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Phosphorylation and membrane dissociation of the ARF exchange factor GBF1 in mitosis

Yuichi MOROHASHI[1], Zita BALKLAVA[2], Matthew BALL, Helen HUGHES[3] and Martin LOWE[4]

Faculty of Life Sciences, University of Manchester, The Michael Smith Building, Oxford Road, Manchester M13 9PT, U.K.

Secretory protein trafficking is arrested and the Golgi apparatus fragmented when mammalian cells enter mitosis. These changes are thought to facilitate cell-cycle progression and Golgi inheritance, and are brought about through the actions of mitotically active protein kinases. To better understand how the Golgi apparatus undergoes mitotic fragmentation we have sought to identify novel Golgi targets for mitotic kinases. We report in the present paper the identification of the ARF (ADP-ribosylation factor) exchange factor GBF1 (Golgi-specific brefeldin A-resistant guanine nucleotide-exchange factor 1) as a Golgi phosphoprotein. GBF1 is phosphorylated by CDK1 (cyclin-dependent kinase 1)–cyclin B in mitosis, which results in its dissociation from Golgi membranes. Consistent with a reduced level of GBF1 activity at the Golgi membrane there is a reduction in levels of membrane-associated GTP-bound ARF in mitotic cells. Despite the reduced levels of membrane-bound GBF1 and ARF, COPI (coat protein I) binding to the Golgi membrane appears unaffected in mitotic cells. Surprisingly, this pool of COPI is dependent upon GBF1 for its recruitment to the membrane, suggesting that a low level of GBF1 activity persists in mitosis. We propose that the phosphorylation and membrane dissociation of GBF1 and the consequent reduction in ARF-GTP levels in mitosis are important for changes in Golgi dynamics and possibly other mitotic events mediated through effectors other than the COPI vesicle coat.

Key words: ADP-ribosylation factor (ARF), Golgi apparatus, Golgi-specific brefeldin A-resistant guanine nucleotide-exchange factor 1 (GBF1), mitosis, phosphorylation.

INTRODUCTION

When mammalian cells enter mitosis the Golgi apparatus undergoes extensive fragmentation, converting from a characteristic ribbon of stacked cisternae into tubulovesicular clusters and numerous free vesicles dispersed in the cytoplasm [1–3]. Golgi disassembly is concomitant with an arrest in secretory protein trafficking, which occurs at the level of both ER (endoplasmic reticulum)-to-Golgi and intra-Golgi trafficking [4]. Golgi disassembly and trafficking arrest are dependent upon the action of several protein kinases, including CDK1 (cyclin-dependent kinase 1), Plk1 (polo-like kinase 1) and MEK1 [MAPK (mitogen-activated protein kinase)/ERK (extracellular-signal-regulated kinase) kinase 1], which in turn phosphorylate downstream targets to bring about changes in Golgi organization and function [2,5]. Several Golgi targets for these kinases have been identified, with the most prominent being members of the GRASP (Golgi reassembly and stacking protein) and golgin families of proteins that are primarily responsible for linking or tethering Golgi membranes during membrane traffic and Golgi assembly [6]. Allied to protein phosphorylation, ubiquitylation of certain proteins is also required for mitotic Golgi disassembly, although the details remain unclear [7].

Prior to mitosis, in late G_2-phase, the Golgi ribbon is unlinked through phosphorylation of the GRASPs, which occurs downstream of MEK1 [8,9], and membrane scission by the CtBP (C-terminal binding protein)/BARS (brefeldin A-ribosylated substrate) protein [10]. When cells enter mitosis, the Golgi stacks are converted into vesicles and tubular remnants in a CDK1 and Plk1-dependent manner [1,11,12]. An attractive model to explain this fragmentation is that COPI (coat protein I) vesicles continue to bud from the cisternal rims, but cannot fuse, thereby converting Golgi cisternae into vesicles [13,14]. Phosphorylation of GRASPs by CDK1 and Plk1 is thought to promote cisternal unstacking, allowing better access to the COPI budding machinery and hence more extensive vesiculation [15,16]. Phosphorylation of golgins probably contributes to this process by promoting unstacking, as well as preventing the tethering and subsequent fusion of COPI vesicles [17,18]. In addition to the COPI pathway, there is also a less well understood COPI-independent fragmentation that has been observed both *in vitro* and *in vivo* [19,20]. This pathway, which is thought to fragment the central core region of the Golgi, proceeds via transient tubular intermediates that undergo membrane scission to form smaller breakdown products. In contrast with the COPI budding model, it has been proposed that COPI-independent fragmentation represents the major breakdown pathway [21]. Here, it is thought that membrane dissociation of COPI in mitosis leads to extensive membrane tubulation in a manner similar to treatment with the fungal metabolite BFA (brefeldin A). It has been reported that the Golgi-derived

