 2002). Similarly, like expression of ARF1 or GBF1, expression of the active Q67L mutant of Rab1b prevented Golgi fragmentation and $\beta$-COP dissociation in BFA-treated cells. Together, our data suggest a role for Rab1b in the GBF1/ARF1-mediated pathway for COPI recruitment. Future work will be necessary to address how Rab1b modulates the COPI recruitment machinery.

## MATERIALS AND METHODS

### *Antibodies*

Rabbit polyclonal antibodies against p115 and mouse polyclonal antibodies against GM130 have been described (Barroso *et al.*, 1995; Nelson *et al.*, 1998). Monoclonal anti-giantin G1/133 (Linstedt and Hauri, 1993) and monoclonal anti-ERGIC53 G1/93 (Schweizer *et al.*, 1988) antibodies were provided by Dr. Hans-Peter Hauri (University of Basel, Basel, Switzerland). Polyclonal antibodies against Mann II (Velasco *et al.*, 1993) were provided by Dr. Marilyn Farquhar (University of California, San Diego, CA). Rabbit polyclonal anti-Rab1b and anti-myc antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Monoclonal anti-myc antibodies were purchased from Invitrogen (Carlsbad, CA). Monoclonal anti-VSV-G (P5D4) antibodies (Roman *et al.*, 1988) were provided by Dr. Kathryn Howell (University of Colorado, Denver, CO). Goat anti-rat and anti-mouse antibodies conjugated with Oregon Green or Texas Red-X were purchased from Molecular Probes (Eugene, OR). Antibodies against Sec13 and Sec31 (Tang *et al.*, 2000) were provided by Dr. Bor Luen Tang (National University of Singapore, Republic of Singapore).

### *Generation of Constructs*

The GalT-GFP construct was provided by Dr. Brian Storrie (Virginia Tech, Blacksburg, VA) (Storrie *et al.*, 1998). ARF1 wild-type and ARF1Q71L were gifts from Dr. Julie Donaldson (National Institutes of Health, Bethesda, MD). Full-length Rab1b was cloned into the *Eco*RI-*Not*I restriction sites of the pEF6/*Myc*-His B 6P vector (Invitrogen, Carlsbad, CA). Full-length Rab1b was obtained by PCR using as a template a human cDNA library. The primers used for rab1b were 5′-CCGGAATTCCCATGAACCCCGAATATGAC-3′ (forward primer) and 5′-TGCCCGCGGCCGCAACAGCCACCGC-CAGCG-3′ (reverse primer) to amplify full-length rab1b (sequence data available from Gen Bank under accession number NM030981). Point mutations were introduced with a Quick Change Site-Directed Mutagenesis Kit according to the manufacturer's protocol (Stratagene Corp., La Jolla, CA). All mutated Rab1 sequences were verified by sequencing. GFP-rab1b versions were subcloned from their respective rab1-myc constructs into pEGFP-vector. GBF1 was obtained from a partial human GBF1 cDNA (KIAA0248) from the Kazusa DNA Research Institute in Chiba, Japan. The missing fragment was amplified from a human lung cDNA library. The PCR product was then subcloned into the KIAA0248 clone using the internal *Eco*RI site at base 1124 and an engineered external *Xho*I site. To generate GBFmyc, wtGBF1 was amplified by PCR and subcloned into pcDNA4.0/TO/myc-his (Invitrogen, Carlsbad, CA).

### *Morphological Analysis of VSV-G Transport*

Analysis was performed as described previously (Alvarez *et al.*, 1999). Briefly, HeLa cells plated on coverslips were transfected with Rab1 constructs. After 24 h, cells were infected with VSVtsO45 at 32°C for 30 min, followed by incubation at 42°C for 3 h to accumulate VSV-G in the ER. The cells were then shifted to 32°C for different amounts of time to allow VSV-G transport to the Golgi and the plasma membrane. Transport was terminated by transferring coverslips to ice and fixing them in 3% formaldehyde/PBS for 10 min. The coverslips were then processed for double-label immunofluorescence.

### *Cell Culture and Immunofluorescence Microscopy*

Cells grown on glass coverslips were washed three times in PBS and fixed in 3% paraformaldehyde in PBS for 10 min at room temperature. Paraformaldehyde was quenched with 10 mM ammonium chloride, and cells were permeabilized with PBS, 0.1% Triton X-100 for 7 min at room temperature. The coverslips were washed (three times, 2 min per wash) with PBS, then blocked in PBS, 0.4% fish skin gelatin, 0.2% Tween 20 for 5 min, followed by blocking in PBS, 2.5% goat serum, 0.2% Tween for 5 min. Cells were incubated with primary antibody diluted in PBS, 0.4% fish skin gelatin, 0.2% Tween 20 for 45 min at 37°C. Coverslips were washed (five times, 5 min per wash) with PBS, 0.2% Tween 20. Secondary antibodies coupled to Oregon Green or Texas Red-X were diluted in 2.5% goat serum and incubated on coverslips for 30 min at 37°C. Coverslips were washed with PBS, 0.2% Tween 20 as above and mounted on slides in 9:1 glycerol:PBS with 0.1% *q*-phenylenediamine. Fluorescence patterns

were visualized with an Olympus IX70 epifluorescence microscope. Optical sections were captured with a CCD high-resolution camera equipped with a camera/computer interface. Images were analyzed with a power Mac using IPLab Spectrum software (Scanalytics Inc., Fairfax, VA).

## RESULTS

### *Generation and Expression of Mutant Forms of Rab1b*

To generate reagents for our studies, a myc-his tag was added to the C terminus of wild-type and three mutant forms of Rab1b (Q67L, S22N, and N121I). Because Rab1b is prenylated on C-terminal cysteine residues (Khosravi-Far *et al.*, 1991), and this modification is important for its membrane association, we also generated wild-type and mutant forms tagged at the N-terminus with the green fluorescent protein (GFP). As shown in Figure 1, A and B, myc- or GFP-tagged proteins of the appropriate molecular weight (~27 and ~55 kDa, respectively) were detected in lysates from transfected HeLa cells but not in control lysates. Approximately the same amounts of wild-type Rab1b and each mutant were detected 24 h after transfection, compared with the loading baseline provided by calnexin.

The myc- and GFP-tagged proteins show analogous localization (Figure 1, C and D). The myc- and GFP-tagged wild-type and Q67L mutants localize predominantly to morphologically normal Golgi membranes. In contrast, the S22N mutants show more diffuse patterns, most likely representing cytosolic and reticular ER localization. The N121I mutants show diffuse cytosolic staining. Our results with myc- and GFP-tagged Rab1b are identical to previously reported localization of GFP-tagged Rab1a (Moyer *et al.*, 2001b).

