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Rab1b Interacts with GBF1 and Modulates both ARF1 Dynamics and COPI Association[D] [V]

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Assembly of the cytosolic coat protein I (COPI) complex at the ER–Golgi interface is directed by the ADP ribosylation factor1 (Arf1) and its guanine nucleotide exchange factor (GBF1). Rab1b GTPase modulates COPI recruitment, but the molecular mechanism underlying this action remains unclear. Our data reveal that in vivo expression of the GTP-restricted Rab1b mutant (Rab1Q67L) increased the association of GBF1 and COPI to peripheral structures localized at the ER exit sites (ERES) interface. Active Rab1b also stabilized Arf1 on Golgi membranes. Furthermore, we characterized GBF1 as a new Rab1b effector, and showed that its N-terminal domain was involved in this interaction. Rab1b small interfering RNA oligonucleotide assays suggested that Rab1b was required for GBF1 membrane association. To further understand how Rab1b functions in ER-to-Golgi transport, we analyzed GFP-Rab1b dynamics in HeLa cells. Time-lapse microscopy indicated that the majority of the Rab1b-labeled punctuated structures are relatively short-lived with limited-range movements. FRAP of Golgi GFP-Rab1bwt showed rapid recovery ($t_{1/2}$ 120 s) with minimal dependence on microtubules. Our data support a model where Rab1b-GTP induces GBF1 recruitment at the ERES interface and at the Golgi complex where it is required for COPII/COPI exchange or COPI vesicle formation, respectively.

INTRODUCTION

Rab GTPases are essential regulators of most membrane transport events between various compartments within eukaryotic cells (Segev, 2001; Zerial and McBride, 2001). Rabs cycle between Rab-GDP "inactive" and Rab-GTP "active" forms. Activation of a Rab is coupled to its association with a specific membrane compartment and allows the recruitment of downstream effector proteins. Rabs regulate formation, tethering, docking and fusion of vesicles through the interaction with their effectors.

Rab1a and Rab1b isoforms are localized at the endoplasmic reticulum (ER)–Golgi interface and also within the Golgi complex (Plutner *et al.*, 1991; Saraste *et al.*, 1995), being required for transport of membranes from the ER to the Golgi (Tisdale *et al.*, 1992). Rab1 effectors identified are the tethering factors p115 (Allan *et al.*, 2000), GM130 (Moyer *et al.*, 2001; Weide *et al.*, 2001), and Golgin 84 (Satoh *et al.*, 2003); and the intermediate filament-binding protein MICAL-1 (Weide *et al.*, 2003), as well as Iporin and Giantin (Bayer *et al.*, 2005; Beard *et al.*, 2005). GM130 and p115 are also essential for ER-to-Golgi transport (Alvarez *et al.*, 1999, 2001). In addition, Rab1b modulates coat protein I (COPI) recruitment (Alvarez *et al.*, 2003). In mammalian cells, COPI coat is associated with the *cis*-side of the Golgi complex and with peripheral punctuated structures adjacent to the COPII sites on the ER, where COPI segregates and moves toward the Golgi apparatus (Stephens *et al.*, 2000). Recruitment of COPI is mediated by the GTPase Arf1 (Donaldson *et al.*, 1992; Bonifacino and Lippincott-Schwartz, 2003). The interchange of GDP for GTP on Arfs is mediated by guanine nucleotide exchange factors (GEF). GBF1 (Golgi-specific brefeldin A [BFA] resistance factor 1) is an Arf-GEF that regulates Arf1-mediated COPI recruitment at the ER–Golgi interface (Garcia-Mata *et al.*, 2003).

In this report, GBF1 was identified as a new Rab1b effector. Additionally, we show that Rab1b in its GTP form increases the amount of β-COPI and GBF1 localized at ER exit sites (ERES) and modulates the membrane association/dissociation properties of Arf1 at the Golgi. These data, together with the Rab1b dynamics presented here, allow us to propose a model where Rab1b is loaded independently at the ERES interface and Golgi complex, via direct exchange with the cytosolic pool, where it then interacts with GBF1 to enable the Arf1 activation required for COPI vesicle formation.

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Abbreviations used: Arf, ADP-ribosylation factor; BFA, brefeldin A; COP, coat protein complex; ERES, endoplasmic reticulum exit sites; GBF1, Golgi-specific BFA resistance factor 1; GEF, guanine nucleotide exchange factors; GS4B, glutathione-Sepharose 4B; VTC, vesicular tubular cluster; IF, immunofluorescence.

MATERIALS AND METHODS

DNA Constructs and Antibodies

All versions of GFP-Rab1b and Rab1b-myc were described in Alvarez *et al.* (2003). GST-Rab1b versions were subcloned from their respective Rab1b-myc constructs into pGEX4T-2-(Amersham Biosciences, Piscataway, NJ). GFP-GBF1, GST-GBF1 (1430-1859), GBF1-E794K-Myc, GBF1-ΔN-E794K-Myc, and GST-p115 were provided by Dr. Elizabeth Sztul (University of Alabama at Birmingham, AL). GST-GBF1 (1-380) fragment was subcloned from the full-length GFP-GBF1 construct into pGEX4T-3-(Amersham Biosciences). Arf1-GFP and p58-YFP were provided by Dr. Jennifer Lippincott-Schwartz (National Institutes of Health, Bethesda, MD). Sec13-YFP was supplied by Dr.

Benjamin Glick (University of Chicago, IL). The GST-Rab5 vector was provided by Dr. Marisa Colombo (Universidad de Cuyo, Argentina).

The following antibodies were used: rabbit polyclonal antibodies to p115 and GBF1 (Garcia-Mata *et al.*, 2003; a gift from Dr. Elizabeth Sztul, University of Alabama at Birmingham, AL); rabbit polyclonal antibodies against α-COP, β-COP, calreticulin, and Sec23 (Affinity BioReagents, Golden, CO); mouse mAb to calnexin (Affinity BioReagents); rabbit polyclonal antibodies to Rab1b and ERK1 (Santa Cruz Biotechnology, Santa Cruz, CA); mouse mAb to GM130 (Abcam, Cambridge, MA); goat anti rabbit Alexa Fluor 594 (Invitrogen, Carlsbad, CA); mouse mAb to c-Myc (Invitrogen); rabbit polyclonal anti-ERGIC53 antibody (Schweizer *et al.*, 1988), a gift from Dr. Hans-Peter Hauri (University of Basel, Switzerland).

Expression and Purification of Glutathione S-Transferase Fusion Proteins

BL21 cells were transformed with plasmids encoding different proteins fused to glutathione *S*-transferase (GST). GST protein expression, and binding to glutathione-Sepharose 4B (GS4B) beads were performed according to the manufacturer's instructions (Amersham Biosciences). Beads containing immobilized GST-Rab1b or GST-Rab5 were processed as described in Christoforidis and Zerial (2000).

Rat Liver Cytosol Preparation and Pulldown Assays

Rat liver cytosol (RLC) was prepared as described previously (Alvarez *et al.*, 1999) and precleared with GS4B beads. For pulldown assays, GST-Rabs: GTPγS or GST-Rabs:GDP attached to the beads was incubated with RLC for 2 h at 4°C in the presence of 100 μM GTPγS or 100 μM GDP. Beads were then washed four times with NS buffer containing 0.1% Triton X-100, 1 mM dithiothreitol (DTT), 100 μM GTPγS or GDP. Interacting proteins were analyzed by Western blot using anti-p115, anti-α-COP, and anti-GBF1 antibodies.

Purification of Recombinant Rab1b

Recombinant GST-Rab1b was purified with GS4B. The GST moiety was detached using thrombin protease (Amersham Biosciences). The excess of thrombin was removed following manufacturer's instructions, incubating supernatants with benzamidine-Sepharose (Amersham Biosciences). Recombinant Rab1b was cleared with GS4B beads to remove GST traces and incubated in NS buffer (Christoforidis and Zerial, 2000) containing 1 mM GTPγS for 30 min at room temperature and stored at −80°C.