Abbreviations used: ARF, ADP-ribosylation factor; AOTF, acousto-optic tunable filter; BFA, brefeldin A; CASP, cytohesin-associated scaffolding protein; CDK1, cyclin-dependent kinase 1; COPI, coat protein I; DMEM, Dulbecco's modified Eagle's medium; DTT, dithiothreitol; ER, endoplasmic reticulum; ERGIC, ER-to-Golgi intermediate compartment; FCS, fetal calf serum; FRAP, fluorescence recovery after photobleaching; GBF1, Golgi-specific BFA-resistant guanine-nucleotide-exchange factor 1; GEF, guanine-nucleotide-exchange factor; GFP, green fluorescent protein; GM130, *cis*-Golgi membrane protein of 130 kDa; GRASP, Golgi reassembly and stacking protein; GST, glutathione transferase; HRP, horseradish peroxidase; MAPK, mitogen-activated protein kinase; MEK1, MAPK/ERK (extracellular-signal-regulated kinase) kinase 1; NRK, normal rat kidney; PBD, polo box domain; Plk1, polo-like kinase 1; ZFPL1, zinc-finger protein-like 1.

[1] Present address: Department of Neuropathology and Neuroscience, The Graduate School of Pharmaceutical Sciences, The University of Tokyo, 7-3-1 Hongo, Bunkyo-ku, Tokyo, Japan

[2] Present address: School of Life and Health Sciences, Aston University, Aston Triangle, Birmingham B4 7ET, U.K.

[3] Present address: Department of Biochemistry, University of Bristol, School of Medical Sciences, University Walk, Bristol BS8 1TD, U.K.

[4] To whom correspondence should be addressed (email martin.lowe@manchester.ac.uk).

tubular intermediates then fuse with the ER, merging the two compartments [22,23]. It should be noted, however, that several studies have failed to observe the merging of Golgi and ER in mitosis [24–27]. Nevertheless, it remains possible that COPI-independent tubulation is a major disassembly pathway.

COPI is recruited to the membrane through binding to active GTP-bound ARF (ADP-ribosylation factor). Several GEFs (guanine-nucleotide-exchange factors) for ARFs exist in mammalian cells, including GBF1 (Golgi-specific BFA-resistant GEF 1), which is localized to the ERGIC (ER-to-Golgi intermediate compartment) and *cis*-Golgi [28–32]. Like other ARF GEFs, GBF1 has a central Sec7 domain that catalyses nucleotide exchange on ARF, resulting in its association with the membrane and subsequent recruitment of downstream effectors [33]. Several ARF GEFs, including GBF1, are targets for BFA, which locks the GEF in an inactive membrane-bound complex with ARF–GDP [32–34]. A major effector regulated by GBF1 is the COPI vesicle coat [30,31,35–39]. Indeed GBF1 appears to be the sole ARF GEF responsible for COPI recruitment to the membrane, and consequently it is vital for membrane trafficking at the ER/Golgi interface [30,35–39]. Although GBF1 can bind to several proteins, including Rab1b [40], the vesicle docking protein p115 [41] and the γ-COP subunit of the COPI coat itself [42], how it is targeted to the ERGIC and *cis*-Golgi remains unclear.

In the present paper we report the identification of GBF1 as a mitotic phosphoprotein. Phosphorylation of GBF1 by CDK1 results in its reduced association with the membrane and a concomitant decrease in levels of membrane-associated GTP-bound ARF. Despite this, COPI remains membrane-associated in mitosis, and surprisingly this binding appears to retain GBF1-dependancy. Our findings suggest a role for mitotic phosphorylation of GBF1 in regulating ARF effector proteins, which in turn might alter Golgi membrane dynamics or other ARF-dependent processes in mitotic cells.

