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The phosphatidylinositol 4-kinase PI4KIIIα is required for the recruitment of GBF1 to Golgi membranes

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Summary

Sorting from the Golgi apparatus requires the recruitment of cytosolic coat proteins to package cargo into trafficking vesicles. An important early step in the formation of trafficking vesicles is the activation of Arf1 by the guanine nucleotide exchange factor GBF1. To activate Arf1, GBF1 must be recruited to and bound to Golgi membranes, a process that requires Rab1b. However, the mechanistic details of how Rab1 is implicated in GBF1 recruitment are not known. In this study, we demonstrate that the recruitment of GBF1 also requires phosphatidylinositol 4-phosphate [PtdIns(4)*P*]. Inhibitors of PtdIns(4)*P* synthesis or depletion of PI4KIIIα, a phosphatidylinositol 4-kinase localized to the endoplasmic reticulum and Golgi, prevents the recruitment of GBF1 to Golgi membranes. Interestingly, transfection of dominant-active Rab1 increased the amount of PtdIns(4)*P* at the Golgi, as detected by GFP-PH, a PtdIns(4)*P*-sensing probe. We propose that Rab1 contributes to the specificity and timing of GBF1 recruitment by activating PI4KIIIα. The PtdIns(4)*P* produced then allows GBF1 to bind to Golgi membranes and activate Arf1.

Key words: GBF1, GGAs, Golgi apparatus, Phosphatidylinositol 4-phosphate, Rab1

Introduction

The intracellular sorting and trafficking of proteins is necessary to establish and maintain organelle function. The Golgi apparatus is responsible for efficiently sorting and trafficking proteins to various cellular destinations, including the lysosomal compartment and plasma membrane. The Golgi accomplishes this function by packaging cargo destined for various destinations into trafficking vesicles that are formed by the recruitment of various cytosolic components, a process that cells tightly regulate (Bonifacino and Lippincott-Schwartz, 2003). ADP ribosylation factors (Arfs) are required for the recruitment of proteins to various cellular membranes, including the Golgi (Dell'Angelica et al., 2000b), endosomes (Ooi et al., 1998) and plasma membrane (Cohen et al., 2007). To initiate recruitment, Arfs must be converted from a GDP-bound to a GTP-bound state by a guanine nucleotide exchange factor (GEF) (Jackson and Casanova, 2000), whereas the GTPase-activating proteins (GAPs) induce hydrolysis of GTP to GDP to deactivate Arfs (Nie et al., 2003). The GEFs are a family of proteins that are thought to play a crucial role in regulating the specificity, location and timing of recruitment of proteins to membranes. The ArfGEF family was originally identified in *Saccharomyces cerevisiae* as the Sec7 protein. Sec7 is common to all members of this family and acts as the catalytic domain of the ArfGEFs, responsible for the activation of Arf (Jackson and Casanova, 2000). In mammalian cells, the smaller family members, such as ARF nucleotide-binding site opener (ARNO), play a role in membrane signal transduction from the plasma membrane (Cohen et al., 2007), whereas the larger members of the family, such as Golgi-specific brefeldin A resistance guanine nucleotide exchange factor 1 (GBF1) and brefeldin-A-inhibited guanine nucleotide exchange proteins 1 and 2 (BIG1 and BIG2), play a role in membrane trafficking (Lefrancois and McCormick, 2007; Manolea et al., 2008; Shinotsuka et al., 2002). Arf1 is involved in the recruitment of COPI (Dascher and Balch, 1994), the Golgi-localized γ-ear-containing, Arf-binding proteins (GGAs) and adaptor protein (AP)-1 to Golgi membranes (Dell'Angelica et al., 2000a; Traub et al., 1993), and AP-3 to endosomal membranes (Ooi et al., 1998). The specificity of this recruitment depends on the GEFs; recent evidence has shown that BIG2 is involved in the recruitment of AP-1 (Shinotsuka et al., 2002), whereas GBF1 is required for the recruitment of COPI (Alvarez et al., 2003; Deng et al., 2009) and the GGAs to Golgi membranes (Lefrancois and McCormick, 2007). The interaction of GBF1 with membranes of the Golgi is required for the conversion of Arf1-GDP to Arf1-GTP (Niu et al., 2005). Although it has been shown that the small G protein Rab1 is involved in the recruitment of GBF1 (Monetta et al., 2007), the mechanism of GBF1 interaction with Golgi membranes has not been elucidated, as efforts to identify a protein receptor for GBF1 on Golgi membranes have been unsuccessful.

Interestingly, the recruitment of ARNO to the plasma membrane requires Arf6 and the phosphoinositide phosphatidylinositol (4,5)-bisphosphate [PtdIns(4,5)P_2] (Cohen et al., 2007). Phosphoinositides are generated by various kinases that esterify a hydroxyl group on phosphatidylinositol with a phosphate at the 3, 4 or 5 position (in any combination) of the inositol ring (Balla and Balla, 2006). We hypothesized that the recruitment of GBF1 to Golgi membranes, which requires Rab1, might also require a phosphoinositide. Because PtdIns(4)*P* is a predominant phosphoinositide in the Golgi (Balla and Balla, 2006), we tested our hypothesis using inhibitors of PtdIns(4)*P* synthesis – wortmannin and phenylarsine oxide (PAO). To determine specificity, we used small interfering RNA (siRNA) to deplete

PI4KIIIα, a phosphatidylinositol 4-kinase (PI4K) localized to the endoplasmic reticulum and early Golgi, which is sensitive to these inhibitors (Balla and Balla, 2006). In this paper, we report that the recruitment of GBF1 is mediated by PtdIns(4)*P* and identify PI4KIIIα as the enzyme required for this recruitment. Moreover, we provide evidence that the role of Rab1 in the recruitment of GBF1 is to activate the production of PtdIns(4)*P*.