Protein Interactions

Similar quantities of GST-GBF1(1-380), GST-GBF1(1430-1859), GST-p115, or GST attached to GS4B beads, as well as glutathione-Sepharose alone, were incubated with 1 μg of recombinant Rab1b for 2 h at 4°C in the presence of 100 μM GTPγS. Beads were washed four times with 500 μl of NS buffer containing 0.1% Triton X-100, 1 mM DTT, and 10 μM GTPγS. Interactions were analyzed by Western blot using anti-Rab1b antibody.

Cell Culture and Immunofluorescence Assays

HeLa cells were grown in DMEM high glucose supplemented with 10% fetal bovine serum (FBS) and plated onto either sterile microscopy glass coverslips (Fisher Scientific, Pittsburgh, PA) or WillCo glass-bottom dishes (Warner Instruments, Hamden, CT). Transfection was performed using Transit LTI (Mirus, Madison, WI). After 48 h, cells were fixed for immunofluorescence or processed for in vivo studies. Immunofluorescence was performed using an Olympus IX70 epifluorescence microscope (Melville, NY). Fixation and staining of cells was performed as described (Alvarez *et al.*, 2003).

Labeled area quantification was performed using ImageJ processing software (www.rsb.info.nih.gov/ij). Briefly, RGB images were converted to 8-bit grayscale, and a threshold was applied to eliminate background. Total labeled area and peripheral labeled area were measured, and a ratio was calculated according to the following function: (peripheral structures area)/(total area) × 100%.

Cell Fractionation

Fractionation was performed as described in Szul *et al.* (2005). Cells were washed with phosphate-buffered saline (PBS) and disrupted in 300 μl of homogenization buffer (50 mM HEPES-KOH, pH 7.5, 100 mM KCl, 1 mM $MgCl_2$, 1 mM DTT, and protease inhibitors) by repeated passage through a 27-gauge needle. The homogenate was centrifuged at 1000 × *g* for 15 min at 4C. The postnuclear supernatant (PNS) was centrifuged at 100,000 × *g* for 60 min. The supernatant fraction was designated cytosolic fraction, and the pellet was rinsed once with homogenization buffer and recentrifuged under the same conditions. The resulting pellet (membrane fraction) was solubilized with 300 μl of homogenization buffer. PNS and equivalent amounts of cytosol and membrane fractions were separated by 8% SDS-PAGE and analyzed by Western blot. The blots were scanned and the density of bands was quantified using ImageJ software (www.rsb.info.nih.gov/ij).

Live Cell Imaging

All live cell imaging was done at 25°C (for times shorter than 20 min) using a Zeiss LSM Pascal confocal scanning microscope (Carl Zeiss, Thornwood, NY). Images were captured with a 63× 1.4 NA Zeiss plan apochromat oil immersion objective (z-stack 1 μm). For quantitative experiments, single line scans were acquired to increase temporal resolution. Single frames were processed with Adobe Photoshop 6.0 (San Jose, CA) for presentation. For green fluorescent protein (GFP) analysis in cyan fluorescent protein (CFP)-expressing cells, excitation was done at 488-nm wavelength, and GFP emission was recovered with an LP 530 filter. Under these conditions, CFP emission level recovery was not detected (see Supplementary Figure S1).

Fluorescence Recovery after Photobleaching Assays

For fluorescence recovery after photobleaching (FRAP) analysis, a prebleach image was taken at 0.05% laser intensity. Then, the ROI was bleached for 3 s at 100% laser intensity, fluorescence recovery in the bleached area was monitored by scanning at 0.05% laser intensity at every 10- or 20-s interval. At the same time periods, the intensity of a reference region (RER) was considered. The RER was an area selected out of the ROI. Average fluorescence intensities were measured using Metamorph processing software (Universal Imaging, West Chester, PA). At each time point, the percentage of fluorescence recovered in the ROI was plotted using the following equation: $F(t) = (F_{ROI}/F_{RER})/(F_{preROI}/F_{preRER}) \times 100$, where F_{preRER} is the fluorescence intensity of the RER before bleaching, F_{RER} is the fluorescence intensity in the RER at time t, F_{preROI} represents the ROI intensity before bleaching, and F_{ROI} is the ROI intensity at time t. The number of cells imaged was indicated by "n." FRAP assays were performed in cells expressing similar levels of GFP constructs. To depolymerize microtubules, cells were chilled on ice for 30 min and then warmed in complete medium containing 5 μg/ml nocodazole.

Time-Lapse and BFA Experiments

For time-lapse experiments, images were taken at 4-s intervals during 10 min at 0.05% laser power. Movement quantification was performed using Zeiss LSM Pascal software. For BFA experiments, a pre-BFA image was taken, and then the cells were incubated with a medium containing 5 μg/ml BFA. The dissociation rate was monitored by scanning the Golgi at 0.05% laser power at 30-s intervals for 10 min. Average fluorescence intensities were measured using Metamorph processing software (Universal Imaging).

Small Interfering RNA–mediated Depletion of Human Rab1b

HeLa cells cultured on a 35-mm dish were transiently transfected with Rab1b small interfering RNA (siRNA; 120885, Ambion, Austin, TX) using siLentFect (Mirus, Madison, WI) according to the manufacturer's instructions. Control transfections were carried out using a 19-nucleotide scrambled oligonucleotide (6201, Cell Signaling, Beverly, MA). Seventy-two hours after transfection, cells were subjected to immunofluorescence assays. To validate quantitatively the knockdown effect of siRNA, transfected cells were lysed and tested for expression of Rab1b by Western blot assays. The same membrane was blotted with anti-GBF1 and anti-calreticulin antibodies (loading controls). Western blots were quantified using ImageJ software (www.rsb.info.nih.gov/ij).

RESULTS

Rab1b-GTP Enhances Membrane Association of GBF1 and COPI at the ERES Interface

In mammalian cells, Rab1b modulates COPI recruitment (Alvarez *et al.*, 2003). Specifically, expression of a dominant negative mutant of Rab1b (Rab1N121I) interfered with COPI recruitment, whereas expression of the active Rab1b-GTP restricted mutant (Rab1Q67L) conferred resistance to BFA. Moreover, the effect induced by Rab1N121I was reversed by expression of GBF1 (Alvarez *et al.*, 2003). To further explore the role of Rab1b on COPI recruitment, we performed immunofluorescence (IF) assays in order to compare GBF1 distribution between GFP-Rab1Q67L–transfected cells and untransfected cells. Previous reports indicate that GBF1 localizes to ERES, vesicular tubular clusters (VTCs), and early Golgi compartments (Claude *et al.*, 1999; Kawamoto *et al.*, 2002; Zhao *et al.*, 2002; Garcia-Mata and Sztul, 2003). As shown in Figure 1, A–C, the Rab1b-GTP–restricted mutant modified the GBF1-punctuated pattern. GBF1-labeled peripheral structures in transfected cells were more intensive and more diffusely distributed than in untransfected cells (Figure 1B). Similarly, β-COP–labeled peripheral structures