EXPERIMENTAL

Materials and antibodies

All materials were from Sigma or Merck unless otherwise stated. Protease inhibitor cocktail set III (used at a 1:250 dilution), golgicide A, roscovitine, CGP74514A, U0126 and purvalanol A were obtained from Calbiochem. [γ-^{32}P]ATP was purchased from NEN. Mouse monoclonal antibodies against GBF1 and GS28 were obtained from BD Biosciences. Mouse anti-Myc antibody 9B11 was purchased from Cell Signaling Technology. Rabbit polyclonal anti-GM130 (*cis*-Golgi matrix protein of 130 kDa) antibody (MLO7) has been described previously [18]. Sheep anti-golgin-84 and rabbit anti-CASP (cytohesin-associated scaffolding protein) antibodies were described previously [43,44]. Sheep anti-ZFPL1 (zinc-finger protein-like 1) antibodies have been described previously [45]. Rabbit anti-p115 and anti-ARF antibodies were gifts from Professor Elizabeth Sztul (Department of Cell Biology, University of Alabama at Birmingham, Birmingham, AL, U.S.A.) and Professor Shamshad Cockcroft (Department of Neuroscience, Physiology and Pharmacology, University College London, London, U.K.) respectively. Mouse and rabbit anti-β-COP antibodies M3A5 and EAGE were previously raised in the laboratory of Professor Thomas Kreis (deceased). Rabbit anti-mannosidase I was a gift from Professor Francis Barr (University of Liverpool, Liverpool, U.K.). Sheep anti-GST (glutathione transferase) antibodies have been described previously [46]. Fluorophore-conjugated (Alexa Fluor® 594 and Alexa Fluor® 488) and HRP (horseradish peroxidase)-conjugated secondary antibodies were purchased from Molecular Probes and Tago Immunologicals respectively.

Molecular biology and protein expression

Standard molecular biology techniques were used for all constructs; primer sequences are available upon request from the corresponding author. All constructs were verified by DNA sequencing. Full-length human GBF1 cDNA in pcDNA4/Myc-His was kindly provided by Professor Elizabeth Sztul. cDNA encoding full-length GBF1 and a truncation lacking the C-terminal 59 amino acids were cloned into the EcoRI and XhoI sites of pcDNA3.1 and an N-terminal Myc-tag was added using QuikChange® mutagenesis (Stratagene). DNA encoding N-terminally Myc-tagged wild-type full-length and the truncated version (ΔC59) of GBF1 were inserted into EcoRI and XhoI sites of pMXs-puro (a gift from Professor Toshio Kitamura, University of Tokyo, Tokyo, Japan) for retroviral expression. cDNA encoding Myc-tagged full-length or the ΔC59 mutant were cloned into the EcoRI and NotI sites of pAcGHLT for baculovirus-driven expression of GST-tagged protein in insect cells. cDNA encoding Plk1 PBD (polo box domain; amino acids 305–603) was cloned from a human liver cDNA library into the EcoRI and XhoI sites of pGEX 4T-1. The VHS-GAT domain of GGA3 (amino acids 1–313) was cloned from a human liver cDNA library into the EcoRI and XhoI sites of pGEX 4T-1. Bacterial expression and purification of GST-tagged Plk1 PBD and GGA3 constructs was performed using standard methods. GST-tagged GBF1 was expressed in Sf9 cells using the BacMagic system (Novagen). Cells were harvested from four 162 cm^2 flasks 1 day post-infection, and lysed in 20 ml of 50 mM sodium phosphate (pH 8.0), 0.3 M NaCl and 1 % Triton X-100 containing protease inhibitors by sonication. Lysates were clarified by centrifugation (100000 ***g*** for 1 h at 4 °C) and incubated with glutathione–Sepharose for 1 h at 4 °C. After washing, GST–GBF1 was eluted from the beads using 20 mM reduced glutathione, 50 mM Tris/HCl (pH 7.4) and 150 mM NaCl. Glycerol was added to 10 % and the purified protein was snap-frozen and stored at −80 °C. ε-COP–GFP (GFP is green fluorescent protein) [47] (kindly provided by Dr Theresa Ward, London School of Hygeine and Tropical Medicine, University of London, London, U.K., and Professor Jennifer Lippincott-Schwartz, National Institutes of Health, Bethesda, MD, U.S.A.) was amplified by PCR and subcloned into pLVX-Puro (Clontech) using EcoRI-XhoI sites, generating a lentivirus from which ε-COP–GFP was stably expressed in NRK (normal rat kidney) cells. Plasmid encoding GFP-tagged wild-type ARF1 was kindly provided by Professor Jennifer Lippincott-Schwartz.