Results

PtdIns(4)*P* is required for the recruitment of GBF1 to Golgi membranes

To test whether PtdIns(4)*P* plays a role in the recruitment of GBF1 to Golgi membranes, we treated cells with DMSO, 5 μM LY294002, 5 μM wortmannin or 5 μM PAO for 10 minutes. LY294002 is a potent inhibitor of phosphoinositide 3-kinases (PI3Ks), with an IC_{50} (half-maximal inhibitory concentration) of 1.4 μM; wortmannin is an inhibitor of PI3Ks and type III PI4Ks, with an IC_{50} of 50-300 nM; PAO is an inhibitor of type III PI4Ks, with greater specificity for PI4KIIIα (IC_{50} of 1-5 μM) than for PI4KIIIβ (IC_{50} of 30 μM). Following the inhibitor treatment, we performed immunofluorescence staining of the cells with the Golgi marker giantin to verify whether the inhibitors affected early Golgi morphology. This was necessary to ensure that any differences observed in GBF1 immunofluorescence staining were not due to disturbed Golgi morphology. Cells treated with DMSO and any of the three inhibitors showed strong perinuclear staining for giantin (Fig. 1A,E,I,M), suggesting that the Golgi was intact in the treated cells. To examine whether the inhibitors had an effect on the recruitment of GBF1, we stained cells for endogenous GBF1. Cells treated with either DMSO (Fig. 1B, arrow) or LY294002 (Fig. 1F, arrow) had a strong perinuclear staining pattern. However, in cells treated with wortmannin (Fig. 1J, asterisk) and PAO (Fig. 1N, asterisk), we found a diffuse staining pattern that lacked strong perinuclear staining, suggesting that PtdIns(4)*P* was necessary for the recruitment of GBF1 to Golgi membranes. Because we have previously shown that the recruitment of the GGAs requires GBF1 (Lefrancois and McCormick, 2007), we stained cells for endogenous GGA3 to examine the effect of blocking the recruitment of GBF1 on GGA3 recruitment to Golgi membranes. We found that wortmannin (Fig. 1K, asterisk) and PAO (Fig. 1O, asterisk) blocked the recruitment of GGA3, because we found a diffuse staining pattern compared with a strong perinuclear staining pattern in cells treated with DMSO (Fig. 1C, arrow) and LY294002 (Fig. 1G, arrow). To verify that the inhibitors were not adversely affecting the recruitment of proteins to the Golgi in general, we stained the cells for AP-1, a cytosolic protein complex recruited to the *trans*-Golgi (Zhu et al., 1998). We found no difference in the staining pattern of AP-1 at the Golgi in the cells treated with the inhibitors (Fig. 1H,L,P) compared with DMSO-treated cells (Fig. 1D). In an apparent contradiction, previous data demonstrated that PtdIns(4)*P* is required for the recruitment of AP-1 to Golgi membranes (Wang et al., 2003). However, the enzyme that was shown to be responsible for this recruitment, PI4KIIα, which is also implicated in the recruitment of AP-3 to endosomes (Craige et al., 2008), is insensitive to wortmannin and PAO at the concentrations used in our study (Balla and Balla, 2006). To verify more closely whether wortmannin and PAO altered Golgi morphology, which then resulted in GBF1 not being recruited, we co-stained cells with giantin and GBF1 antibodies (supplementary material Fig. S1). We found that giantin (supplementary material Fig. S1A,C) and GBF1 (supplementary material Fig. S1B,C) co-localized in DMSO-treated cells, but did not in cells treated with wortmannin (supplementary material Fig. S1D-F) or PAO (supplementary material Fig. S1G-I), because GBF1 was not recruited to Golgi membranes even though giantin staining seemed normal. To ensure that the inhibitors were not causing the degradation of GBF1, GGA3 and AP-1, we performed a western blot analysis to compare the absolute amount of these proteins in the various drug-treated cells. We found no large differences in the amount of these proteins (Fig. 1Q), regardless of drug treatment, suggesting that the lack of immunofluorescence staining for GBF1 and GGA3 was due to lack of recruitment to Golgi membranes and not to degradation of the proteins. Actin was used a loading control.

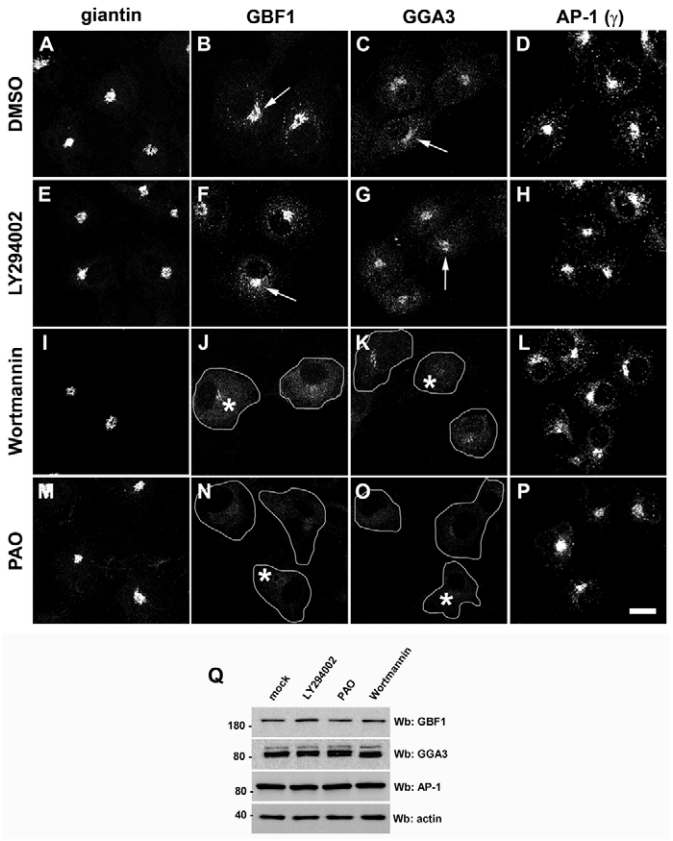

Fig. 1. PtdIns(4)*P* inhibitors block the recruitment of GBF1 to Golgi membranes. COS-7 cells grown on coverslips overnight were treated with (**A-D**) DMSO, (**E-H**) LY294002, (**I-L**) wortmannin or (**M-P**) PAO for 10 minutes and fixed in 4% paraformaldehyde. Immunofluorescence staining for giantin (A,E,I,M), GBF1 (B,F,J,N), GGA3 (C,G,K,O) or AP-1 (D,H,L,P) was then performed. Arrows in panels demonstrate the normal staining pattern of GBF1 (B,F) and GGA3 (C,G) in mock- or LY294002-treated cells, whereas asterisks show the diminished staining pattern of GBF1 (J,N) and GGA3 (K,O) in wortmannin- or PAO-treated cells. Scale bar: 10 μm. (**Q**) Western blot (Wb) analysis of the absolute amount of GBF1, GGA3, AP-1 and actin in cells treated with the inhibitors as indicated.