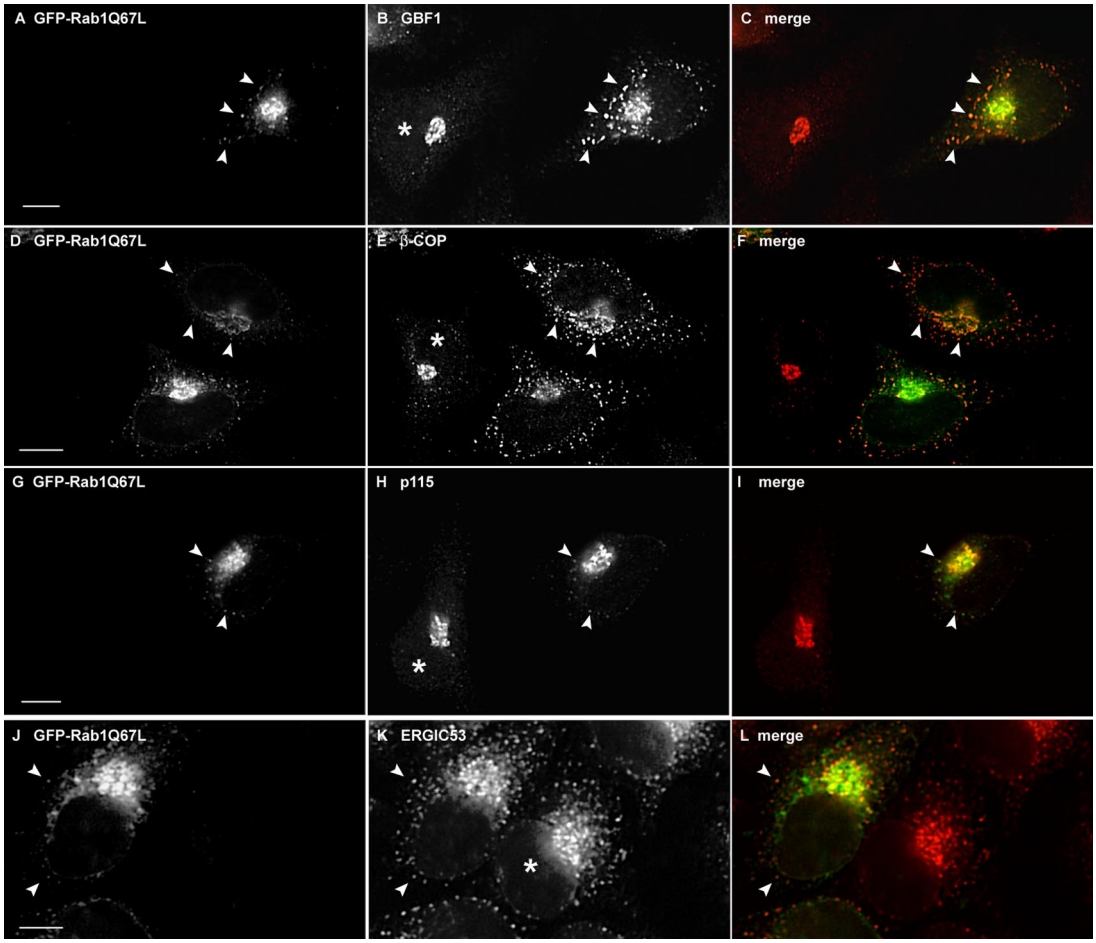

Figure 1. Rab1b-GTP enhances membrane association of GBF1 and COPI at the ERES interface. HeLa cells were transfected with GFP-Rab1Q67L, and analyzed 60 h after transfection. Panels show IF assays to detect GFP-Rab1Q67L and GBF1 (A–C), β-COP (D–F), p115 (G–I), and ERGIC53 (J–L). Asterisks label untransfected cells. Bars, 10 μm.

in transfected cells (Figure 1, D–F) were also more intensive and diffusely distributed than in untransfected cells. GBF1 and β-COP colocalized with GFP-Rab1Q67L in peripheral structures (Figure 1, A–F, arrowheads). To test how specific the Rab1Q67L effect was, we analyzed the intracellular distribution of p115, a Rab1b effector (Figure 1, G–I), and the transmembrane protein ERGIC53 (Figure 1, J–L). Unlike GBF1 or β-COP, p115 and ERGIC53 showed the same pattern in both Rab1Q67L transfected and untransfected cells. In addition, GFP-Rab1Q67L peripheral punctuated structures colocalized with VTC and ERGIC structures labeled with p115 and ERGIC53, respectively (Figure 1, G–L, arrowheads). Quantitative IF analysis for each marker was performed, and the ratio of peripheral structure labeled area to the total labeled area (peripheral structures plus Golgi) in transfected and untransfected cells was calculated. Analysis indicated that the calculated ratio for GBF1 increased from 13% in untransfected cells to 42% in Rab1Q67L-transfected cells. Similarly, for β-COP, the ratio increased from 25% in untransfected cells to 48% in Rab1Q67L-transfected cells. However, there was no significant modification in the ratio calculated for p115 or ERGIC53 between transfected and untransfected cells. To examine the identity of peripheral structures labeled by Rab1b, we analyzed colocalization of GFP-Rab1b wild type with Sec23, ERGIC53, β-COP, and GBF1. As shown in Figure 2, A–L (arrowheads), Rab1b-labeled peripheral structures colocalized with ERES, VTCs, and COPI markers. Taken together, our data suggest that active Rab1b induces GBF1 and COPI recruitment at the ERES-VTC and early Golgi interface.

Rab1b Modulates Arf1 Membrane Association–Dissociation at the Golgi Complex

GBF1 directly interacts with Arf1 (Niu *et al.*, 2005) and catalyzes the exchange of GDP for GTP on it. Arf1 rapidly cycles on and off Golgi membranes (Vasudevan *et al.*, 1998; Presley *et al.*, 2002); hence, because Rab1b-GTP enhances membrane association of GBF1, we aimed to determine whether the cycling dynamic of Arf1 depended on Rab1b GTPase activity. FRAP assays in HeLa cells transfected with Arf1-GFP were performed to measure Arf1 dynamics. Arf1-GFP was distributed on Golgi membranes, in the cytoplasm and a small amount could also be seen associated with peripheral structures (Figure 3A; Presley *et al.*, 2002). Because of the low intensity of peripheral structures, it was difficult to perform Arf1-GFP FRAP assays on them. Therefore, FRAP assays were performed on Golgi membranes in cells expressing Arf1-GFP and in cells coexpressing Arf1-GFP and CFP-Rab1Q67L. As shown in Figure 3, A and C, Arf1-GFP in the Golgi bleached area recovered 100% of the initial fluorescence over a short time period (with a $t_{1/2}$ ~30 s). The half-time of recovery of Arf1-GFP binding to Golgi

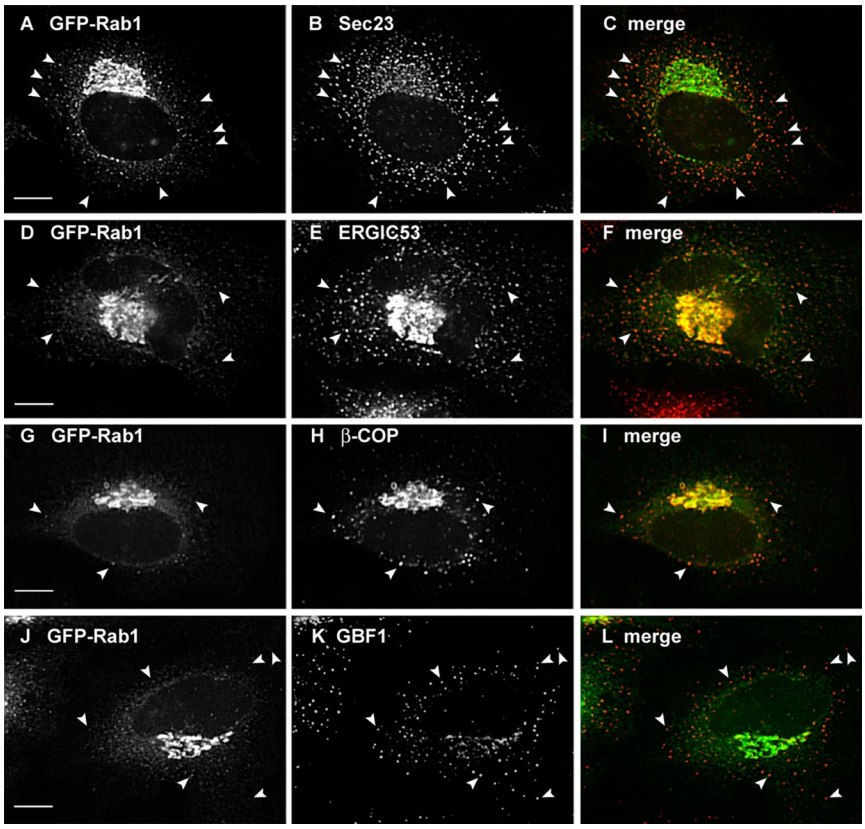

Figure 2. Immunofluorescence of GFP-Rab1b–transfected cells. IF assays of HeLa cells transiently transfected over 24 h. Panels show IF assays to detect GFP-Rab1bwt and Sec23 (A–C); ERGIC53 (D–F), β-COP (G–I), and GBF1 (J–L). Arrowheads in the respective merged panel show colocalization in between GFP-Rab1b and each marker. Bars, 10 μm.

membranes was similar to those reported previously (Vasudevan *et al.*, 1998; Presley *et al.*, 2002). However, in cells coexpressing Arf1-GFP and CFP-Rab1Q67L, only ~55% of the initial Arf1-GFP fluorescence on the Golgi was recovered during the time of the experiment (Figure 3, B and C). To test whether slow fluorescence recovery was a consequence of an overexpression of Rab1b rather than impaired GTPase activity, FRAP in cells coexpressing Arf1-GFP and CFP-Rab1bwt was also performed and presented similar kinetics to noncotransfected cells (Figure 3C). Our results indicate that membrane association-dissociation kinetics of Arf1 associated with Golgi membranes is delayed by impaired Rab1b GTPase activity and suggest that Rab1b-GTP stabilizes Arf1 in membranes.