### *Effects of Mutant Forms of Rab1b on Cargo Transport*

Previous studies have shown that the S22N and the N121I mutants of Rab1a cause dominant negative effects on secretory traffic (Tisdale *et al.*, 1992; Nuoffer *et al.*, 1994; Pind *et al.*, 1994). To functionally characterize our Rab1b mutants, we analyzed the transport of a transmembrane glycoprotein (VSV-G) of the vesicular stomatitis virus (VSV) in HeLa cells transfected with wild-type or mutant Rab1b. The infection was performed at the nonpermissive temperature of 42°C to accumulate VSV-G in the ER (Figure 2, 42°C, insets). The cells were then shifted to the permissive temperature of 32°C for 40 min to allow VSV-G to be transported to the Golgi (Figure 2, 32°C, 40 min panels) or shifted to the permissive temperature of 32°C for 120 min to allow VSV-G to be transported to the PM (Figure 2, 32°C, 120 min panels). The localization of VSV-G was monitored by immunofluorescence. VSV-G is transported to the Golgi at 40 min and to the PM at 120 min in cells expressing wild-type Rab1b or the Q67L mutant (transfected cells in this and following figures are denoted with asterisks). These results are consistent with previous biochemical findings on Rab1a showing that VSV-G becomes endo-H resistant in cells transfected with the Q67L mutant (Tisdale *et al.*, 1992). In cells expressing the S22N mutant, VSV-G is found in perinuclear punctate structures and does not transit to the PM, even after 120 min at the permissive temperature. This is consistent with biochemical data on Rab1a showing that expression of the S22N mutant in cells prevents endo-H resistance of VSV-G (Tisdale *et al.*, 1992). In cells expressing the N121I mutant, VSV-G is retained in the ER, consistent with biochemical data on Rab1a showing Endo-H sensitivity of VSV-G in transfected cells (Tisdale *et al.*, 1992; Pind *et al.*, 1994).

### *Effects of Mutant Forms of Rab1b on Golgi Structure*

The effects of expressing wild-type or mutant Rab1b in vivo have not been reported previously. As shown in Figure 3, cells expressing the wild-type or the Q67L mutant have normal Golgi, as visualized by the localization of two resident Golgi proteins (mannosidase II and giantin), and normal ERGIC, as visualized by the distribution of ERGIC53. In contrast, transient expression of the S22N mutant leads to partial Golgi disruption and relocation of mannosidase II and giantin into perinuclear elements. Such elements seem to be concentrated in the Golgi region, but some more peripheral structures are also detected. The ERGIC53 pattern also shows partial redistribution to more peripheral elements. The Golgi is completely disrupted in cells expressing the N121I mutant, with mannosidase II and giantin disappearing from the Golgi and redistributing to the ER. The disruption seems to be progressive, and in some cells (arrowhead), punctate perinuclear structures containing giantin can be seen. An adjacent cell, presumably expressing higher levels of the mutant or for a longer period, has a completely diffuse giantin pattern. The distribution of ERGIC53 seems to be less disturbed, although relocation of the protein from a peri-Golgi region to peripheral structures is observed. Quantitative analysis shows that the vast majority of cells expressing the S22N mutant (88% of transfected cells) or the N121I mutant (85% of transfected cells) present the described phenotypes. The disruption was observed even at moderate and low levels of expression, attesting to the strong dominant effects of these Rab1b mutants in cells.

The effects of the N121I mutant resembled those caused by BFA treatment, and representative images of cells treated with BFA are shown in Figure 3, BFA panels. BFA treatment causes Golgi disassembly and the relocation of resident Golgi proteins to the ER. BFA also induces relocation of ERGIC53 to punctate structures, shown by others to represent ER exit sites and immature VTCs (Ward *et al.*, 2001).

We also explored the localization of two Rab1 effectors, GM130 and p115, in cells expressing wild-type and mutant Rab1b. As shown in Figure 4, the localization of GM130 and p115 is not influenced by the expression of either the wild-type Rab1b or the Q67L mutant. In cells expressing the S22N mutant, GM130 and to a greater extent p115 are redistributed from the Golgi to punctate peripheral structures. In cells expressing the N121I mutant, GM130 and p115 localization is disrupted, and both are found in small punctate structures dispersed throughout the cell. The observed patterns are analogous to those induced by BFA treatment (Figure 4, BFA panels). BFA inhibits the activity of guanine nucleotide exchange factors (GEFs) for ARFs and prevents COPI recruitment (Donaldson *et al.*, 1992; Helms and Rothman, 1992). Because expression of the N121I mutant causes BFA-like phenotype, we explored whether Rab1b could be involved in COPI recruitment.

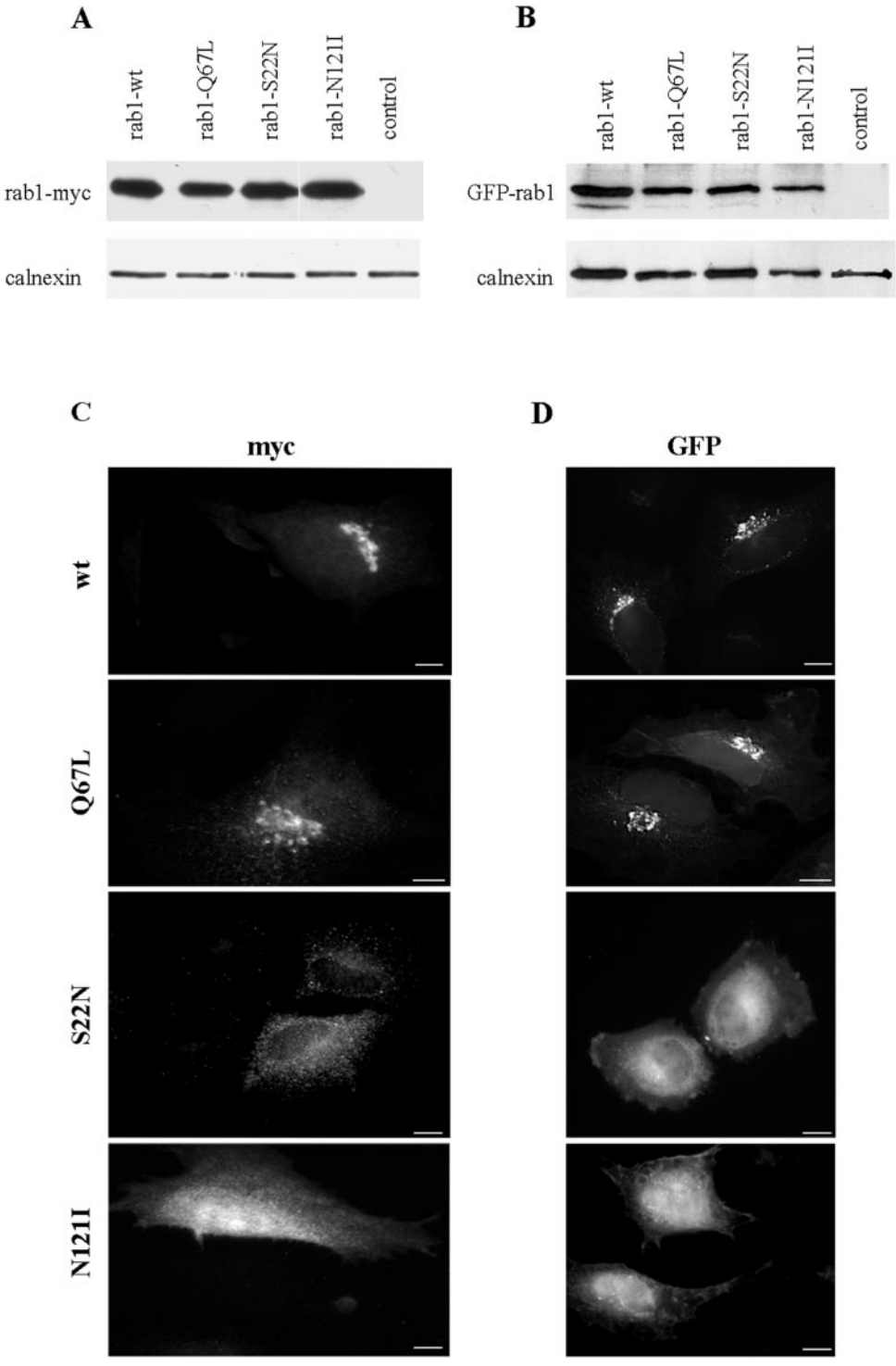


**Figure 1.** Expression and localization of wild-type and mutant Rab1b. HeLa cells were transfected with myc- or GFP-tagged wild-type Rab1b, the Q67L mutant, the S22N mutant, or the N121I mutant and analyzed 24 h later by Western blotting (A and B) or immunofluorescence (C and D). Cell lysates were obtained and processed by SDS-PAGE and immunoblotted with anti-myc and anti-calnexin antibodies (A) or with anti-GFP and anti-calnexin antibodies (B). Control lanes contain lysates from untransfected cells. Levels of expression of all constructs are similar. Cells were processed for immunofluorescence by use of anti-myc antibodies (C) or GFP fluorescence (D). Wild-type Rab1b and the Q67L mutant localize to the Golgi. The S22N mutant is detected in the cytosol and also in a reticulate pattern. The N121I mutant shows diffuse cytosol staining.