PI4KIIIα is required for GBF1 recruitment

Although inhibitors provide an efficient method to investigate the role of phosphatidylinositol 3-phosphate [PtdIns(3)*P*] and PtdIns(4)*P* in biological systems, they suffer from potential off-target effects. Because wortmannin and PAO block the production of PtdIns(4)*P* by blocking the type III PI4Ks, with a preference for PI4KIIIα over PI4KIIIβ at the concentrations used in this study

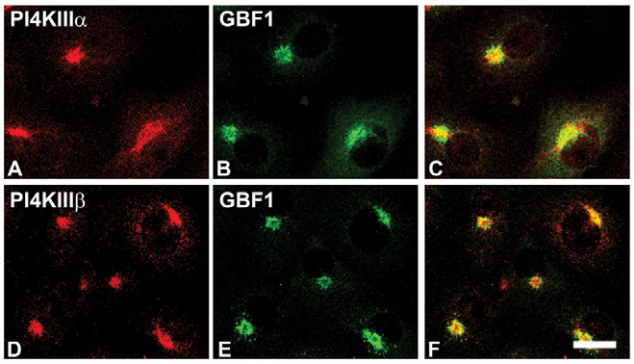

Fig. 2. GBF1 colocalizes with the PI4K PI4KIIIα. COS-7 cells were grown on coverslips overnight and fixed in 4% paraformaldehyde. Immunofluorescence staining was performed with antibodies against either PI4KIIIα (**A**) or PI4KIIIβ (**D**) and GBF1 (**B**,**E**). The merge of the images is shown in (**C**,**F**). Scale bar: 10 μm.

(Balla and Balla, 2006), we depleted PI4KIIIα and PI4KIIβ using siRNA to determine whether or not PI4KIIIα was indeed the enzyme responsible for the production of the PtdIns(4)*P* required for the recruitment of GBF1 to Golgi membranes. We first examined whether PI4KIIIα and GBF1 colocalized. We performed immunofluorescence microscopy and found that GBF1 (Fig. 2B,E) colocalized with both PI4KIIIα (Fig. 2A,C) and PI4KIIIβ (Fig. 2D,F). We next compared the recruitment of GBF1 to Golgi membranes in mock-, PI4KIIIα- and PI4KIIIβ-depleted cells by immunofluorescence. We found that in mock- (Fig. 3A, arrow) and PI4KIIIβ- (Fig. 3C, arrow) depleted cells, GBF1 was efficiently recruited to Golgi membranes, as evidenced by strong perinuclear staining for this protein in all cells. However, in PI4KIIIα-depleted cells, we found no perinuclear staining for GBF1 in more than 70% of cells (Fig. 3B, asterisk). Similarly, in mock- (Fig. 3D, arrow) and PI4KIIIβ- (Fig. 3F, arrow) depleted cells, we found strong perinuclear staining for GGA3. In support of our previous work implicating GBF1 in the recruitment of GGAs to Golgi membranes (Lefrancois and McCormick, 2007), we found that, in the PI4KIIIα-depleted cells, significantly less GGA3 was recruited to Golgi membranes, as we observed reduced perinuclear staining (Fig. 3E, asterisk). To verify that the depletion of PI4KIIIα was not having an effect on the recruitment of all proteins to the Golgi membranes, we tested for the recruitment of AP-1. We found that in mock- (Fig. 3G, arrow), PI4KIIIβ- (Fig. 3I, arrow) and PI4KIIIα- (Fig. 3H, arrow) depleted cells, AP-1 was equally recruited to the perinuclear compartment. To verify the effect of the siRNA on Golgi morphology, we stained mock-, PI4KIIIβ- and PI4KIIIα-depleted cells with the late Golgi marker TGN46 and the early Golgi marker giantin. We found no appreciable differences in the staining pattern for TGN46 in the mock (Fig. 3J, arrow), PI4KIIIβ- (Fig. 3L, arrow) or PI4KIIIα- (Fig. 3K, arrow) depleted cells. However, we did notice that, compared with mock- (Fig. 3M, arrow) and PI4KIIIβ- (Fig. 3O, arrow) depleted cells, the giantin staining was dispersed in the PI4KIIIα-depleted cells (Fig. 3N). This was expected, as depletion of Rab1b (Monetta et al., 2007) or treatment of cells with brefeldin A (Alvarez et al., 2003) produced a similar result. We verified the efficiency of PI4KIIIα and PI4KIIIβ depletion by western blot. We obtained a substantial depletion (more than 80%) of PI4KIIIα (Fig. 4A, upper panel) and PI4KIIIβ (Fig. 4C, upper panel) in COS-7 cells, whereas the level of actin