BFA inhibits GBF1 activity and induces Arf1 dissociation from membranes. When GBF1 is overexpressed, Arf1 dissociation induced by BFA is significantly decreased because of the formation of a stable complex composed of GBF1-Arf1-BFA (Szul *et al.*, 2005; Niu *et al.*, 2005). To test whether in the Golgi Rab1Q67L induces an effect similar to GBF1 overexpression, cells expressing Arf1-GFP or coexpressing Arf1-GFP and CFP-Rab1Q67L were treated with BFA, and Arf1-GFP fluorescence in the Golgi region was monitored. In agreement with previously reported results (Presley *et al.*, 2002; Niu *et al.*, 2005), Arf1-GFP was rapidly lost from Golgi membranes after treatment with BFA (Figure 3D), and ~50% of the Golgi pool of Arf1-GFP was redistributed into the cytoplasm during the first 300 s. In cells overexpressing CFP-Rab1Q67L, a higher level of Arf1-GFP was retained on Golgi membranes in the presence of BFA (Figure 3D), and more than 80% of the Golgi pool of Arf1-GFP remained associated with membranes during the first 300 s. This result indicates that in the presence of BFA, expression of both Rab1Q67L and GBF1 produced a similar effect on Arf1 dissociation, suggesting that more GBF1 and therefore more GBF1-Arf1-BFA complex is membrane associated at the Golgi complex.

Rab1b-GTP Interacts with GBF1

Rab1b interacts with p115 (Allan *et al.*, 2000), which also binds GBF1 (Garcia-Mata and Sztul, 2003). At present, it is unknown if the role of Rab1b in COPI recruitment is due to either an indirect effect of Rab1b on GBF1 (mediated by the interaction of Rab1b with p115) or a direct effect of Rab1b on GBF1. We postulate that active Rab1b directly interacts with GBF1 to modulate its membrane association because in vivo expression of Rab1b dominant negative mutant (Rab1N121I) compromises GBF1 binding to membranes (but not p115; Alvarez *et al.*, 2003). In addition, Rab1b-GTP amplified GBF1 and COPI membrane association without increasing p115 association (Figure 1, A–I).

To explore if Rab1b interacts with GBF1, a GST-Rab1b fusion construct was generated to perform protein–protein interaction assays. Pulldown assays were performed to test whether GST-Rab1b (loaded with GTPγS or GDP) could bind GBF1 from RLC. As shown in Figure 4A, GBF1 bound to Rab1b loaded with GTPγ-S and weakly bound to Rab1b loaded with GDP (GBF1 panel, lanes 2 and 3). GBF1 did not bind to controls performed with GST-Rab5, GST, or glutathione-Sepharose alone (GBF1 panel, lanes 4–7). As a positive control we blotted for p115, which preferentially binds to the GTP form of Rab1b (Allan *et al.*, 2000). As expected, p115 bound to Rab1b loaded with GTPγ-S (p115 panel, lane 2); the minimal amount of p115 detected in the Rab5-GTPγ-S fraction could be considered nonspecific (being similar to the amount bound to the Rab1b GDP form). As a specificity control, we blotted for α-COP, which was not detected in any of the fractions (α-COP panel, lines 2–7). These data

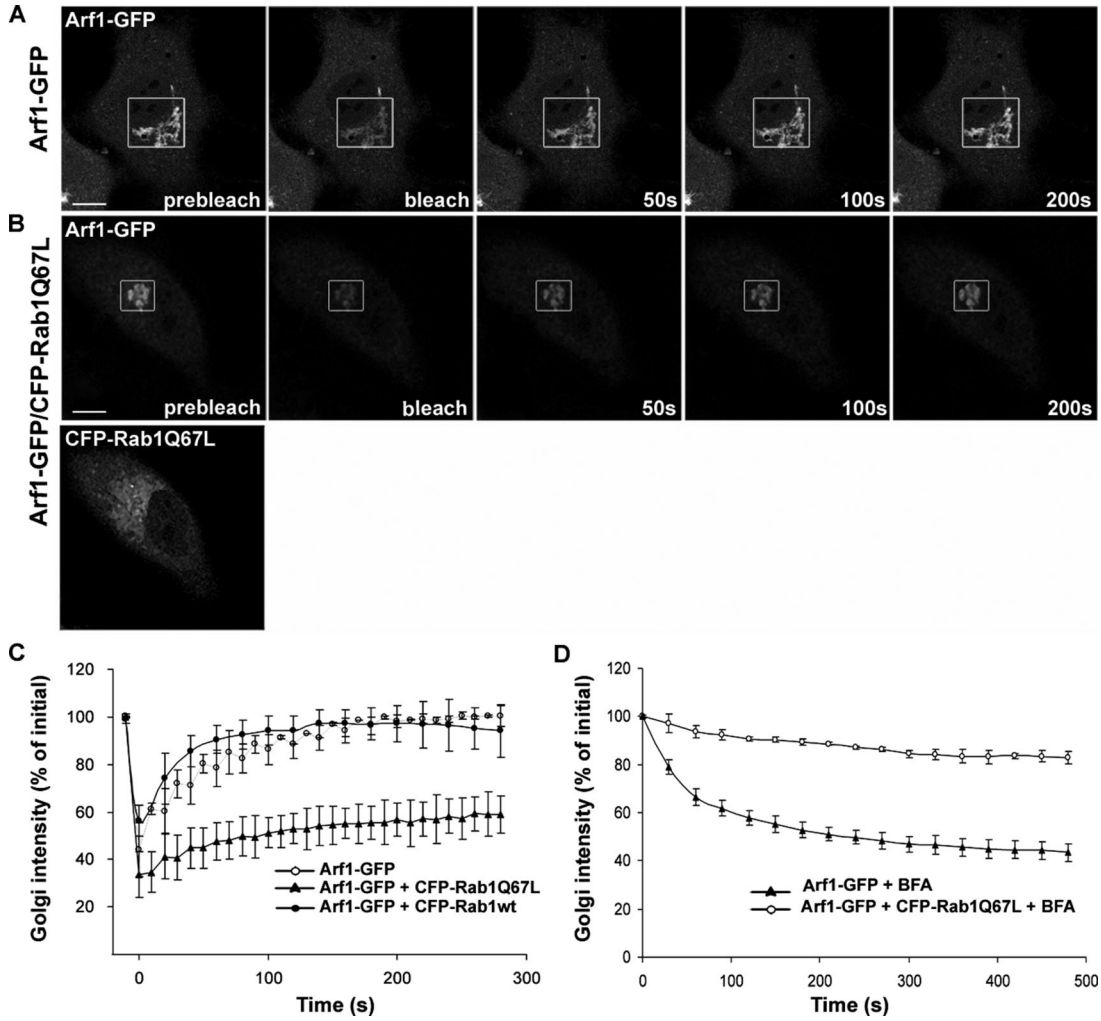

Figure 3. Kinetics of Arf1-GFP binding to and dissociation from Golgi membranes in cells expressing Rab1b-GTP. (A and B) Representative FRAP experiments in HeLa cells transiently expressing Arf1-GFP or coexpressing Arf1-GFP and CFP-Rab1Q67L. The Golgi was selectively photobleached (white square). The first frames show an initial prebleached image. After photobleaching, images frames were selected at times 0, 50, 100, and 200 s. Bars, 10 μm. Bottom panel in B shows expression of CFP-Rab1Q67L in the same cell. Note that the CFP signal is not recognized in the GFP detection conditions. (C) Quantification of Golgi intensity in Arf1-GFP–expressing cells (○; n = 5), Arf1-GFP/CFP-Rab1Q67L (▲; n = 4), and Arf1-GFP/CFP-Rab1bwt–coexpressing cells (●; n = 3). Error bars, SD. (D) Quantification of Golgi intensity in Arf1-GFP–expressing cells (▲; n = 7) and in Arf1-GFP/CFP-Rab1Q67L–coexpressing cells (○; n = 4) during 500 s after BFA (5 μg/ml) treatment. Error bars, SD.

suggest that GBF1 specifically binds Rab1b, with a clear preference for the GTP-loaded form.