### *A Mutant Form of Rab1b Perturbs COPI Coat Assembly*

We first examined the effects of Rab1b mutants on the assembly of COPII coats, because these have been reported to be unaffected by BFA treatment (Ward *et al.*, 2001). As shown in Figure 5, the localization of the Sec13 and the Sec31components of the COPII coat seems to be normal in cells expressing the wild-type or the Q67L mutant. Sec13 and Sec31 are present in punctate structures concentrated in the Golgi region and also in more peripheral sites. In cells

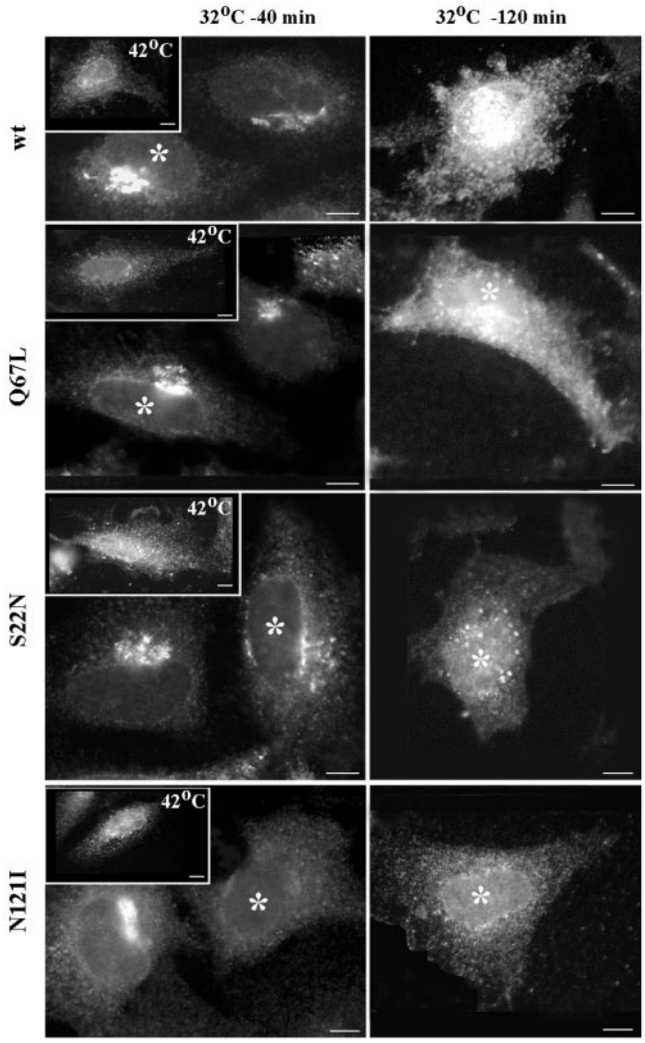


**Figure 2.** Rab1b mutants block cargo transport. HeLa cells were transfected with wild-type Rab1b, the Q67L mutant, the S22N mutant, or the N121I mutant. After 24 h, cells were infected with VSVtsO45 at 42°C. After 3 h, cells were either fixed (insets), incubated at 32°C for 40 min (32°C-40 min panels), or incubated at 32°C for 120 min (32°C-120 min panels) before fixation. Cells were analyzed by immunofluorescence with anti-myc antibodies to detect transfected cells (asterisks) and with anti-VSV-G antibodies. In cells expressing wild-type Rab1b or the Q67L mutant, VSV-G is transported to the Golgi at 40 min and to the cell surface at 120 min. In cells expressing the S22N mutant, VSV-G is transported out of the ER into punctate structures at 40 min and remains in such structures at 120 min. In cells expressing the N121I mutant, VSV-G is retained in the ER at 40 min and at 120 min. Bars, 10 $\mu$m.

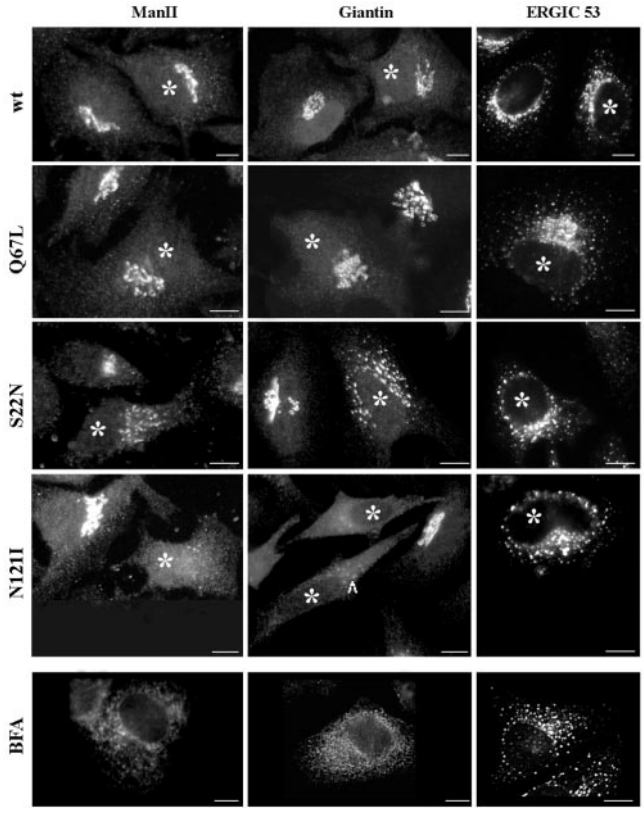


**Figure 3.** Rab1b mutants induce Golgi disruption. HeLa cells were transfected with the wild-type Rab1b, the Q67L mutant, the S22N mutant, or the N121I mutant. Cell were analyzed 24 h later by immunofluorescence with anti-myc antibodies to detect transfected cells (asterisks) and with anti-mannosidase II (anti-MannII), anti-giantin, or anti-ERGIC53 antibodies (see respective panels). Wild-type Rab1b and the Q67L mutant do not affect the structure of the Golgi or ERGIC. The S22N mutant partially disrupts Golgi and induces the formation of punctate structures in the Golgi region. The N121I mutant causes complete Golgi disruption, with MannII and giantin redistributing to the ER and ERGIC53 relocating to peripheral punctate structures. The distribution of Golgi and ERGIC markers in cells expressing the N121I mutant is similar to that induced by a 30-min BFA (5 $\mu$g/ml) treatment (BFA panels). Bars, 10 $\mu$m.

expressing the S22N or the N121I mutant, the overall Sec13 and Sec31 pattern still appears normal, but with fewer peri-Golgi structures. Distribution of ER exit sites has been shown to be influenced by the Golgi (Ward *et al.*, 2001). Significantly, even in cells expressing the N121I mutant, both Sec13 and Sec31 are efficiently recruited to ER exit sites.