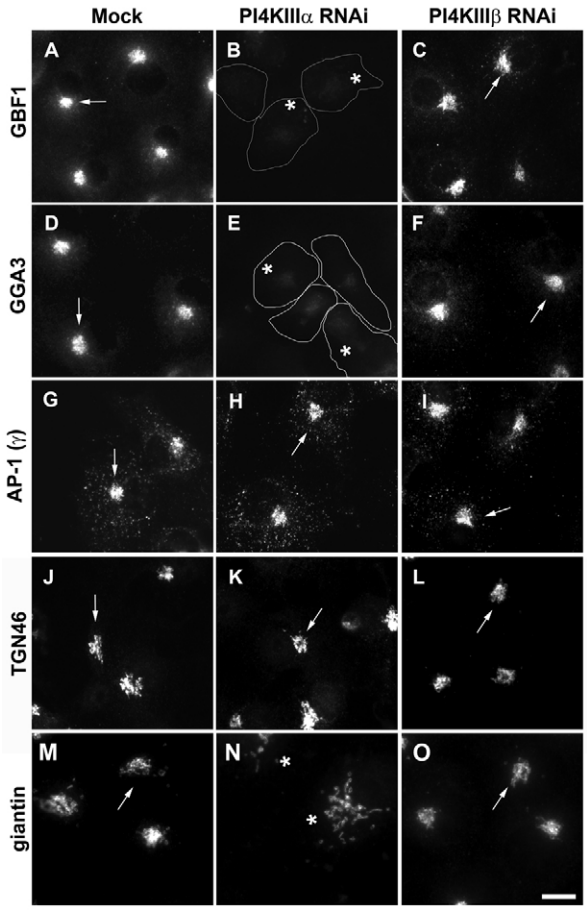

Fig. 3. Depletion of PI4KIIIα prevents the recruitment of GBF1 to Golgi membranes. COS-7 cells were mock (**A**,**D**,**G**,**J**,**M**), PI4KIIIα (**B**,**E**,**H**,**K**,**N**) or PI4KIIIβ (**C**,**F**,**I**,**L**,**O**) depleted and stained for GBF1 (A-C), GGA3 (D-F), AP-1 (G-I), TGN46 (J-L) or giantin (M-O). Arrows highlight the normal staining patterns of GBF1, GGA3, AP-1, TGN46 and giantin, whereas asterisks highlight the diminished staining pattern of GBF1 (B) and GGA3 (E) or the redistributed staining of giantin (N) in PI4KIIIα-depleted cells. Scale bar: 10 μm.

remained similar (Fig. 4A,C, bottom panel). We compared the expression levels of PI4KIIIα, GBF1, GGA3 and AP-1 in mock- versus PI4KIIIα-depleted cells (Fig. 4A). Although the levels of PI4KIIIα were significantly reduced, as expected, the level of actin remained the same, as did the absolute amount of GBF1, GGA3 and AP-1. This suggested that the lack of immunofluorescence staining of GBF1 and GGA3 (Fig. 3B,E) was due to lack of recruitment and not to degradation of the proteins. To confirm this, we compared the amount of GBF1, GGA3 and AP-1 associated with membranes in mock- versus PI4KIIIα-depleted cells (Fig. 4B). We found significantly less membrane-associated GBF1 and GGA3 in PI4KIIIα-depleted cells compared with mock-depleted cells, whereas the amount of membrane-associated AP-1 was not as affected.

Depletion of Rab1a and Rab1b decreases recruitment of GBF1

It was recently shown that the association of GBF1 with Golgi membranes required Rab1b, as Rab1b-depleted HeLa cells had no perinuclear immunofluorescence staining for GBF1, even though

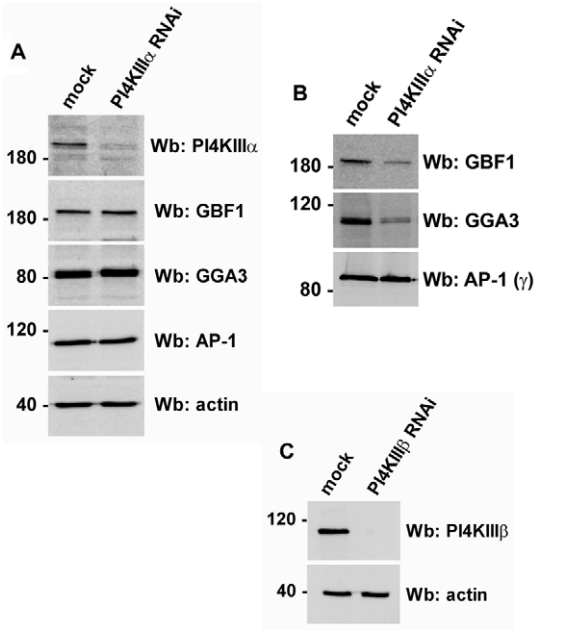

Fig. 4. Depletion of PI4KIIIα blocks the membrane association of GBF1. (**A**) HeLa cells were either mock or PI4KIIIα depleted, and western blot (Wb) analysis was performed to examine the total cellular amount of PI4KIIIα, GBF1, GGA3, AP-1 and actin. (**B**) Western blot analysis was performed to examine the amount of membrane-associated GBF1, GGA3 and AP-1 on membranes isolated from HeLa cells that were either mock or PI4KIIIα depleted. (**C**) Western blot analysis was performed on HeLa cell lysate to examine the total cellular levels of PI4KIIIβ in cells that were either mock or PI4KIIIβ depleted. Actin was used as a loading control.

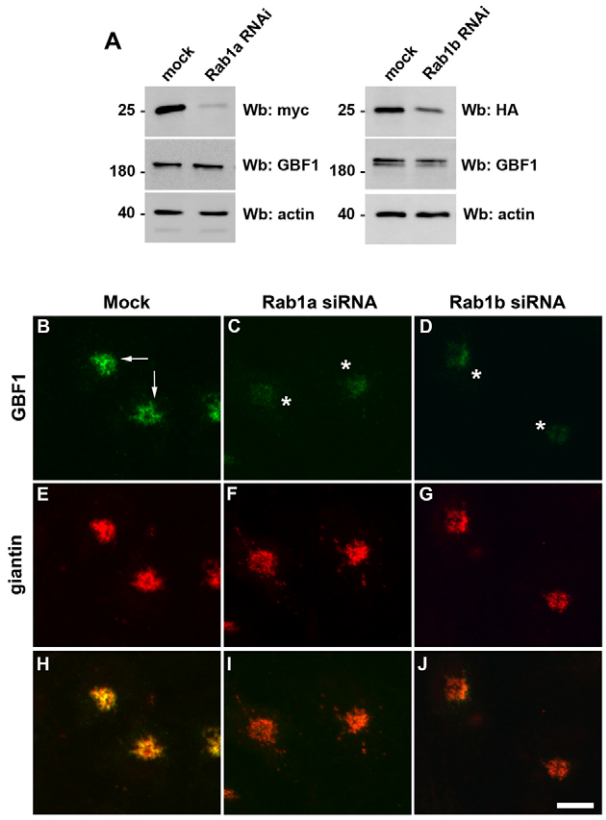

Fig. 5. Rab1a and Rab1b both participate in the recruitment of GBF1 to Golgi membranes. (**A**) Absolute amounts of myc-Rab1a, HA-Rab1b, GBF1 and actin from whole cell lysates were examined by western blot (Wb) after Rab1a RNAi (left panels) or Rab1b RNAi (right panels). COS-7 cells were mock (**B**,**E**,**H**), Rab1a (**C**,**F**,**I**) or Rab1b (**D**,**G**,**J**) depleted and immunostained for GBF1 (B-D) and giantin (E-G). The arrows show the normal staining pattern of GBF1 and colocalization with giantin in mock-depleted cells (H), whereas asterisks show diminished staining intensity for GBF1 in Rab1a- (I) and Rab1b-(J) depleted cells with little colocalization. Scale bar: 10 μm.