To test if the Rab1b and GBF1 interaction was direct we performed pulldown assays using GST-proteins and purified Rab1b loaded with GTPγ-S. Two GST-GBF1 constructs were used, one including 380 aa from the N-terminal domain [GST-GBF1(1-380)] and the second one containing the last 429 aa from the C-terminal domain [GST-GBF1(1430-1859)]. GST-p115 full-length and GST alone were used as controls. As shown in Figure 4B, Rab1b was recovered from both the GST-GBF1(1-380) and the GST-p115 full-length bound fractions. However, Rab1b was not detected in either GST-GBF1(1430-1859) or GST-bound fractions. These results indicate that Rab1b directly interacts in vitro with GBF1 and that the N-terminal domain of GBF1 is required for Rab1b-GBF1 interaction.

GEF activity of GBF1 resides in a conserved Sec7 domain (Munro, 2002), and the point mutation (E794K) in this domain generates an inactive, membrane-associated protein that behaves as a dominant negative. GFP-E794K expressed in cells caused a disassembling of the Golgi similar to that induced by BFA (i.e., Golgi proteins redistribute to the ER and to peripheral structures; Figure 5, A–C; Garcia-Mata *et al.*, 2003). To test if E794K requires the N-terminal domain to act as a dominant negative, an E794K construct without the first 294 aa (ΔN-E794K) was expressed into cells. Unlike E794K, ΔN-E794K was mostly cytosolic and did not induce Golgi disassembly (Figure 5, D–F). This result suggests that E794K needs the N-terminal domain (necessary for Rab1b interaction) to behave as a dominant negative. We cannot overlook the possibility that lack of membrane localization of the N-truncated GBF1 construct is due to improper folding. However, in GBF1 constructs containing only the N-terminal domain encoding either the first 380 aa or the first 294 aa were mostly cytosolic (data not shown). Together, these data suggest that the N-terminal domain of GBF1 is required but is not sufficient for its membrane localization.

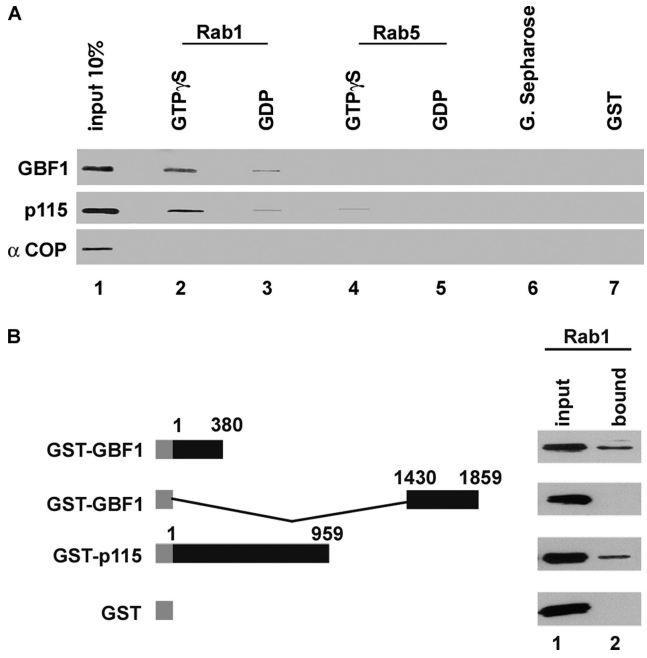

Figure 4. GBF1 preferentially binds to Rab1b-GTP. (A) GST pull-down assays performed with similar quantities of GST-Rab1b and GST-Rab5 loaded with GDP or GTPγS incubated in the presence of rat liver cytosol (lanes 2–5). Glutathione-Sepharose (lane 6) and GST alone (lane 7) were used as controls. Panels show Western blot analysis of bound fractions probed with anti-GBF1, anti-p115, and anti-αCOP. (B) Purified recombinant Rab1b loaded with GTPγS was incubated with truncations of GBF1 (1-380 or 1430-1859 aa) or full-length p115 fused to GST, as well as GST alone. Rab1b bound to GST proteins was detected by Western blot probed with anti-Rab1b (lane 2). Lanes 1 (A and B) represent 10% of input.

Rab1b Is Required for GBF1 Membrane Association

To analyze the effect of Rab1b on GBF1 membrane association, we established experimental conditions to knock down endogenous Rab1b expression levels using specific siRNA oligonucleotides. As shown in Figure 6A, transfections with 50–200 nM of Rab1b siRNAs, over 72 h, induced an 80–90% reduction in Rab1b levels (Rab1 panel, lanes 1–3) compared with Rab1b levels observed in cells treated with the control siRNA (Rab1 panel, lane 4). Moreover, expression levels of GBF1 and Calreticulin were not altered in either Rab1b specific siRNA or in control siRNA-treated cells (panels GBF1 and Calreticulin, lanes 1–4).

IF assays performed on control siRNAs treated cells showed normal Rab1b patterns, with Rab1b concentrated in a juxtanuclear region (that colocalized with GM130) and in peripheral punctuated structures (Figure 6, B–D). On the other hand, in Rab1b-silenced cells, the Rab1b signal was almost undetectable, presenting a diffuse background (Figure 6E). Rab1b silencing induced Golgi disruption (Figure 6, E–G), with GM130 being redistributed from the Golgi to peripheral punctuated structures and displaying a similar pattern to that observed after BFA treatment (Alvarez *et al.*, 2003).

The effect of Rab1b silencing on GBF1 and COPI membrane association was analyzed. In control cells, GBF1 (Figure 6, H–J) showed a normal pattern, associated with peripheral structures and concentrated in the juxtanuclear region that colocalized with GM130. In Rab1b-depleted cells, GBF1 redistributed to a diffuse, cytosolic pattern (Figure 6, K–M), with a very small proportion remaining attached to peripheral structures. Similarly, in Rab1b-depleted cells, β-COP redistributed to a diffuse, cytosolic pattern with a minimal amount attached to peripheral structures (Supplementary Figure S2, B–G). GBF1 and β-COP redistribution to cytosol was not complete, probably because approximately 10% of Rab1b remained after siRNA treatment (Figure 6A and Supplementary Figure S2A). To evaluate how much GBF1 remained membrane associated after Rab1b depletion, cells treated with Rab1b siRNA or with control siRNA were subjected to cell fractionation assays. As shown in Supplementary Figure S2H, the amount of GBF1 associated with membranes was significantly decreased in Rab1b-depleted cells (30%) compared with control cells (70%).

To assess whether the GBF1 cytosolic redistribution observed in Rab1b-silenced cells was also correlated with p115 localization, we evaluated the intracellular distribution of p115 after Rab1b siRNA treatment. As shown in Figure 6, N–P, p115 remained mainly associated with membranes colocalizing with GM130. Similar results were obtained when Rab1b dominant negative mutant (N121I) was expressed in cells; p115 and GM130 remained associated with membranes, whereas GBF1 presented a mainly cytosolic pattern (Alvarez *et al.*, 2003). Taken together, these results indicate a specific requirement of Rab1b for GBF1 membrane association.

How Does Rab1b Participate in Transport?