Retroviral infection of mammalian cells

Retroviral infection was carried out as reported previously [48]. The packaging cell line Plat-A (from Professor Toshio Kitamura, University of Tokyo, Tokyo, Japan) was kept in DMEM (Dulbecco's modified Eagle's medium) containing 1 μg/ml puromycin and 10 μg/ml blasticidin. Retroviral transfer plasmids were transfected using FuGENE® 6 (Roche Biochemicals) into Plat-A cells. After 48 h, conditioned media were filtered through a 0.45 μm filter and used as a virus stock. For infection, target cells were cultured with a virus stock diluted into an equal volume of DMEM/10 % FCS (fetal calf serum) containing 8 μg/ml Polybrene. Stable infectants of HeLa cells were generated by selection in DMEM containing 2 μg/ml puromycin. Cells were analysed at 10–14 days after infection.

Fluorescence microscopy

Adherent HeLa or NRK cells were grown on glass coverslips at 37 °C and 5 % CO_2 in DMEM containing 10 % FCS. For the enrichment of mitotic NRK cells, 2.5 μg/ml aphidicolin was added to the medium for 14–16 h to arrest cells at the G_1/S boundary. Cells were washed five or six times to remove the residual drug and incubated in fresh medium for 5–7 h to allow progression into mitosis. Unsynchronized HeLa cells were transfected with ARF1–GFP 16–20 h before fixation using FuGENE® 6 according to the manufacturer's instructions. Cells were fixed in paraformaldehyde [3 % (w/v) in PBS] for 20 min at room temperature (22 °C), quenched with 10 mM glycine (pH 8.5) in PBS and permeabilized with 0.1 % Triton X-100 in PBS for 5 min at room temperature. Labelling was performed by sequentially incubating with primary then secondary antibodies diluted in PBS containing 0.5 mg/ml BSA for 20 min at room temperature. Images were acquired using an Olympus BX60 microscope equipped with a coolSNAP EZ CCD (charge-coupled device) camera using Metaview software (Universal Imaging) and processed using Adobe Photoshop CS.

Incubations with interphase and mitotic cytosol

Incubations of rat liver Golgi with interphase or mitotic cytosol prepared from HeLa S3 cells was carried as described previously [44]. Briefly, 10 μg of Golgi membranes were incubated with 40 μl of interphase or mitotic cytosol [at 9 mg/ml in buffer A: 20 mM 2-glycerophosphate, 15 mM EGTA, 10 mM magnesium acetate, 50 mM potassium acetate, 2 mM ATP, 1 mM DTT (dithiothreitol) and 0.2 M sucrose] in the presence of an ATP-regeneration system (10 mM creatine phosphate and 20 μg/ml creatine kinase) for various times at 30 °C. Membranes were re-isolated by centrifugation (100 000 ***g*** for 10 min at 4 °C) through a 0.4 M sucrose cushion. In certain experiments, 10 μM staurosporine or 2 μl (20 units) of calf intestinal alkaline phosphatase (NEB) was included in the incubation. In some experiments the ATP-regeneration system was omitted and [γ-^{32}P]ATP was added at 0.5 μCi/μl. *In-vitro*-translated ^{35}S-labelled GBF1 was made using the TNT®-coupled reticulocyte lysate system (Promega) according to the manufacturer's instructions. *In-vitro*-translated protein (0.5 μl) was incubated with 20 μl of interphase or mitotic cytosol (2 mg/ml in buffer A with ATP-regeneration system) for 30 min at 30 °C. GST-tagged GBF1 (1 μg) or Protein S-tagged GM130 was bound to 10 μl of glutathione or Protein S-coupled beads and incubated with 20 μl of interphase or mitotic cytosol at 2 or 9 mg/ml containing 0.5 μCi/μl [γ-^{32}P]ATP for 30 min at 30 °C. In some cases, parallel incubations were performed by adding 10 μg of histone type III-S (Sigma). Inhibitors were added from 100×concentrated stock solutions in DMSO, while controls contained DMSO vehicle only. Cyclin B/CDK1 was purified from insect cells and added to give 0.4× and 1.0× of added histone kinase activity of mitotic cytosol. Proteins were solubilized in SDS sample buffer and analysed by SDS/PAGE and autoradiography on BioMax MS film (Kodak).