its expression was similar in mock- and Rab1b-depleted cells (Monetta et al., 2007). This suggested that the lack of perinuclear staining was due to a block in recruitment and not degradation (Monetta et al., 2007). To verify whether only Rab1b or both Rab1a and Rab1b were implicated in the recruitment of GBF1, we depleted cells of either Rab1a or Rab1b using siRNA. We were able to effectively deplete Rab1a (Fig. 5A, left panels) and Rab1b (Fig. 5A, right panels). Actin was used as a loading control (Fig. 5A). As expected, we found less Golgi-localized GBF1 in Rab1b-depleted cells (Fig. 5D, asterisks), but we also observed the same phenotype in Rab1a-depleted cells (Fig. 5C, asterisks) compared with mock-depleted cells (Fig. 5B, arrow), whereas staining for the early Golgi marker giantin was not appreciably affected in the Rab1a- (Fig. 5F) or Rab1b- (Fig. 5G) depleted cells compared to mock-depleted cells (Fig. 5E). Furthermore, we found no appreciable changes in the absolute amount of GBF1 in Rab1a- (Fig. 5A, left panels) and Rab1b- (Fig. 5A, right panels) depleted cells, suggesting lack of recruitment to Golgi membranes and not degradation of GBF1. To validate our observational data on the effect of Rab1 depletion on the recruitment of GBF1 to Golgi membranes, we quantified the intensity of GBF1 in the Golgi of the mock-, Rab1a- and Rab1b-depleted cells using a custom-made algorithm that first detected the presence of a cell and then calculated the fluorescence intensity of the Golgi within that cell. A representative image is shown of the immunostaining for GBF1 (Fig. 6A, arrow) and the areas identified as 'Golgi' by the algorithm are shown outlined in red (Fig. 6B, arrow). We found that the mean fluorescence intensity of GBF1, as quantified by the computer, was reduced by 55% in Rab1a-depleted cells (Fig. 6C, white bar) and 42% in Rab1b-depleted cells (Fig. 6C, gray bar) compared with mock-depleted cells (Fig. 6C, black bar).

Rab1 stimulates the production of PtdIns(4)*P* at the Golgi

Because both Rab1 and PtdIns(4)*P* seem to be implicated in the recruitment of GBF1 to Golgi membranes, we tested whether the role of PtdIns(4)*P* was to localize Rab1a and Rab1b to the Golgi. We treated cells with LY294002 (Fig. 7B,F), wortmannin (Fig. 7C,G) or PAO (Fig. 7D,H) after they had been transfected with myc-Rab1a or hemagglutinin (HA)-Rab1b. When compared with DMSO-treated cells (Fig. 7A,E), we found no substantial changes in the localization of myc-Rab1a or HA-Rab1b, as both Rab1a and Rab1b were recruited to the Golgi. This suggested that the membrane association of Rab1 was not dependant on PtdIns(3)*P* or PtdIns(4)*P*. Because PtdIns(4)*P* did not seem to play a role in Rab1 localization, we sought to further explore the relationship between Rab1 and PtdIns(4)*P* in GBF1 recruitment to Golgi membranes. Retromer is a protein complex implicated in the endosome to Golgi trafficking pathway of several proteins, including the lysosomal sorting receptors sortilin and cation-independent mannose 6-phosphate receptor (Aguilar et al., 1997;

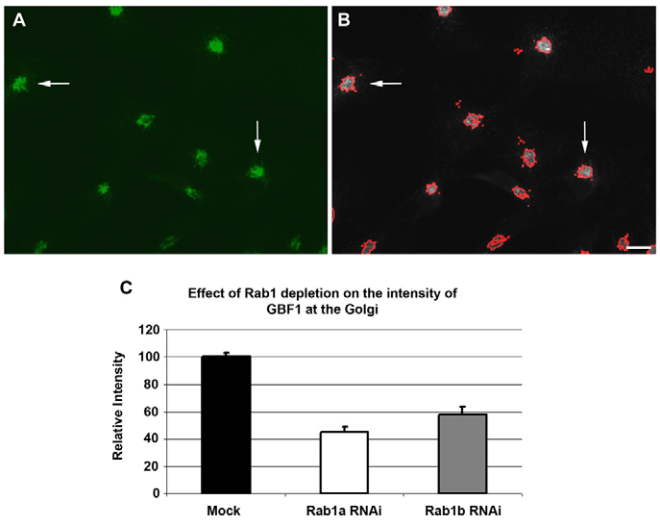

Fig. 6. Depletion of Rab1a and Rab1b reduces the membrane association of GBF1 at the Golgi. An automated algorithm was used to measure the intensity of GBF1 immunofluorescence at the Golgi of mock-, Rab1a- or Rab1b-depleted cells. A representative immunofluorescence microscopy image shows the localization of GBF1 at the Golgi (**A**, arrow) and the areas the software identified as Golgi to be quantified (**B**, arrow). (**C**) The intensity of GBF1 staining was set at 100% in mock-depleted cells (black bar). Rab1a and Rab1b depletion resulted in a 55% (white bar) and 42% (gray bar) decrease in GBF1 intensity, respectively. Bars represent average intensity from 226, 205 and 88 cells, respectively, with the standard error of the mean.