Rab1b is required for ER-to-Golgi transport, and it also colocalizes with COPII/ERES, VTCs, and COPI structures. These structures also participate in ER-to-Golgi transport, despite having different dynamics. COPII structures are stable and generally immobile (Hammond and Glick, 2000; Stephens *et al.*, 2000), whereas COPI and VTC structures segregate from COPII/ERES and move toward the Golgi in

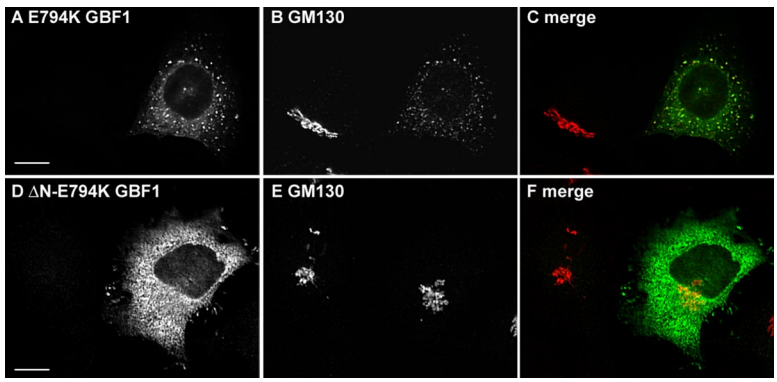

Figure 5. Role of the N-terminal domain in GBF1-E794K mutant intracellular localization. (A–C) Cell expressing GBF1 dominant negative construct E794K-Myc. (D–F) Cell expressing E794K-Myc without 294 aa from the N-terminal domain (ΔN-E794K GBF1). (B and E) The Golgi pattern, labeled with GM130, in transfected and untransfected cells. Bars, 10 μm.

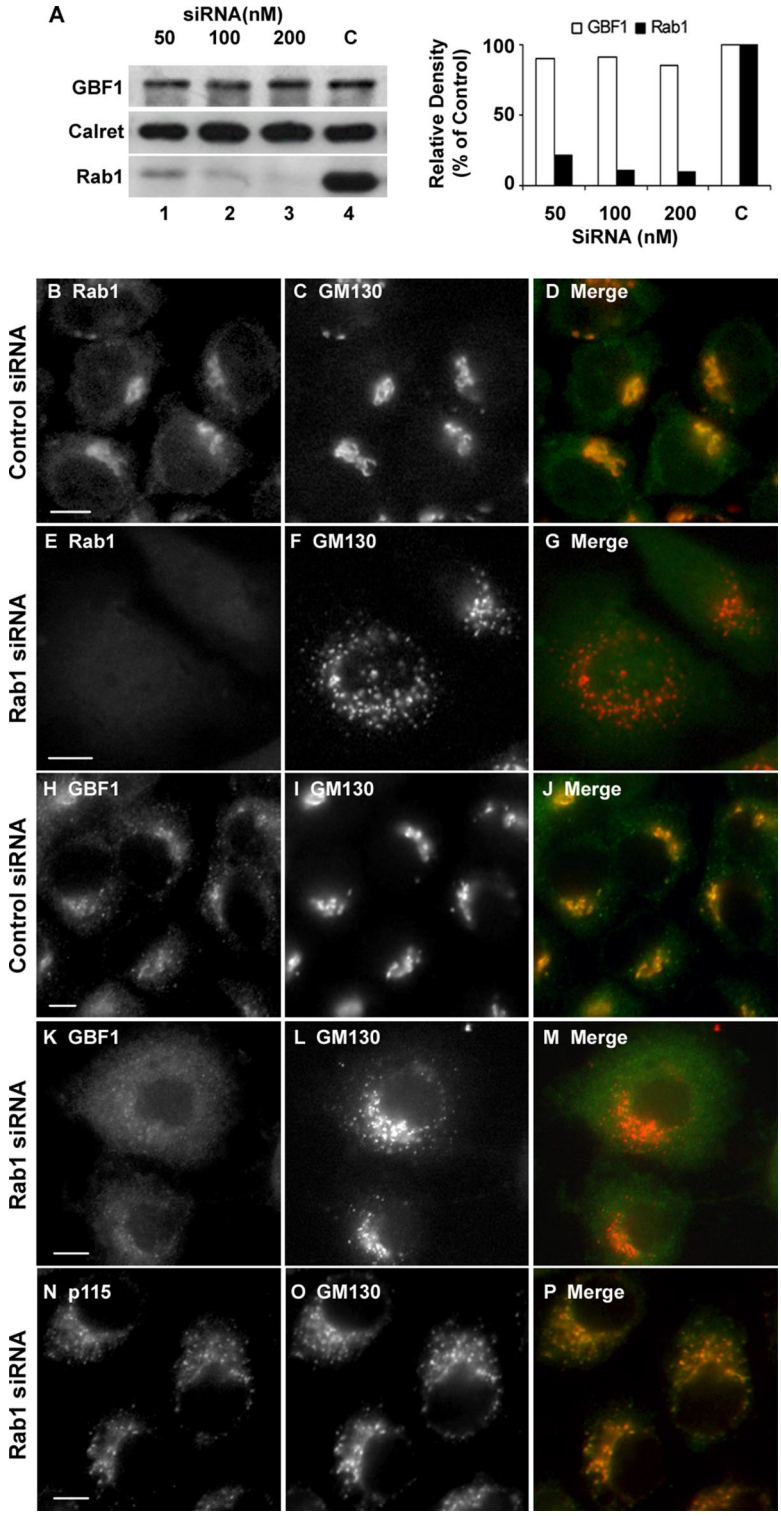

Figure 6. Rab1b requirement for GBF1 membrane association. RNAi treatment to knock down Rab1b levels in HeLa cells. (A) Western blot (and its quantification) performed after 72 h of transfection with different amounts of Rab1b siRNAs (lanes 1–3) or with control siRNAs (lane 4), probed with anti-Rab1b, anti-GBF1, or anti-Calreticulin as a loading control. The density of Rab1b and GBF1 bands relative to the density of Calreticulin was calculated. Relative density in controls was taken as 100%. (B–P) Subcellular localization of Rab1b, GBF1, GM130, and p115 in either Rab1b-silenced (100 nM Rab1b siRNA) or control cells (indicated at left). Bars, 10 μm.

a microtubule dependent manner (Stephens *et al.*, 2000; Presley *et al.*, 2002). Our results indicate that Rab1b interacted with GBF1, which is required for COPI recruitment. Therefore, we consider it important to analyze Rab1b dynamics in order to understand how Rab1b functions in ER-to-Golgi transport. Time-lapse experiments were performed, and the movement of GFP-Rab1bwt–labeled peripheral structures was analyzed (Supplementary Video 1). Three different dynamic patterns (Supplementary Table S1) were defined to describe the behavior of Rab1b-labeled structures: Immobile (Figure 7A, arrowheads), mobile (Figure 7B, Region 1 and 2, arrowheads), or transient (Figure 7B, Region 3, arrowheads). The GFP-Rab1bwt–transient pattern could have been caused by the short permanence of Rab1b in punctuated structures or by movement across the focal plane. We think that the first option is more likely, because