The results indicate that cells expressing the N121I mutant recruit COPII and ERGIC53 to ER exit sites and immature VTCs but are unable to sort and deliver resident Golgi proteins into those structures (compare Figures 5 and 3). To ensure that this is not because of different N121I expression levels in distinct cells, we compared the localization of Sec31 and ERGIC53 to the localization of a resident Golgi protein (galactosyl transferase, Gal-T) in the same cell. Cells were cotransfected with the N121I mutant and GFP-tagged Gal-T, and the localization of Sec31 or ERGIC53 was compared with the localization of GalT-GFP. As shown in Figure 6, in N121I-transfected cells (see insets), GalT-GFP is detected in a diffuse ER pattern. Gal-T-GFP is not recruited into ER exit sites or immature VTCs, even though Sec31 and ERGIC53 are efficiently sorted and maintained in those structures.

We next explored the distribution of the $\beta$-COP component of COPI in cells expressing wild-type or mutant Rab1b. As shown in Figure 7, $\beta$-COP panels, in cells expressing the

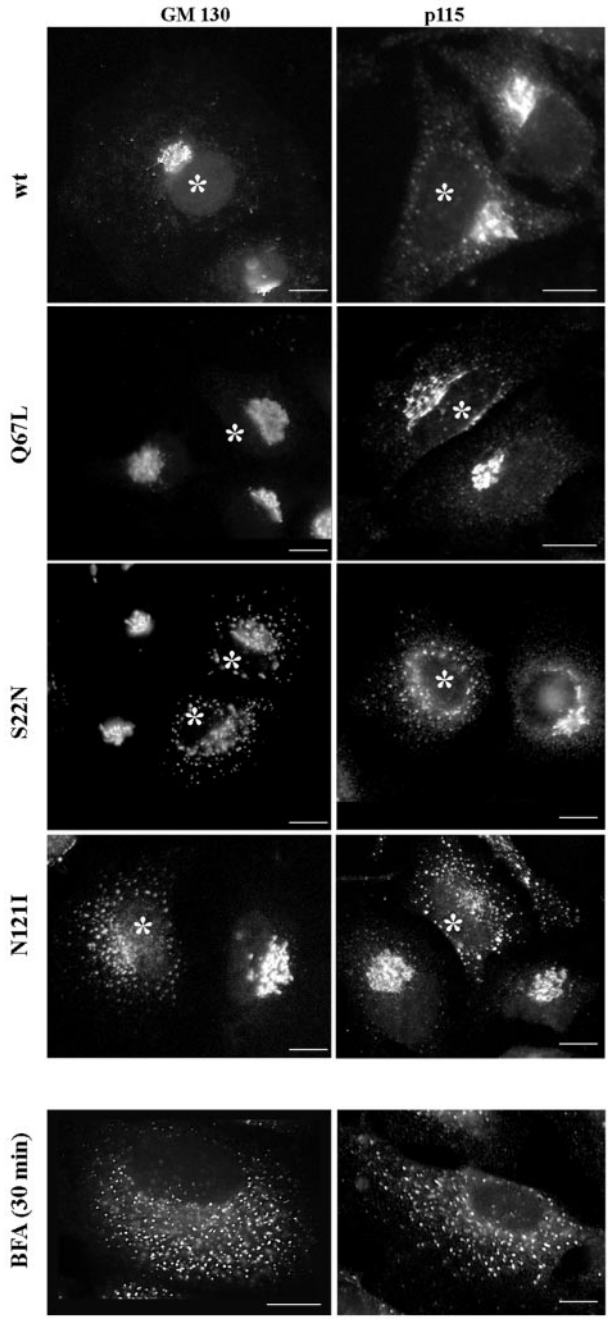


**Figure 4.** Rab1b mutants induce redistribution of its effectors GM130 and p115. HeLa cells were transfected with wild-type Rab1b, the Q67L mutant, the S22N mutant, or the N121I mutant and analyzed 24 h later by immunofluorescence with anti-myc antibodies to detect transfected cells (asterisks) and with either anti-GM130 or anti-p115 antibodies (see respective panels). In cells expressing wild-type Rab1b or the Q67L mutant, GM130 localizes to the Golgi, whereas p115 is found in the Golgi and in peripheral punctate structures. In cells expressing the S22N mutant, GM130 and p115 are partially localized to the Golgi but also show redistribution to peripheral punctate structures. In cells expressing the N121I mutant, both GM130 and p115 are completely redistributed to peripheral punctate structures. The redistribution of GM130 and p115 is similar to that induced by a 30-min BFA (5 $\mu$g/ml) treatment (BFA panels). Bars, 10 $\mu$m.

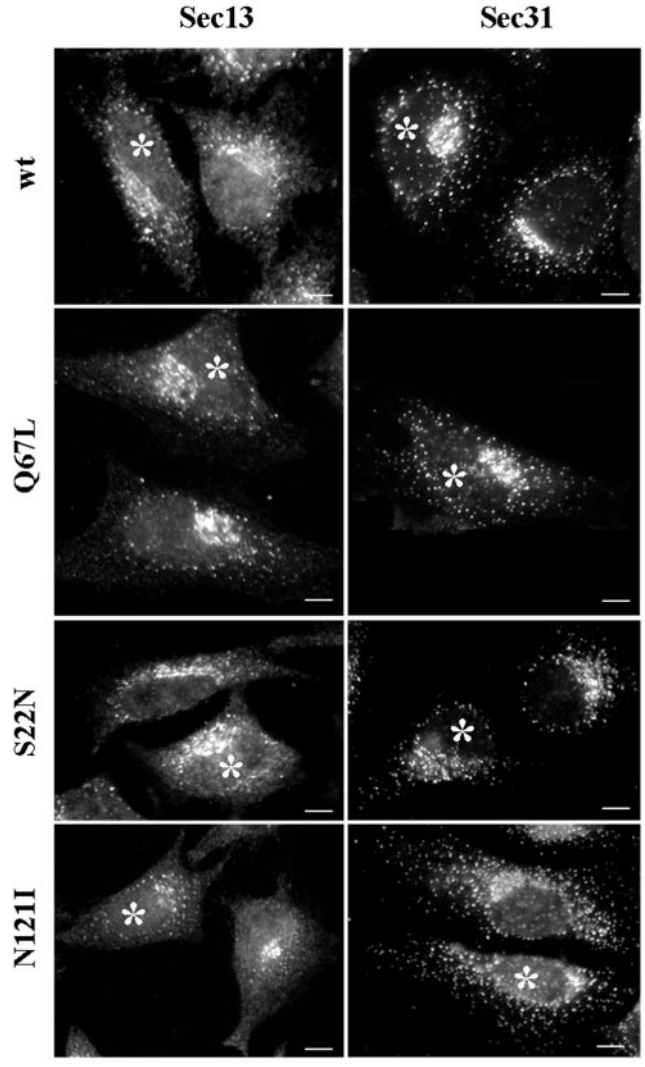


**Figure 5.** Rab1b mutants do not perturb COPII recruitment. HeLa cells were transfected with wild-type Rab1b, the Q67L mutant, the S22N mutant, or the N121I mutant and analyzed 24 h later by immunofluorescence with anti-myc antibodies to detect transfected cells (asterisks) and with either anti-Sec13 or anti-Sec31 antibodies (see respective panels). In cells expressing wild-type Rab1b or the Q67L mutant, COPII markers distribute in normal patterns. In cells expressing the S22N mutant or the N121I mutant, COPII markers present a relatively normal pattern but with fewer punctate structures in the Golgi region. Bars, 10 $\mu$m.

wild-type or the Q67L mutant, $\beta$-COP distributes to the Golgi and to peripheral sites. The pattern is indistinguishable from that in untransfected cells. In cells expressing the S22N mutant, the $\beta$-COP pattern is more dispersed and parallels the redistribution observed for ERGIC53. A more dramatic effect is seen in cells expressing the N121I mutant, with the majority of $\beta$-COP being in a diffuse cytosolic pattern. The N121I-induced $\beta$-COP dissociation is analogous to that seen in BFA-treated cells (Figure 7, BFA panel).