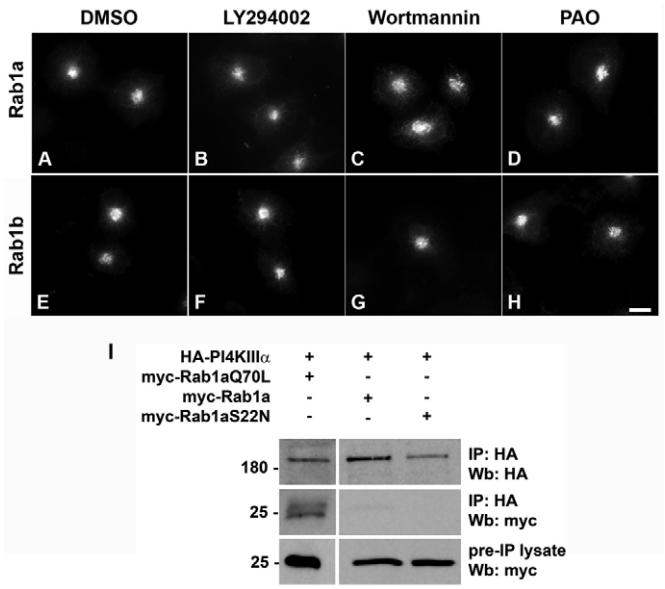

Fig. 7. PtdIns(4)*P* is not required for Rab1 association with Golgi membranes. COS-7 cells were transfected with either myc-Rab1a (**A-D**) or HA-Rab1b (**E-H**), treated with DMSO (A,E), LY294002 (B,F), wortmannin (C,G) or PAO (D,H) for 10 minutes, fixed in 4% paraformaldehyde and then immunostained with either anti-myc or anti-HA antibodies. Scale bar: 10 µm. (**I**) Total cell lysate from HeLa cells co-transfected with HA-PI4KIIIα and myc-Rab1aQ70L, myc-Rab1a or myc-Rab1aS22N was immunoprecipitated (IP) with anti-HA antibody. Western blotting (Wb) was performed with polyclonal anti-myc antibody and anti-HA antibody.

Arighi et al., 2004; Canuel et al., 2008; Seaman, 2004). To function, retromer is recruited to endosomes, a process that requires the PI3K VPS34 (Burda et al., 2002) and the small G protein Rab7 (Rojas et al., 2007). Rab7 has been shown to activate VPS34, as dominant-active Rab7 (Rab7Q67L) transfected into BHK cells increased the intensity of a PtdIns(3)*P* reporter probe, 2X-FYVE^{hrs}-myc (Stein et al., 2003). Based on these data, we asked whether the role of Rab1 in the recruitment of GBF1 to Golgi membranes was to activate PtdIns(4)*P* synthesis. First, to test whether Rab1 interacts with PI4KIIIα, HeLa cells were co-transfected with HA-PI4KIIIα and myc-Rab1aQ70L (dominant-active Rab1a), myc-Rab1a or myc-Rab1aS22N (dominant-negative Rab1a). The total cell lysate was then immunoprecipitated with anti-HA antibody. We observed an interaction between HA-PI4KIIIα and myc-Rab1aQ70L, but not myc-Rab1 or myc-Rab1aS22N, suggesting that the interaction between Rab1a and PI4KIIIα is GTP dependent (Fig. 7I). To test whether Rab1 could increase the amount PtdIns(4)*P* in the Golgi, we used GFP-PH (a generous gift from Maria Antonietta De Matteis, Consorzio Mario Negri Sud, Italy), a probe derived from the pleckstrin homology (PH) domain of FAPP1 that specifically binds to Golgi-localized PtdIns(4)*P* (Godi et al., 2004). We imaged GFP-PH in COS-7 cells (Fig. 8A,D) transfected with myc-Rab1a (Fig. 8B) or myc-Rab1aQ70L (Fig. 8E). We found that dominant-active Rab1a could increase the intensity of GFP-PH, and hence the amount of PtdIns(4)*P* in the Golgi compared with wild-type Rab1a. To vigorously quantify that dominant-active Rab1 could increase the amount of PtdIns(4)*P* in the Golgi, we co-transfected GFP-PH with sortilin-myc (as a transfection control), myc-Rab1aQ70L or HA-Rab1bQ67L (dominant-active Rab1b). We then quantified the intensity of GFP-PH in the Golgi of the co-transfected cells using a custom-made algorithm that first detected the presence of a cell and then calculated the fluorescence intensity of the Golgi within that cell. An example of the quantified GFP-PH probe images is shown (Fig. 8G, arrow). The areas identified as 'Golgi' are outlined in pink on the image (Fig. 8H, arrow) and the mean intensity was calculated. We analyzed 65, 73 and 76 cells per group, respectively, using the software we developed and found that, compared to sortilin (Fig. 8I, black bar), Rab1aQ70L (Fig. 8I, white bar) and Rab1bQ67L (Fig. 8I, gray bar) increased the intensity of GFP-PH in the perinuclear region by 55% and 41%, respectively. This suggested that dominant-active Rab1a and Rab1b increased the amount of PtdIns(4)*P* in the Golgi compared with sortilin.

Discussion

GEFs are thought to be important in the spatio-temporal regulation of the activation of Arfs (Jackson and Casanova, 2000), the small G proteins that are involved in the recruitment of cytosolic membrane components required for vesicular trafficking. For example, Arf1 is required for the recruitment of AP-1 (Traub et al., 1993), the GGAs (Dell'Angelica et al., 2000a) and AP-3 (Ooi et al., 1998). The question is how cells regulate the activation of Arf1 to specifically recruit AP-1 and the GGAs to the Golgi, and AP-3 to the endosomes. GEFs are localized to and recruited to specific membranes, and it is this step that is believed to control the specificity of the activation of Arfs. The large ArfGEF GBF1 is an activator of Arf1, and is required for the recruitment of both the COPI complex (Alvarez et al., 2003; Deng et al., 2009) and the GGAs (Lefrancois and McCormick, 2007), whereas the GEF BIG2, which also activates Arf1, has been implicated in the recruitment