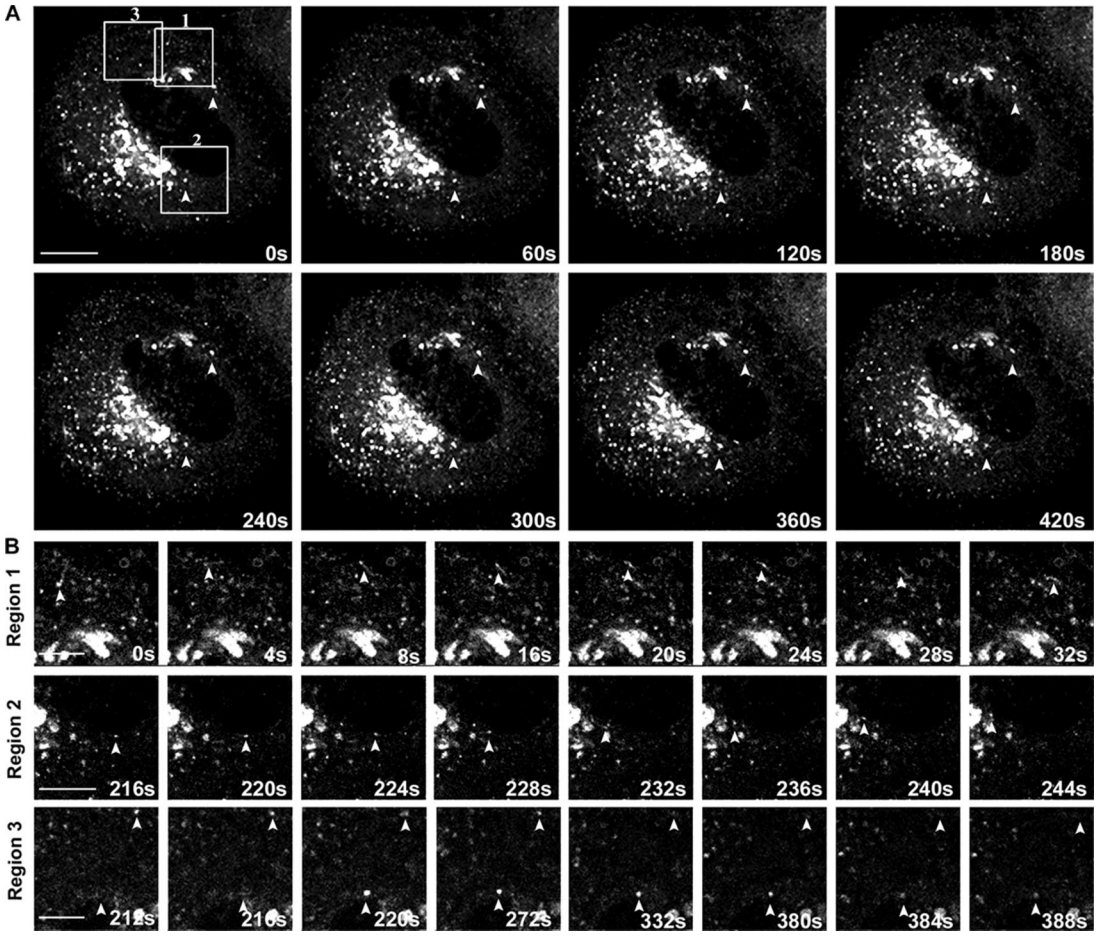

Figure 7. Dynamics of Rab1b labeled peripheral structures. (A and B) Time-lapse imaging of peripheral structures labeled with GFP-Rab1bwt transiently expressed in HeLa cells. (A) A representative cell is shown. Image frames were selected from the associated Quick Time movie every 60 s from time 0 to 420 s. Arrowheads indicate two immobile structures. White boxes indicate selected regions shown in B. Bars, 10 μm. (B) Different behaviors of mobile and transient peripheral structures. Regions 1 and 2 show mobile structures that either described peripheral long-range random movements or emerged from the cell periphery and moved toward the Golgi (arrowheads). Region 3 shows a transient structure that appeared and then vanished (arrowheads) during a short time period. Image frames were selected from the associated Quick Time movie at the indicated times. Bars, 5 μm.

there was a small number of Rab1b-labeled structures (6%) that described a measurable trajectory. To corroborate this, the dynamics of the GFP-Rab1bwt-labeled structures were compared with dynamics of other peripheral structure markers. We performed time-lapse imaging with the bona fide VTCs/ERGIC marker p58-YFP (rat homolog of the human ERGIC53; Supplementary Video 3) and the COPII marker, Sec13-YFP (Supplementary Video 2). Their dynamic behavior (Supplementary Table S1) was similar to the one previously described for GFP-ERGIC53 (Ben-Tekaya *et al.*, 2005), GFP-Sec13 (Hammond and Glick, 2000), and GFP-Sec24 (Stephens *et al.*, 2000). Taken together, these data support our dynamic results showing that Rab1b-labeled peripheral structures were mostly transient, suggesting that the majority of Rab1b associates and dissociates rapidly to peripheral structures, without presenting a preferred trajectory.

The dynamics of the isoform Rab1a was reported recently (Sannerud *et al.*, 2006). In contrast to the Rab1b dynamic presented here, Rab1a elements were highly mobile, presenting multiple tubular and globular elements moving bidirectionally between the Golgi and cell periphery. It is probable that, despite Rab1a and Rab1b seeming to be both required for ER-to-Golgi transport, they may play slightly different roles and therefore present dissimilar dynamics. In support of this, a recent report showed that the Golgi-associated Rab6 isoforms (Rab6A and Rab6A′) performed different functions within the cell (Del Nery *et al.*, 2006). Alternatively, variations during image acquisition could be the reason for the differences between Rab1b and Rab1a dynamics. However, the detected variations in experimental conditions do not seem to be relevant in this case, because YFP-Sec23 and p58-YFP dynamics concur well with published data.

Furthermore, FRAP analysis on HeLa cells transfected with GFP-Rab1bwt were performed to determine the association rate of Rab1b to Golgi membranes. As shown in Figures 8, A and B, 100% of GFP-Rab1bwt initial fluorescence in the Golgi bleached area recovered exponentially with a half-time ($t_{1/2}$) of 120 s.

To study the contribution of microtubule dependent membrane transport, a GFP-Rab1bwt FRAP assay was performed in the presence of nocodazole (to depolymerize microtubules). About 80% of GFP-Rab1bwt fluorescence was recovered on Golgi membranes (Figure 8B), suggesting, in agreement with our time-lapse studies, that a small proportion of Rab1b reached the Golgi by delivery via microtubule-

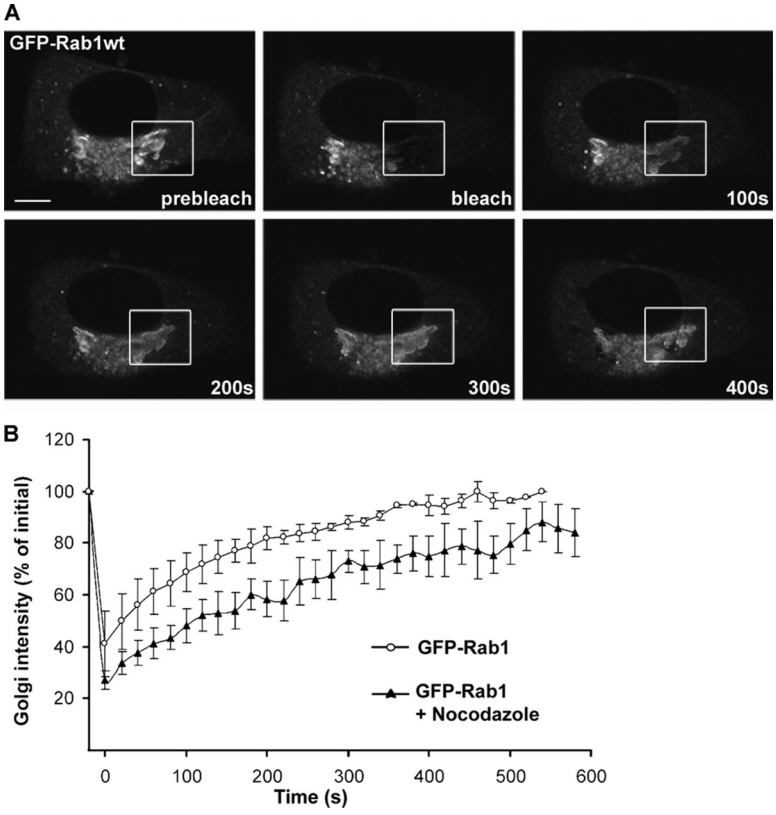

Figure 8. Kinetics of Rab1b binding to and dissociation from Golgi membranes. (A) Representative FRAP experiment in HeLa cell transiently expressing GFP-Rab1bwt. (B) Quantification of GFP-Rab1bwt FRAP from two independent experiments, with 5 μg/ml nocodazole (▲; n = 4 cells) or without nocodazole (○; n = 5 cells). Error bars, SD.

dependent transport. To evaluate how impaired GTPase activity affected Rab1b cycling, we performed FRAP assays with the GFP-Rab1Q67L mutant. Our results indicated that GFP recovery did not reach 100% of initial fluorescence (~70% was recovered), suggesting that an important fraction of the mutant remained irreversibly bound to membranes (Supplementary Figure S3).

DISCUSSION

In this study, we have analyzed the mechanism by which Rab1b modulates Arf1-mediated COPI recruitment as well as Rab1b in vivo dynamic behavior.