Because $\beta$-COP recruitment requires membrane-associated ARF, and GBF1 has been shown to recruit ARFs to

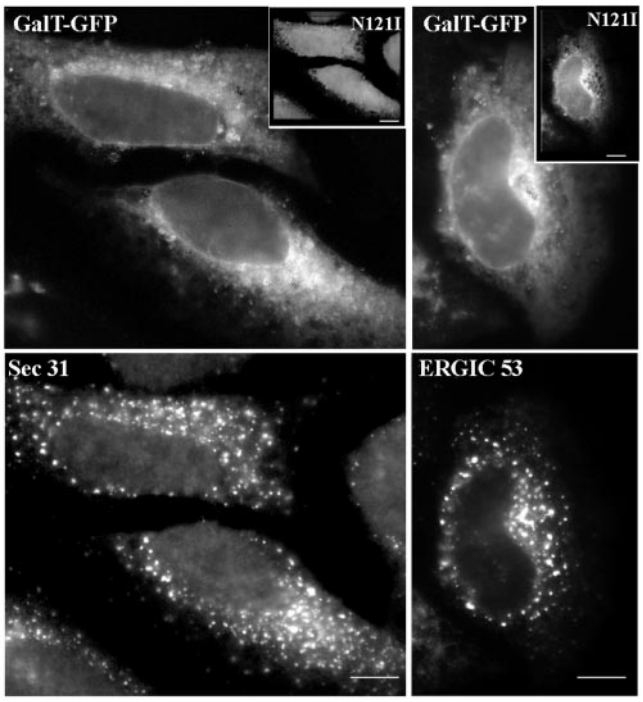


**Figure 6.** The N121I mutant prevents sorting of a Golgi enzyme. HeLa cells were cotransfected with the N121I mutant and GFP-tagged Gal-T and analyzed 24 h later by immunofluorescence with anti-myc antibodies to detect transfected cells (insets) and with either anti-Sec31 or anti-ERGIC53 antibodies. Gal-T was detected by green fluorescence. Gal-T is localized to the ER in the same cells in which Sec31 or ERGIC53 present punctate patterns. Bars, 10 $\mu$m.

membranes (Kawamoto *et al.*, 2002), we examined the distribution of GBF1 in cells expressing the wild-type Rab1b and the various mutants. As shown in Figure 7, GBF1 panels, in cells expressing the wild-type or the Q67L mutant (transfected cells denoted by asterisks), GBF1 distributes to the Golgi and to peripheral sites. The pattern is indistinguishable from those in neighboring untransfected cells. In cells expressing the S22N mutant, GBF1 localization is altered, with GBF1 relocating to punctate structures concentrated in the Golgi region. A dramatic redistribution of GBF1 is seen in cells expressing the N121I mutant. Although the distribution of GBF1 appears diffuse, the nuclear membrane is labeled (arrowhead), indicative of ER localization. The N121I-induced GBF1 pattern is analogous to that seen in BFA-treated cells (Figure 7, BFA panel), in which GBF1 relocates to the ER.

### *Expression of ARF1 or GBF1 Rescues β-COP Dissociation Induced by a Mutant Form of Rab1b*

Golgi disruption and β-COP dissociation induced by the N121I mutant are analogous to those induced by BFA. BFA inhibits the activity of a GEF for ARFs and prevents ARF activation (Donaldson *et al.*, 1992; Helms and Rothman, 1992). To determine whether Rab1b-catalyzed events might participate in COPI recruitment, we explored the possibility that the N121I mutant causes its effects through an inhibi-

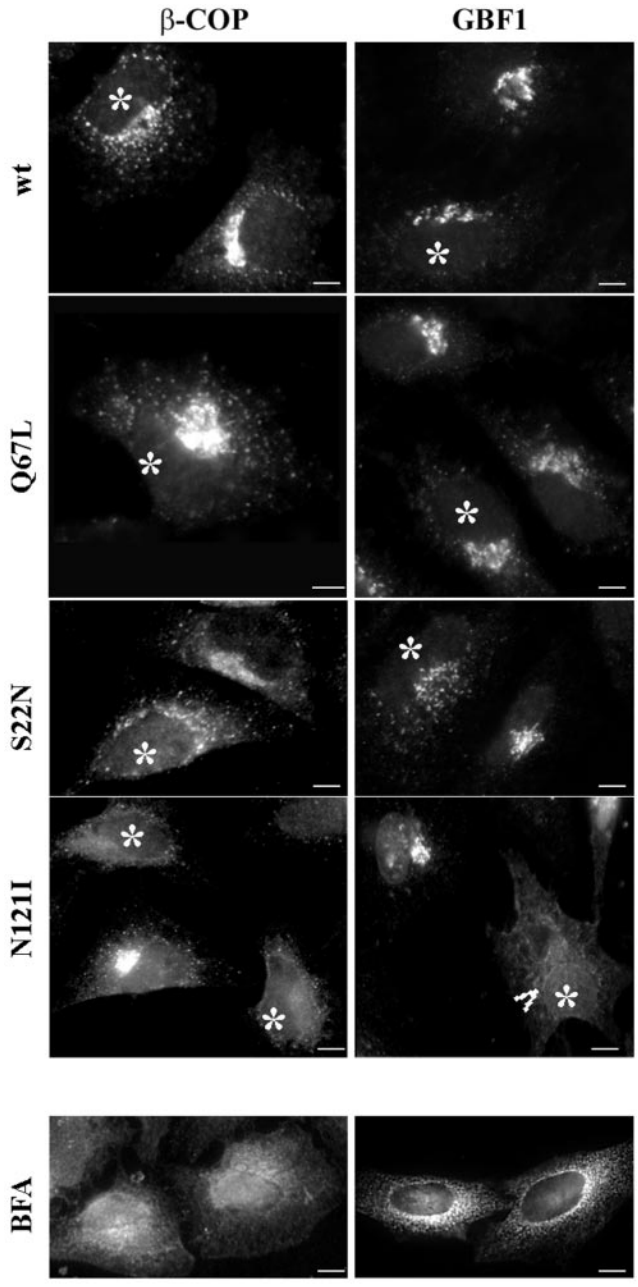


**Figure 7.** The N121I mutant induces β-COP dissociation and relocation of GBF1. HeLa cells were transfected with wild-type Rab1b, the Q67L mutant, the S22N mutant, or the N121I mutant (see respective panels) and analyzed 24 h later by immunofluorescence with anti-myc antibodies to detect transfected cells (asterisks) and with either anti-β-COP or anti-GBF1 antibodies. In cells expressing wild-type Rab1b or the Q67L mutant, β-COP and GBF1 distribute in normal patterns. In cells expressing the S22N mutant, β-COP and GBF1 present a more punctate peri-Golgi pattern. In cells expressing the N121I mutant, β-COP and GBF1 are not associated with a distinguishable compartment and appear diffuse. GBF1 is detected in the nuclear membrane (arrowhead), indicating ER localization. The redistribution of β-COP and GBF1 is similar to that induced by a 30-min BFA (5 $\mu$g/ml) treatment (BFA panels). Bars, 10 $\mu$m.