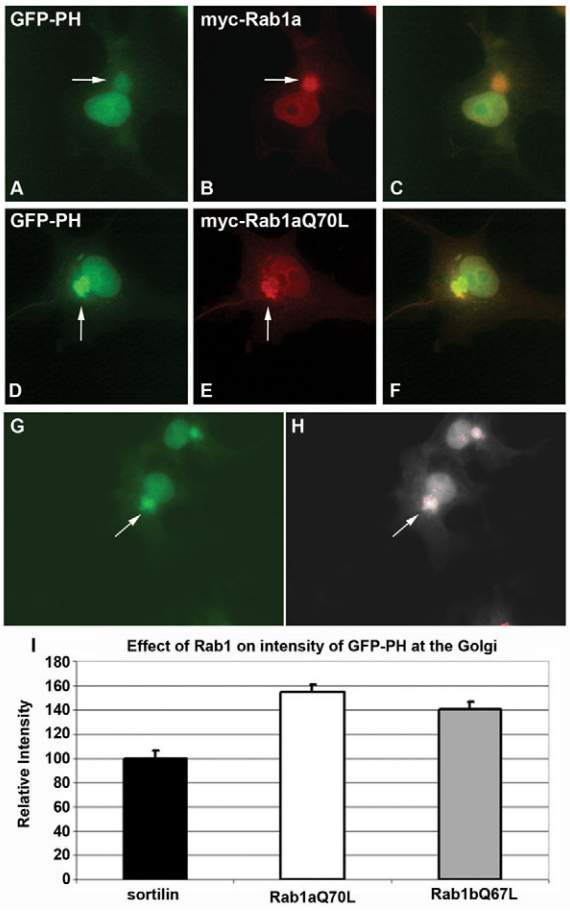

Fig. 8. Dominant-active Rab1 increases the amount of PtdIns(4)*P* in the Golgi. COS-7 cells were transfected with GFP-PH (**A-F**) and myc-Rab1a (A-C) or myc-Rab1aQ70L (D-F) and immunostained with anti-myc antibody (B,C,E,F). Arrows highlight the localization of GFP-PH (A,D), myc-Rab1a (B) and myc-Rab1aQ70L (E). Panels C and F show the merges of images. COS-7 cells were co-transfected with GFP-PH and sortilin-myc, myc-Rab1aQ70L or HA-Rab1bQ67L. An automated algorithm was used to measure the intensity of the GFP-PH probe at the Golgi. A representative immunofluorescence microscopy image shows the localization of the GFP-PH probe (**G**, arrow) and the region the software identifies as Golgi to be quantified (outlined in pink) (**H**, arrow). (**I**) The average intensity of the GFP-PH probe in the Golgi of cells transfected with sortilin (black bar), Rab1aQ70L (white bar) or Rab1aQ67L (gray bar) is shown. Bars represent the average intensity from 65, 73 and 76 cells, respectively, with the standard error of the mean.

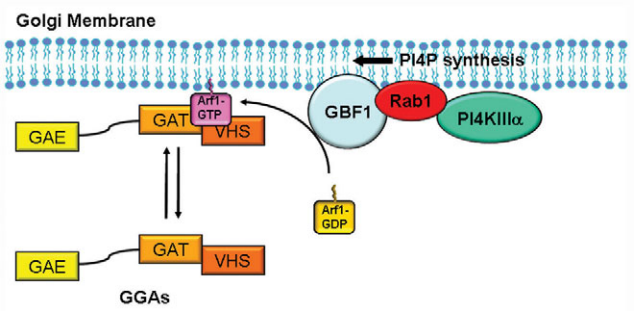

Fig. 9. Model of GBF1 recruitment to Golgi membranes. The recruitment of the GGAs is initiated by Rab1, which stimulates the production of PtdIns(4)*P* (PI4P) by PI4KIIIα. PtdIns(4)*P* then enables GBF1 to be recruited, which in turn can activate Arf1-GDP to Arf1-GTP. Arf1-GTP then recruits GGAs, which bind to specific motifs in the cytosolic tails of receptors (not shown) to sort them into trafficking vesicles.

of AP-1 (Ishizaki et al., 2008). Therefore, understanding how GEF recruitment to membranes is controlled is of vital importance for vesicular trafficking. Although GBF1 interacts with several proteins of the Golgi membrane, none is absolutely required for its recruitment (Garcia-Mata and Sztul, 2003). Given that the recruitment of ARNO requires PtdIns(4,5)P_2 (Cohen et al., 2007), we tested whether a phosphoinositide is involved in the recruitment GBF1 to Golgi membranes. We found that PtdIns(4)*P* but not PtdIns(3)*P* was required for GBF1 recruitment to Golgi membranes. Moreover, we have shown that PI4KIIIα, a PI4K localized to the Golgi, is necessary for GBF1 recruitment. We have previously shown that GBF1 is required for the recruitment of the GGAs (Lefrancois and McCormick, 2007). In support of this, cells depleted of PtdIns(4)*P* or PI4KIIIα had little or no Golgi-localized GGA3. Because GBF1 is also implicated in the recruitment of COPI to Golgi membranes (Alvarez et al., 2003; Deng et al., 2009), we predict that but have not tested whether the depletion of PtdIns(4)*P* would also affect the recruitment of the COPI complex. A previous study has shown that PtdIns(4)*P* produced by PI4KIIα, a Golgi-localized PI4K, is required for GGA recruitment (Wang et al., 2007). However, PI4KIIα is not sensitive to wortmannin or PAO. Our data do not exclude a role for PI4KIIα in the recruitment of the GGAs; it is possible that two recruitment mechanisms exist or both enzymes are required, as PI4KIIIα is localized towards the early Golgi whereas PI4KIIα is localized to the later Golgi and endosomes. GBF1 does not have a defined lipid-binding region, such as a PH domain. We attempted to generate domains of GBF1 tagged with GST to perform a lipid overlay assay and test whether GBF1 interacts directly with PtdIns(4)*P*. However, under several different conditions, we were unable to generate the required amount of protein to perform our assay. Although we can not rule out the possibility that GBF1 binds to another protein, which in turn interacts with PtdIns(4)*P*, we suggest that the interaction is direct and mediated by a potential polybasic region of GBF1, such as residues 226 to 231 (KWKKQK), which are known to bind lipids (Yeung et al., 2008) and localize Drs2p (RMKKQR) to the Golgi by interacting with PtdIns(4)*P* (Natarajan et al., 2009).

The small G protein Rab1b has previously been implicated in the recruitment of GBF1 to membranes. Recruitment of GBF1 requires its N-terminal region, as deletion mutants did not interact with Rab1b and were not recruited (Monetta et al., 2007). In addition to Rab1b, we show that Rab1a and PtdIns(4)*P* are involved in the recruitment of GBF1. To resolve how both Rab1 and PtdIns(4)*P* participate in the recruitment of GBF1, we tested two possibilities. One possible mechanism we explored was whether Rab1 localization to the Golgi depended on the synthesis of PtdIns(4)*P*. To test this hypothesis, we treated cells with PtdIns(4)*P* inhibitors (wortmannin and PAO) and performed immunofluorescence staining. Rab1a and Rab1b were localized to the Golgi, and we could not detect any changes in the intensity of the fluorescence staining in the perinuclear area compared with DMSO-treated cells. Because it was previously shown that Rab7 could activate VPS34 to produce PtdIns(3)*P* (Stein et al., 2003), we tested whether Rab1 could increase the amount of PtdIns(4)*P* in the Golgi. To accomplish this, we used GFP-PH, a probe that has been shown to bind PtdIns(4)*P* in the Golgi (Godi et al., 2004).