Immunoprecipitation of GBF1 and far-Western blotting

Interphase or mitotic rat liver Golgi membranes were extracted in extraction buffer [20 mM Tris/HCl, 50 mM KCl, 5 mM $MgCl_2$ and 0.5 % Triton X-100 (pH 7.4)] containing protease inhibitors for 1 h on ice prior to clarification by centrifugation (18 000 ***g*** for 10 min at 4 °C). Clarified Golgi membrane extracts, or interphase or mitotic HeLa S3 cytosol (containing 0.5 % Triton X-100 and protease inhbitors) were incubated with 2 μg of mouse anti-GBF1 antibody followed by 20 μl of Protein G–Sepharose for 2 h at 4 °C. After washing, bound proteins were eluted and analysed by SDS/PAGE and autoradiography, Western blotting or far-Western blotting. In some cases, immunoprecitates were incubated with 2 μl (20 units) of calf intestinal alkaline phosphatase in 50 mM Tris/HCl (pH 7.9), 0.1 M NaCl, 10 mM $MgCl_2$ and 1 mM DTT for 30 min at 37 °C prior to elution. Far-Western blotting was performed by transferring proteins on to nitrocellulose membrane, blocking the membrane in PBS containing 0.2 % Tween 20 and 5 % non-fat milk powder, followed by incubation with 1 μg/ml GST-tagged Plk1 PBD in PBS containing 0.2 % Tween 20 and 2 % non-fat milk powder for 3 h at room temperature. Bound Plk1 PBD was detected by incubating with anti-GST antibodies followed by HRP-conjugated secondary antibodies and ECL (enhanced chemiluminescence).

Identification of Plk1 PBD-binding proteins

For large-scale identification of Golgi Plk1 PBD-binding proteins, 200 μg of rat liver Golgi membranes was incubated with 500 μl of mitotic cytosol (9 mg/ml in buffer A) containing 0.5 μCi/μl [γ-^{32}P]ATP for 30 min at 30 °C. Membranes were re-isolated by centrifugation through a 0.4 M sucrose cushion for 10 min at 55 000 rev./min in a TLA55 rotor. Membranes were extracted in extraction buffer for 1 h on ice, clarified by centrifugation (18 000 ***g*** for 10 min at 4 °C), and incubated with 10 μg of GST–Plk1 PBD bound to glutathione–Sepharose for 1 h at 4 °C. Beads were washed, and bound proteins were analysed by SDS/PAGE and autoradiography or Western blotting. Radiolabelled proteins were excised from rehydrated gel slices and identified using MS by the University of Manchester Faculty of Life Sciences Biomolecular Analysis Facility.

Kinase inhibitor treatment of HeLa cells

Treatment with kinase inhibitors was performed on adherent HeLa cells grown in 12-well plates. Cells were arrested in mitosis with 100 ng/ml nocodazole for 22–24 h at 37 °C and treated with the required concentration of kinase inhibitor for an additional 1 h at 37 °C. Cells were harvested, washed in ice-cold PBS and extracted in 100 μl of extraction buffer containing 20 mM 2-glycerophosphate and protease inhibitors. Samples were analysed by SDS/PAGE and Western blotting.