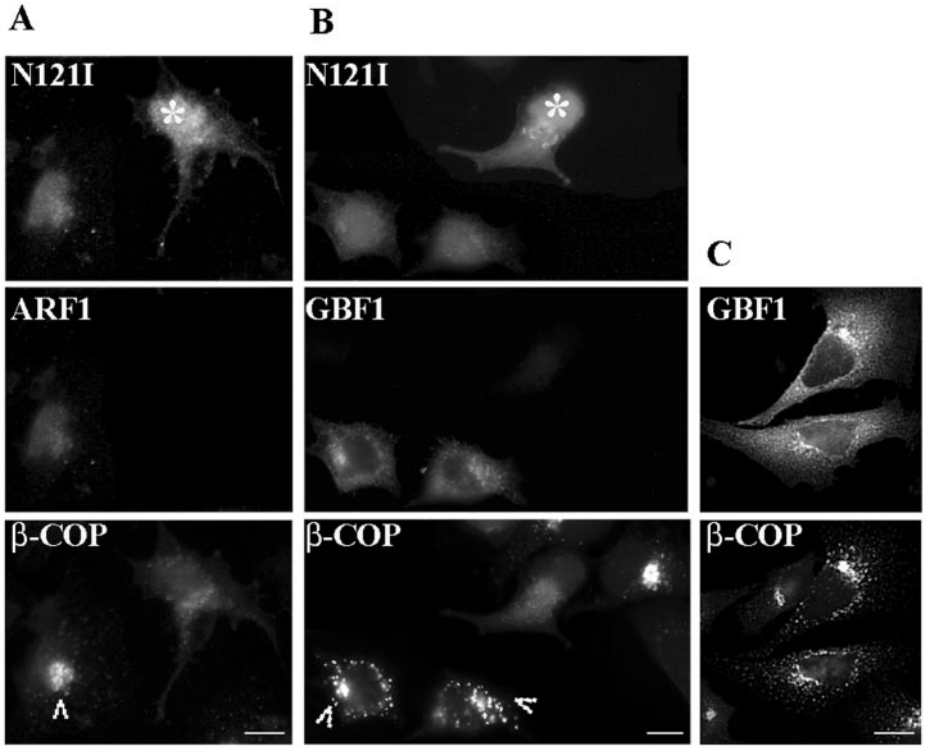


**Figure 8.** Expression of ARF1 or GBF1 rescues β-COP disassembly induced by the N121I mutant. HeLa cells were cotransfected with GFP-tagged N121I mutant and HA-tagged wild-type ARF1 (A) or with GFP-tagged N121I mutant and myc-tagged GBF1 (B). After 24 h, cells were analyzed by immunofluorescence with anti-β-COP antibodies and with either anti-HA or anti-myc antibodies. GFP-N121I was detected by green fluorescence. In cotransfected cells (arrowheads), β-COP is membrane associated. In cells transfected only with N121I (asterisks), β-COP is cytosolic. In C, cells were transfected only with myc-tagged GBF1. Overexpression of GBF1 causes partial Golgi fragmentation. Bars, 10 μm.

tory effect on the ARF1-mediated COPI recruitment pathway. If that were the case, than overexpression of ARF1 should rescue the N121I-induced β-COP dissociation. To test this, we cotransfected the N121I mutant with wild-type ARF1. Expression of ARF1 has no detectable effect on Golgi structure or β-COP localization (our unpublished results) (Teal *et al.*, 1994). As shown in Figure 8A, a cell cotransfected with N121I and ARF1 (arrowhead) shows β-COP in a membrane-associated Golgi-like pattern. In contrast, a cell transfected only with the N121I mutant (asterisk) shows diffuse distribution of β-COP.

The Sec7-family member GBF1 is an exchange factor for ARF1 in vivo (Kawamoto *et al.*, 2002). Because expression of ARF1 rescues the N121I phenotype, we explored whether GBF1 could also reverse the N121I effect. We cotransfected cells with the N121I mutant and wild-type GBF1. As shown in Figure 8B, in cells cotransfected with the N121I mutant and GBF1 (arrowheads), β-COP is retained on membranes. β-COP is associated with the Golgi and with punctate peri-Golgi structures. This pattern is analogous to that in cells expressing only GBF1 (Figure 8C). The peri-Golgi structures are most likely caused by a GBF1-mediated increase in COPI recruitment to membranes. In cells expressing only the N121I mutant (asterisk), β-COP is diffusely distributed in the cytosol. It seems that both ARF1 and GBF1 can antagonize the effect of the N121I mutant and maintain β-COP association with membranes.

### *Expression of the Active Mutant of Rab1b Confers BFA Resistance to Cells*

Previous studies have documented that expression of proteins known to be involved in COPI recruitment, such as the active form of ARF1 and GBF1, confers BFA resistance to transfected cells (Teal *et al.*, 1994; Claude *et al.*, 1999; Kawamoto *et al.*, 2002; Zhao *et al.*, 2002). In agreement, we show that the expression of the active Q71L mutant of ARF1 prevents Golgi redistribution in BFA-treated cells (Figure 9A). As shown in that figure, a cell transfected with ARF1-Q71L shows normal Golgi localization of p115 (arrowhead). An adjacent untransfected cell shows the typical BFA-induced p115 pattern (compare to p115 localization in Figure 4, BFA panel). Similarly, expression of GBF1 confers BFA resistance (Figure 9A), in agreement with previous reports (Claude *et al.*, 1999; Kawamoto *et al.*, 2002; Zhao *et al.*, 2002). Cells expressing GBF1 show Golgi localization of p115 (arrowheads), whereas an untransfected cell shows a dispersed p115 pattern. Quantification of the BFA resistance of transfected cells (defined as Golgi pattern of p115) shows that ~4% of cells transfected with a control plasmid encoding GFP are BFA resistant, whereas ~41% of cells transfected with the ARF1-Q71L plasmid and ~39% of cells transfected with the GBF1 plasmid are BFA resistant (Figure 9B).