The intensity of GFP-PH at the Golgi reflects the amount of PtdIns(4)*P* present. We found an increase in GFP-PH intensity in cells transfected with dominant-active Rab1a and Rab1b compared with cells transfected with sortilin. We propose that Rab1 activates PI4KIIIα to produce PtdIns(4)*P* in the Golgi (Fig. 9). PtdIns(4)*P* serves as a binding site for GBF1, which is recruited to Golgi membranes. When GBF1 is membrane bound, it can then activate Arf1, which recruits COPI and the GGAs. This in turn enables proper vesicular sorting and trafficking.

Materials and Methods

Antibodies and other reagents

All reagents were purchased from Sigma-Aldrich (Oakville, ON, Canada) unless otherwise noted. The following monoclonal antibodies were used in our study: anti-myc (Cedarlane Laboratories, Burlington, ON, Canada), anti-GBF1 (BD Biosciences, Mississauga, ON, Canada), anti-GGA3 (BD Biosciences), anti-AP-1 (Sigma-Aldrich), anti-actin (BD Biosciences). The following polyclonal antibodies were used: anti-myc, anti-giantin, anti-PI4KIIIα, anti-PI4KIIIβ and anti-TGN46 (all Cedarlane Laboratories). LY294002, wortmannin and PAO were purchased from Cedarlane Laboratories. Wild-type myc-tagged Rab1a (myc-Rab1a) and dominant-negative myc-tagged Rab1a (myc-Rab1aS22N) were generous gifts from Terry Hebert, McGill University, Montreal, Canada. We used the QuickChange mutagenesis kit (Stratagene, La Jolla, CA) to generate dominant-active Rab1a (myc-Rab1aQ70L). HA-tagged wild-type Rab1b (HA-Rab1b) and dominant-active Rab1b (HA-Rab1bQ67L) were generous gifts from Angelika Barnekow, University of Münster, Germany.

Immunofluorescence microscopy

COS-7 or HeLa cells were grown in DMEM supplemented with 10% FBS and antibiotics. If required, the cells were grown on glass coverslips overnight and transfected with 1 μg DNA using Lipofectamine (Invitrogen, Burlington, ON, Canada) with the Plus reagent (Invitrogen). Cells were fixed in 4% paraformaldehyde in PBS for 10 minutes and washed twice in PBS for 5 minutes each. The cells were then permeabilized with 0.5% Triton X-100 for 30 minutes, washed twice for 5 minutes in PBS then incubated with the primary antibody in a solution of 0.1% BSA in PBS for 2 hours. The coverslips were then incubated with the appropriate secondary antibody conjugated to either AlexaFluor-488 or AlexaFluor-594 (Invitrogen) diluted in 0.1% BSA in PBS for 1 hour. The coverslips were then mounted on glass slides with Fluoromount G (Fisher Scientific, Ottawa, ON, Canada) and sealed with clear nail polish. A Leica DMRE microscope was used and bright-field fluorescence 16-bit 1360×1036 pixel images were acquired. To compare fluorescence intensity levels from different cells and samples, CCD camera exposure time and gain remained constant.

RNA interference

To deplete cells of PI4KIIIα, PI4KIIIβ, Rab1a or Rab1b, cells were plated and transfected with SmartPool siRNA reagent (Fisher Scientific) using Oligofectamine (Invitrogen) following the manufacturer's instructions. For efficient depletion, we treated cells with 100 nM of the SmartPool on two consecutive days. 48 hours post-transfection, our assays were performed.

Membrane isolation

Cells were lysed in lysis buffer (250 mM sucrose, 20 mM HEPES, 10 mM KCl, 1.5 mM $MgCl_2$ and 1 mM EDTA) by passing them through a 25G needle using a 1 ml syringe. The nuclear pellet was removed by centrifugation at 720***g*** for 5 minutes. Debris was removed from the cytosolic and membrane fractions by centrifugation of the post-nuclear supernatant at 10,000 ***g*** for 5 minutes. Centrifugation of the supernatant at 100,000 ***g*** was used to collect membranes, which were then resuspended in membrane buffer (lysis buffer plus 10% glycerol and 0.1% SDS).

Quantification

Cells were co-transfected following the same protocol as above, with GFP-PH in combination with sortilin-myc, myc-Rab1aQ70L or HA-Rab1bQ67L. 16 hours post-transfection, cells were fixed and immunostained as described above. To accurately measure the expression level of GFP-PH, we developed an algorithm to perform an unbiased quantification of fluorescence microscopy images. The program was implemented in MATLAB (The MathWorks, Natick, MA) and its image analysis toolbox. The algorithm first achieves a segmentation of the Golgi apparatus, and then measures the mean fluorescence intensity and standard deviation for this particular organelle. The Golgi apparatus segmentation is obtained by applying three consecutive automatic thresholding processes to select the most intense region in each cell. The first intensity threshold yields a binary image that separates the fluorescent cells (foreground pixels) from the background. After a 9-pixel dilation, individual cells were considered, taking into account only pixel connectivity. Next, a second intensity threshold was established for each individual cell in the image. This second threshold visually separates the pixels of both the Golgi and the nuclei from the rest of the cell. Finally, a third threshold segments the Golgi and only the intensity of those pixels was considered to establish the brightness in each cell. To determine all these threshold levels automatically and in an objective manner, the Otsu algorithm was used. This algorithm considers the intensity histogram of a region of interest in an image and computes the intensity level that separates the pixels in two distributions, such that the sum of variances of the two distributions is minimal. Overall, the algorithm selects, with no bias, which pixels belong to the Golgi apparatus and computes their mean intensity for each cell in a series of images. All cells were considered equally and the total mean intensity and standard deviation were calculated for the three different protocols considered in the study.

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Supplementary material available online at
http://jcs.biologists.org/cgi/content/full/123/13/2273/DC1

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