Fractionation of HeLa cells

HeLa S3 cells were cultured in 250 ml spinner flasks. Mitotic cells were prepared by incubating in 100 ng/ml nocodazole for 22–24 h at 37 °C. In some experiments BFA was added at 5 μg/ml for 10 min prior to harvesting. Cells were harvested by centrifugation (900 ***g*** for 10 min), washed in PBS and, after swelling in 0.4× lysis buffer [1×lysis buffer is 20 mM Hepes (pH 7.4), 50 mM KCl, 5 mM $MgCl_2$, 1 mM DTT and 0.2 M sucrose], were lysed in 1× lysis buffer containing protease inhbitors by passing through a ball-bearing homogenizer (8.02 mm barrel with 8.01 mm ball). Lysates were then cleared by centrifugation at 1000 ***g*** for 10 min to generate a post-nuclear supernatant. This was subsequently layered on to lysis buffer containing 0.4 M sucrose, and cytosol and membrane fractions were generated by centrifugation at 50 000 rev./min for 15 min in a TLS55 rotor. Retrovirally transduced adherent HeLa cells expressing Myc-tagged GBF1 were scraped into PBS, washed as above and homogenized in lysis buffer by passing through a 25 gauge needle. Resulting homogenates were fractionated as above. Quantification

of bands was performed by densitometry using ImageJ software (NIH).

GST–GGA3 binding experiments

Interphase or mitotic HeLa S3 cells (25 ml of suspension culture) were incubated without or with 10 μg/ml BFA for 1 h at 37 °C prior to washing in ice-cold PBS and lysis in 800 μl of 50 mM Tris/HCl (pH 7.5), 0.1 M NaCl, 5 mM $MgCl_2$ and 1 % Triton X-100 containing protease inhibitors. After clarification by centrifugation (18 000 ***g*** for 10 min at 4 °C), 300 μl of extract was incubated with 30 μl of glutathione–Sepharose containing 50 μg of bound GST or GST–GGA3 VHS-GAT domain for 1 h at 4 °C. After washing the beads, bound ARF was detected by Western blotting. Quantification of bands was performed by densitometry using ImageJ software.

FRAP (fluorescence recovery after photobleaching)

NRK cells stably expressing ε-COP–GFP were grown on MatTek dishes and synchronized at ~40 % confluence with 2 mM thymidine for 16 h. Cells were washed with PBS and incubated in fresh DMEM for 8 h. FRAP was performed between 10 and 12 h after the second synchronization. Cells were imaged at 37 °C with the PerkinElmer UltraVIEW ERS 6FE confocal system attached to a Leica DM I6000 inverted epifluorescence microscope encompassing Yokogawa CSU22 spinning disc technology and a Hamamatsu C9100-50 EM-CCD camera controlled by Volocity 5 (Improvision) acquisition software. Cells were imaged in MEM (minimal essential medium) without Phenol Red, supplemented with 30 mM Hepes (pH 7.4), 0.5 g · l^{-1} sodium bicarbonate and 10 % FCS using a 63× 1.3 NA (numerical aperture) Plan-Apochromat objective. Photobleaching of ε-COP–GFP was performed with a ~500 nm diameter circular region. Pre-bleach images were collected for 5 s and post-bleach images were collected for 60 s using 30 % AOTF (acousto-optic tunable filter) power and low laser intensity. For ε-COP–GFP, FRAP was performed at a rate of five time points per 1 s, and region-of-interest bleaching with five iterations of the 488 and 514 nm lasers at 100 % AOTF power. Punctae that moved out of focus or moved over 500 nm during the photobleaching series could not be analysed. Fluorescence recovery in the bleached region during the time series was quantified using Volocity 5 (Improvision) FRAP analysis software and exported for analysis to GraphPad Prism 4.02. Recycling kinetics were obtained by curve fitting to a one-phase exponential (eqn 1):

$$f(\mathrm{t}) = \mathrm{A} \times (1 - \mathrm{e} - k_t) + \mathrm{B} \quad (1)$$

Where A is the mobile fraction, B is the fluorescence directly after photobleaching (%), and k is the rate of fluorescence recovery from which $t_{1/2}$ is determined [$t_{1/2}$=ln(2)/k]. Statistical significance was tested using S.D. and Student's unpaired t test.