To test whether expression of the active form of Rab1b also confers BFA resistance, we transfected cells with the Q67L mutant and then treated them with BFA. As shown in Figure 9C, cells expressing the Q67L mutant are BFA resistant, as shown by Golgi localization of the Q67L protein and of p115 (arrowhead). Two adjacent untransfected cells show typical BFA-induced p115 pattern. Quantification of the BFA resistance of transfected cells (defined as Golgi pattern of p115) indicates that ~35% of cells transfected with the Q67L mutant are resistant to BFA (Figure 9B). Expression of the Q67L mutant also prevents GBF1 relocation to the ER in BFA-treated cells (Figure 9C). A cell expressing the Q67L

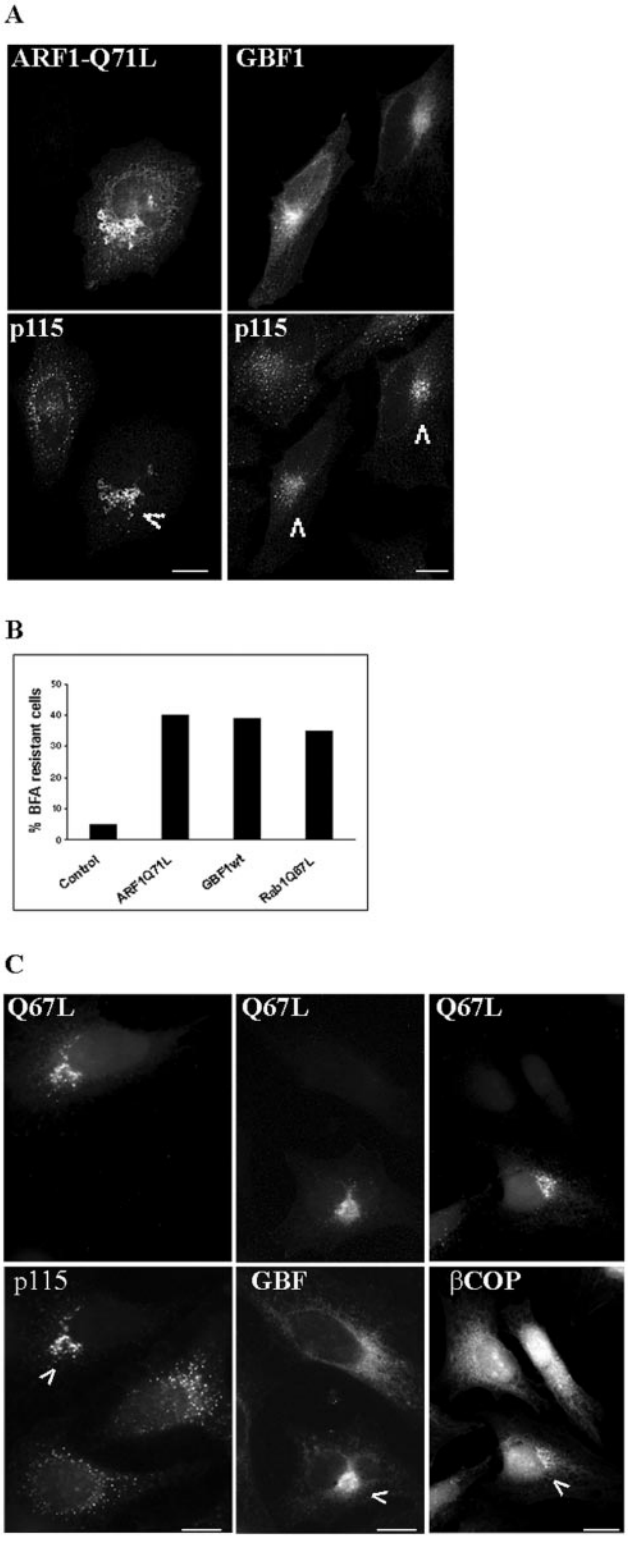


**Figure 9.** Rab1-Q67L-transfected cells are BFA resistant. HeLa cells were transfected with HA-tagged ARF1-Q71L myc-tagged GBF1, or GFP-tagged Rab1b-Q67L. After 24 h, cells were treated with BFA (30 min; 5 $\mu$g/ml) and analyzed by immunofluorescence with either anti-HA or anti-myc antibodies to detect transfected cells and with anti-p115, anti-GBF1, or anti-$\beta$-COP antibodies to detect the Golgi. GFP-Rab1b-Q67L was detected by green fluorescence. Cells transfected with either ARF1-Q71L or GBF1 are BFA resistant, as shown by a Golgi pattern of p115 (arrowheads in A). The percentage of cells transfected with a control plasmid (encoding GFP), ARF1Q71L, GBF1, or Rab1-Q67L that are BFA resistant was quantified and is plotted as a bar graph (B). Bars represent the average of two independent experiments using p115 as a Golgi marker (50 transfected cells were counted in each experiment). Cells transfected with Rab1b-Q67L are BFA resistant, as shown by Golgi pattern of p115 and GBF1 and partial membrane association of $\beta$-COP (arrowheads in C). Bars, 10 $\mu$m.

mutant shows Golgi localization of GBF1 (arrowhead), whereas an adjacent untransfected cell shows redistribution of GBF1 to the ER. Quantification of the BFA resistance of transfected cells (defined as Golgi pattern of GBF1) indicates that ~35% of cells transfected with the Q67L mutant are resistant to BFA (our unpublished results). The level of BFA resistance conferred by the Rab1b-Q67L mutant is analogous to that conferred by ARF1-Q71L (~41%) or by GBF1 (~39%). Expression of wild-type Rab1 also prevents p115 relocation in BFA-treated cells; quantification of the BFA resistance of transfected cells (defined as Golgi pattern of p115) indicates that ~30% of cells transfected with wild-type Rab1 are resistant to BFA (our unpublished results).

Expression of the Q67L mutant partially antagonizes BFA-induced release of $\beta$-COP, and a cell expressing the Q67L mutant shows membrane-associated $\beta$-COP in a Golgi region (arrowhead), in addition to a more diffuse cellular staining (Figure 9C). Adjacent untransfected cells show exclusively diffuse localization of $\beta$-COP. Quantification of the persistence of membrane-associated $\beta$-COP in transfected cells (defined as Golgi pattern of $\beta$-COP) indicates that ~15% of cells transfected with either the Q67L mutant or GBF1 show BFA-resistant $\beta$-COP localization (our unpublished results).

## DISCUSSION

Here, we provide a comprehensive analysis of Rab1b function in cells by examining the effects of mutant forms of Rab1b on (1) ER-Golgi traffic, (2) Golgi and ERGIC structure, (3) the response of Rab1b effectors, (4) the status of the COPII machinery, and (5) the status of the COPI machinery. In our studies, we use Rab1b constructs tagged at the C terminus with myc or at the N-terminus with GFP. Rabs are posttranslationally prenylated (by addition of a 20-carbon geranylgeranyl to the C-terminal GGCC motif) (Farnsworth *et al.*, 1994), and prenylation has been shown to be required for membrane association of the yeast homologue of rab1a, YPT1 (Newman *et al.*, 1992). We did not formally analyze whether our C-terminally myc-tagged constructs are prenylated. However, because the myc-tagged wild-type Rab1b associates efficiently with membranes, we assume that the myc tag does not prevent prenylation. It has been shown that Rabs containing the -CCXXX motif are prenylated (Pereira-Leal and Seabra, 2000), suggesting that addition of C-terminal amino acids might not prevent prenylation. Furthermore, we show that the myc-tagged constructs behave

identically to constructs tagged at the N-terminus with GFP and induce the same phenotypes.

### *Effects of Rab1b Mutants*

***Q67L*** The Q67L mutant localizes to peripheral VTCs and to the Golgi in a pattern indistinguishable from that of a wild-type protein. Expression of the Q67L mutant has no detectable effect on any of the tested parameters, in agreement with previously published data (Tisdale *et al.*, 1992). Although the Q67L mutant is expected to have reduced intrinsic GTPase activity in vitro, it is likely to interact with a GAP that would promote GTP hydrolysis to normal levels in vivo. The lack of visible enlargement of VTCs or the Golgi in transfected cells differs from the effects observed in the endosomal system, in which the expression of GTP-restricted Rab5 increases the rate of internalization and leads to the formation of enlarged endosomes (Barbieri *et al.*, 1996; Seachrist *et al.*, 2001).