Our results indicate that active Rab1b directly interacts with the N-terminal domain of GBF1 (Figure 4). GBF1 is a peripherally associated protein that cycles on and off membranes (Niu *et al.,* 2005; Szul *et al.,* 2005). However, how GBF1 is recruited to membranes is unclear. It is likely that protein–protein interactions mediate GBF1 membrane recruitment. GBF1 binds to p115, but this interaction is not required for GBF1 membrane localization (Garcia-Mata and Sztul, 2003). Gea1p and Gea2p, GBF1 yeast homologues, both bind to Gmh1p, which does not seem necessary for membrane association of Gea proteins because in the absence of Gmh1p there is no redistribution of Gea2p (Chantalat *et al.,* 2003). Our data showing that Rab1b depletion induced a substantial distribution of GBF1 from membranes to cytosol (Figure 6) strongly suggest that Rab1b is necessary for GBF1 membrane association. Moreover, the expression of the Rab1b dominant negative mutant (Rab1N121I) induced GBF1 membrane redistribution (Alvarez *et al.,* 2003).

GBF1 promotes recruitment of COPI at the ERES and Golgi interface (Garcia-Mata *et al.,* 2003) by catalyzing the nucleotide exchange on Arf1 (Niu *et al.,* 2005). In the same way, expression of Rab1Q67L induced accumulation of GBF1 and COPI in peripheral structures at the ERES interface (Figure 1). A likely explanation for this is that the amount of Rab1b-GTP irreversibly bound to membranes (~30%, Supplementary Figure S3) induced a delayed membrane dissociation of GBF1. Although, Rab1Q67L induced accumulation of GBF1 and COPI in peripheral structures, we did not detect any modification of the Arf1-GFP signal in them upon expression of Rab1Q67L. This may be due to the low intensity of peripheral structures in living cells and the low sensitivity in our live imaging system. Alternatively, GBF1 could induce recruitment of a different Arf besides Arf1 in peripheral structures. This is supported by reports showing that GBF1 causes recruitment of more than one class I and class II Arfs onto membranes (Kawamoto *et al.,* 2002) and that the cooperation of two Arfs at the same site is a general feature of Arf signaling (Volpicelli-Daley *et al.,* 2005).

Our data suggest that Rab1b modulates COPI recruitment/vesicle formation as a consequence of its interaction with GBF1. Alternatively, Rab1b could modulate COPI recruitment through the interaction with p115, which also interacts with GBF1. We consider this option unlikely because a GBF1 construct missing the C-terminal domain required for p115 interaction was still recruited to membranes (Garcia-Mata and Sztul, 2003). In support of this, after Rab1b depletion (Figure 6), p115 was associated with membranes, whereas GBF1 showed cytosolic distribution.

Furthermore, we showed that, at Golgi level, membrane association-dissociation of Arf1 was delayed by impaired Rab1b GTPase activity (Figure 3, A–C), with Arf1 dissociation induced by BFA being significantly decreased by expression of Rab1Q67L (Figure 3D). These data suggest that

Rab1b stabilizes activated Arf1 on the membranes. Rab1b may do this by regulating GBF1, a GAP, or an Arf1 effector. On the basis of the fact that a proportion of Rab1b-GTP remains irreversibly bound to membranes and that Rab1b-GTP directly interacted with GBF1 (Figure 4), thereby increasing GBF1 membrane association (Figure 1), plus the observation that overexpression of GBF1 induced Arf1 stabilization (Niu *et al.*, 2005; Szul *et al.*, 2005), we postulate that Rab1b stabilizes Arf1 through its interaction with GBF1. The existence of a Rab1b-Arf GTPase cascade in the secretory pathway has been previously proposed after a genetic interaction between Ypt1 with the yeast homologues of GBF1 (Gea1/2p) was reported (Jones *et al.*, 1999).

In peripheral structures at the ERES interface, assembly of the COPI coat is required for the formation and maturation of pre-Golgi intermediates (also called VTCs) into Golgi elements. The role of COPI on the anterograde-directed VTCs movement is thought to be the sorting of cargo for its transport back to the ER (Letourneur *et al.*, 1994; Lewis and Pelham, 1996; Shima *et al.*, 1999). In Rab1Q67L-expressing cells, GBF1 and β-COP specifically accumulate at peripheral structures in the proximity of COPII structures (Figure 3), suggesting that Rab1b participates by promoting COPI recruitment in the retrieval of proteins from the VTCs required for their maturation. In COPII vesicles, Rab1b also interacts with p115, which forms a complex with SNAREs (Allan *et al.*, 2000). Furthermore, Rab1b interacts with GM130, which also interacts with p115. The GM130-p115 complex allows tethering of ER-derived membranes to the *cis*-Golgi complex. It has been postulated that Rab1 programs fusion of donor and acceptor membranes and also modulates the assembly and/or activity of these tethering complexes (Moyer *et al.*, 2001; Weide *et al.*, 2001). Taken together, these data indicate that at the ERES interface, Rab1b coordinates COPI vesicle formation and the events required for their posterior fusion with the *cis*-Golgi.

At the Golgi level, COPI proteins have multiple functions, participating in maintaining the normal structure of the mammalian interphase Golgi complex (Lippincott-Schwartz *et al.*, 1998) as well as in intra-Golgi retrograde transport of Golgi enzymes (Love *et al.*, 1998; Lanoix *et al.*, 1999, 2001). It was shown that Rab1a interacts with Giantin (Beard *et al.*, 2005), which is concentrated in COPI Golgi-derived vesicles, and also interacts with p115 (Sonnichsen *et al.*, 1998). Giantin–p115 interaction tethers retrograde COPI vesicles to the Golgi. Rab1b, through the interaction with GBF1 could be coordinating COPI vesicle formation and their fusion in intra-Golgi transport. In addition to COPI, Arf1 recruits a variety of proteins, such as ankyrin, spectrin, signaling proteins, and lipids-modifying proteins (Randazzo *et al.*, 2000). Therefore Rab1b-GBF1 interaction may modulate more than one Arf1 activity and consequently various cellular roles. Participation of Rab1b in many cellular functions is supported by its interaction with Golgin-84 (Satoh *et al.*, 2003) and MICAL-1 (Weide *et al.*, 2003), proteins that participate in maintaining the architecture of the Golgi apparatus (Satoh *et al.*, 2003) and in binding to the intermediate filament vimentin, respectively. In agreement, a recent report showed a functional connection between Arf1 and vimentin cytoskeleton (Styers *et al.*, 2006).

Furthermore, we have characterized both the dynamic behavior of Rab1b associated with peripheral punctuated structures as well as the Golgi membrane association-dissociation kinetics of Rab1b in living cells. We found that Rab1b is localized, in peripheral structures, with COPII. Although, labeled Rab1b was as stationary as labeled COPII (Hammond and Glick, 2000; Stephens *et al.*, 2000), it presented a shorter half-life. Perhaps, Rab1b promoted COPII/COPI exchange in the ERES interface. Golgi FRAP observed for GFP-Rab1b ($t_{1/2}$ 120 s) indicated that Rab1b remains associated with membranes for a longer time than Arf1 ($t_{1/2}$ ~30s, Figure 3; Presley *et al.*, 2002) or GBF1 ($t_{1/2}$ ~30 s; Niu *et al.*, 2005; Szul *et al.*, 2005). This suggests that Rab1b needs to spend more time associated with membranes to be able to interact (before and/or after GBF1 interaction) with other effector proteins such as GM130, p115, Giantin, or Golgin-84.

Dynamic data and the Rab1b-GBF1 interaction presented in this report, together with published data, support a model where Rab1b is loaded independently at the ERES interface and Golgi complex via direct exchange with the cytosolic pool, remaining only transiently associated with membranes. Once in the membranes, active Rab1b interacts with a broad category of proteins to orchestrate every step (vesicle formation, tethering, and fusion) required for ER-to-Golgi and intra-Golgi transport. This model postulates that Rab1b is a key molecule required for synchronizing every early secretory transport event. Further analysis should focus on characterizing, in mammalian cells, how Rab1b recruitment and activation are achieved.

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