RESULTS

Identification of GBF1 as a mitotic phosphoprotein

To identify novel mitotic Golgi phosphoproteins we used a previously described *in vitro* assay in which rat liver Golgi membranes are incubated with cytosol from interphase or mitotic HeLa cells [14,43]. Golgi extracts were then prepared and incubated with immobilized Plk1 PBD before MS identification of bound proteins. The Plk1 PBD is a phosphoserine/threonine-binding domain that targets Plk1 to various mitotic substrates [49,50]. We used binding to the Plk1 PBD to simplify the profile of Golgi phosphoproteins, as well as to identify new potential Golgi-localized Plk1-binding partners. As shown in Figure 1(A) (top panel) several mitotically phosphorylated Golgi proteins bound to wild-type Plk1 PBD, but not a H538A/K540M mutant unable to bind phosphorylated ligands [49]. Among the specifically bound proteins were GRASP65, previously identified as a Plk1 PBD ligand [51], and GM130, which may bind Plk1 PBD indirectly via its association with GRASP65 (Figure 1A, open arrowheads). A minor phosphoprotein running at 210 kDa was identified as GBF1, the Golgi-associated ARF nucleotide-exchange factor (Figure 1A, filled arrowhead). The identity of GBF1 as a Plk1 PBD-binding protein was confirmed by Western blotting (Figure 1A, bottom panel). GBF1 bound to wild-type, but not mutant, Plk1 PBD, with stronger binding observed under mitotic conditions. These results suggest that GBF1 undergoes mitosis-specific phosphorylation. This was tested by incubating Golgi membranes with cytosol in the presence of [γ-^{32}P]ATP and immunoprecipitating the protein with specific antibodies. As shown in Figure 1(B), GBF1 was phosphorylated by mitotic cytosol. Phosphorylation is quantitative, causing a molecular-mass shift in the entire pool of cytosolic GBF1 (Figure 1C). Interestingly, we also observed a lower level of interphase phosphorylation (Figures 1B and 1C). Far-Western blotting confirmed that GBF1 can directly bind to the Plk1 PBD, and that it preferentially binds after mitotic phosphorylation (Figure 1C). Treatment of mitotic GBF1 with alkaline phosphatase abolished this binding, indicating that it is dependent upon phosphorylation. The alkaline phosphatase-sensitive molecular-mass shift of mitotic compared with interphase GBF1 was also seen in total cell extracts, confirming that GBF1 is phosphorylated *in vivo* (Figure 1D; see also Figure 3C).

GBF1 is phosphorylated by CDK1

To identify the kinase responsible for mitotic GBF1 phosphorylation, we first prepared recombinant N-terminally GST-tagged full-length protein from insect cells. As shown in Figure 2(A), GST–GBF1 was phosphorylated by mitotic cytosol. The amount of phosphorylation was comparable with that of GM130, one of the major Golgi phosphoproteins [12,52]. As expected, and in contrast with GM130, GST–GBF1 was also phosphorylated by interphase cytosol, although to a lower level than mitotic cytosol (Figure 2A). Many PBD-binding proteins rely on phosphorylation by proline-directed kinases, most notably CDK1 in early mitosis, to generate the PBD recognition site [50]. We therefore tested whether GST–GBF1 phosphorylation was sensitive to the CDK1 inhibitors roscovitine and CGP74154A. Mitotic phosphorylation of GST–GBF1 was sensitive to both inhibitors, with a similar level of inhibition to that seen with the model CDK1 substrate histone III-S (Figure 2B). To examine this further, we used another measure of GBF1 phosphorylation, namely its shift in mobility upon SDS/PAGE. *In-vitro*-translated GBF1 was incubated with interphase or mitotic cytosol in the absence or presence of various kinase inhibitors. The mobility-shift of GBF1, and hence its phosphorylation, was prevented by roscovitine and CGP7415A, but not by the MEK1 inhibitor U0126 (Figure 2C). This is consistent with phosphorylation by CDK1, but not MEK1. To determine whether CDK1 phosphorylates GBF1 *in vivo*, mitotically arrested HeLa cells were treated with the CDK inhibitors CGP7415A and purvalanol A and phosphorylation monitored by mobility-shift. Both CDK inhibitors reduced the mobility-shift of GBF1 in mitotic cells, indicating inhibition of phosphorylation, whereas U0126 had no effect (Figure 2D). The inhibitor effects were similar to those upon the known CDK1 substrate GM130 [12], consistent with CDK1 phosphorylating