***S22N*** The S22N mutant presents a diffuse cellular staining without a clear compartmental staining. In some cells, a faint nuclear and reticular pattern can be discerned, suggesting that S22N might associate with the ER. The partially cytosolic localization of this mutant is consistent with its preferred GDP status and GDI-mediated extraction from membranes (Alexandrov *et al.*, 1994). Expression of the S22N mutant leads to inhibition in transport and the arrest of cargo VSV-G in fragmented perinuclear Golgi structures. This is consistent with biochemical data showing that cargo VSV-G remains endo-H sensitive in cells expressing the analogous Rab1a mutant (Tisdale *et al.*, 1992). Cells transfected with S22N show Golgi disruption and relocation of Golgi proteins to perinuclear fragments. It is likely that those are analogous to Golgi elements induced in cells by microinjection of the S25N Rab1a mutant and shown by electron microscopic analysis to resemble polarized Golgi ministacks formed in the presence of nocodazole (Wilson *et al.*, 1994). The COPII and COPI machinery is not noticeably perturbed in transfected cells. It is likely that the S22N mutant mediates its effect by competing with the endogenous Rab1b for binding to cellular accessory proteins (possibly GDI or GEF) but does not act as an irreversible inhibitor, because its effects can be rescued by overexpression of the wild-type protein (Nuoffer *et al.*, 1994).

***N121I*** The N121I mutant localizes in a diffuse cellular pattern without associating with a clearly defined compartment. Expression of N121I in cells blocks the exit of cargo VSV-G from the ER and causes the complete disassembly of the Golgi as monitored by the disappearance of mannosidase II, giantin, GOS28 (not shown), and gal-T from the Golgi region and their redistribution to the ER. This result is completely reproducible when using either myc-tagged or GFP-tagged N121I mutant, and the redistribution of four different proteins argues against a protein-specific phenotype. The collapse of the Golgi is most easily explained by a block in the anterograde pathway and continuing retrograde recycling, in agreement with the established role of Rab1 in the forward traffic (Plutner *et al.*, 1991; Tisdale *et al.*, 1992). Our findings differ from published data that show arrest in VSV-G transport at the level of VTCs in cells expressing the N121I mutant (Tisdale *et al.*, 1992) or in semi-intact cells supplemented with N121I (Pind *et al.*, 1994), and the relocation of Golgi proteins into perinuclear structures in cells microinjected with the N121I mutant (Wilson *et al.*, 1994). The rationale for the differences in reported results and our data is currently unclear. However, we provide further experimental support for the disruptive effects of N121I by showing that it causes dissociation of β-COP from membranes. The phenotype we describe is analogous to that observed in cells treated with BFA (Klausner *et al.*, 1992) or expressing the inactive mutant of ARF1 (Dascher and Balch, 1994). In each case, β-COP is not associated with membranes, there is no sorting of cargo and Golgi proteins into VTCs, and ER-Golgi traffic is inhibited. Recruitment of COPI is required for the maturation of COPII-differentiated ER exit sites into transport-competent VTCs, and this process seems to be inhibited by N121I. The significantly more severe phenotype induced by N121I versus S22N can be explained by the finding that N121I is expected to act as an irreversible inhibitor in vivo, because its effects cannot be suppressed by overexpression of wild-type Rab1b (Pind *et al.*, 1994).

### *Rab1b and Its Effectors*

GM130 has been identified as a Rab1a and Rab1b effector (Moyer *et al.*, 2001a) (Weide *et al.*, 2001), whereas p115 has been identified as a Rab1a effector (Allan *et al.*, 2000). Rab1a shares 92% identity with Rab1b (Touchot *et al.*, 1987), and we detected p115 interaction with the active Q67L mutant of Rab1b in a yeast dihybrid system (our unpublished results). Surprisingly, expression of Q67L had a limited effect on GM130 and p115 distribution, but expression of S22N, and to an even larger extent, expression of N121I, significantly influenced GM130 and p115 localization. The S22N mutant caused partial relocation of both proteins from the Golgi to peripheral punctate structures, whereas the N121I mutant caused complete redistribution into a BFA-like punctate pattern. Significantly, both proteins remained membrane associated. It has been suggested that the active form of Rab1 is required for the recruitment of p115 to membranes in an in vitro COPII budding assay (Allan *et al.*, 2000), but our in vivo results indicate that p115 can associate with membranes independently of Rab1 activity. Similarly, the Rab3 effector rabphilin3A also associates with membranes in a manner independent of active Rab3 (Shirataki *et al.*, 1994). Although it seems that p115 recruitment to membranes is mediated by other protein(s), subsequent p115 sorting or function might be regulated by the active Rab1.

### *Rab1b and COPI Recruitment*

The ability of the N121I mutant to induce β-COP dissociation from membranes suggests that Rab1b participates in events leading to COPI recruitment. A link between Rabs and COPI is not unexpected, because the active form of Rab2 promotes β-COP recruitment to membranes (Tisdale and Jackson, 1998), and a relationship between Rabs and ARFs has been shown genetically in yeast (Segev, 2001b). The Rab1b yeast homologue YPT1 has been shown to interact genetically with GEA2, a guanine nucleotide exchange factor for ARF1/2 (Jones *et al.*, 1999). Furthermore, there is synthetic lethality between YPT1 and ARF1, suggesting a

functional link between these GTPase families. In agreement, we document a functional relationship between Rab1b and ARF1 by showing that (1) β-COP dissociation induced by N121I can be reversed by overexpression of ARF1; (2) overexpression of GBF1, a mammalian exchange factor for ARF, is also able to reverse β-COP dissociation induced by N121I; and (3) overexpression of the active Q67L mutant reverses β-COP dissociation induced by BFA. The combined data suggest that the Rab and ARF families of GTPases interact in a regulatory cascade mediating COPI recruitment.

A plausible model for Rab1b function is that it might act to recruit or activate GBF1, which in turn activates ARF, which then recruits COPI and allows COPII/COPI exchange on VTCs. This model fits with the BFA-like phenotype induced by N121I, because BFA has been shown to inhibit GEF activity, thus preventing ARF activation and COPI recruitment. The model is also consistent with our findings that Rab1b acts in the COPI pathway upstream of ARF1 and GBF1 and that the block induced by N121I precedes formation of VTCs. Previous work has shown that COPII/COPI exchange is necessary for formation of VTCs (Pepperkok *et al.*, 1993; Peter *et al.*, 1993) and that the process seems to involve at least two stages. The initial differentiation of the ER membrane into ER exit sites occurs through the action of the COPII machinery, and the subsequent maturation to VTCs requires recruitment of the COPI coat. In the absence of active Rab1b, ER exit sites form, as shown by the localization of COPII components and the sorting of ERGIC53, GM130, and p115, but such structures do not differentiate into VTCs, as shown by the retention of cargo and Golgi proteins in the ER. That Rab1b might participate in exit from the ER is also strongly suggested by studies in *Drosophila* expressing the N124I mutant under heat-shock promotor, in which expression of the mutant protein caused extensive ER swelling in addition to the disruption of the Golgi and block in ER-to-Golgi transport of rhodopsin (Satoh *et al.*, 1997). Interestingly, expression of N124I also caused the accumulation of clusters of small vesicles (<150 nm) close to the ER, perhaps indicating that COPI function is required for their differentiation into VTCs.

Our results significantly extend previous studies examining the effects of mutant rab1b on Golgi structure and ER-Golgi transport in vivo and in vitro and provide support for a novel function of rab1b in COPI coat assembly. Future studies are necessary to elucidate the exact mechanism of rab1b function, to identify all its compartment-specific effectors, and to define all the compartments and traffic steps at which it acts.

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