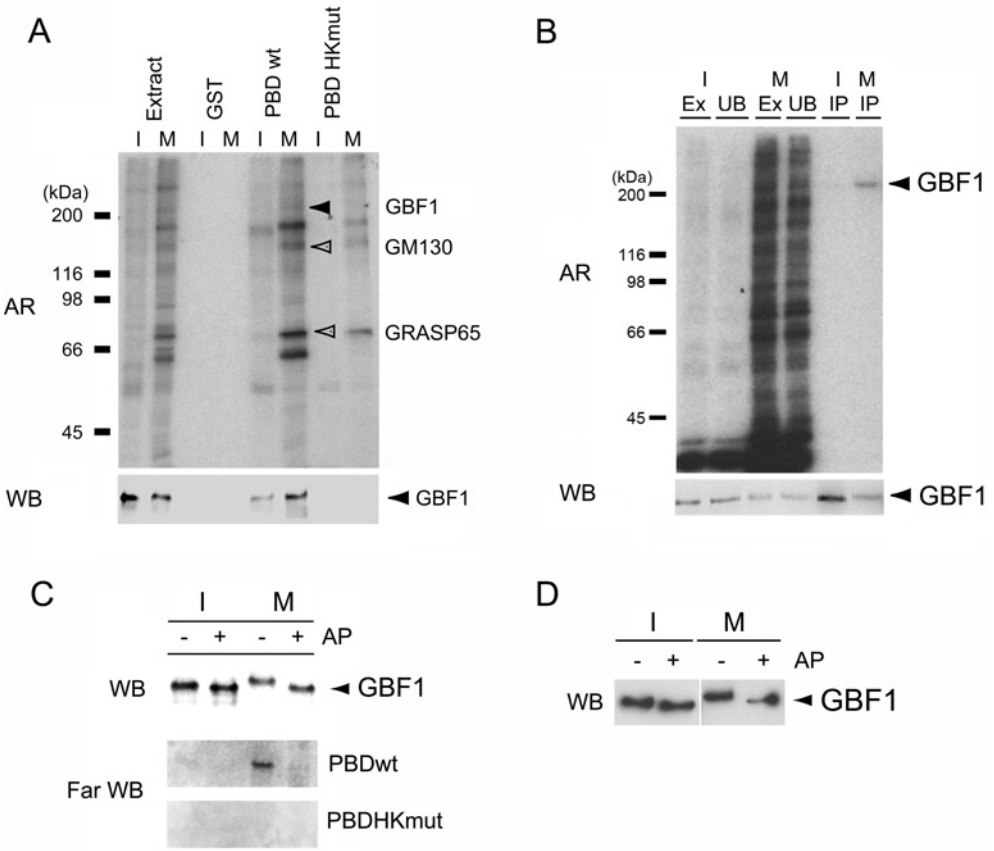

Figure 1 Phosphorylation of GBF1 in mitosis

(**A**) Rat liver Golgi membranes were incubated with interphase (I) or mitotic (M) cytosol in the presence of [γ-^{32}P]ATP for 30 min at 30 °C prior to extraction and incubation with GST alone or GST–Plk1 PBD wild-type (wt) or H538/K540 substrate binding mutant (HKmut). Total extract and bound proteins were analysed by autoradiography (AR, top panel) or Western blotting with an antibody against GBF1 (WB, bottom panel). The positions of GBF1, GM130 and GRASP65 on the autoradiograph, as identified by MS, are indicated. (**B**) Interphase (I) or mitotically (M) phosphorylated Golgi extracts were immunoprecipitated with ant-GBF1 antibody (IP). Total extract (Ex), unbound proteins (UB) and immunoprecipitated GBF were analysed by autoradiography (AR, top panel) or Western blotting with anti-GBF1 antibody (WB, bottom panel). (**C**) GBF1 was immunoprecipitated from interphase (I) or mitotic (M) cytosol and incubated in the absence (−) or presence (+) of alkaline phosphatase (AP) before transfer to nitrocellulose and Western blotting with anti-GBF1 antibody (WB) or far-Western blotting with recombinant PBD wild-type (wt) or HK mutant (HKmut). (**D**) GBF1 was immunoprecipitated from interphase (I) or mitotic (M) sHeLa cells and incubated in the absence (−) or presence (+) of alkaline phosphatase (AP) before Western blotting (WB) with anti-GBF1 antibody. For (**A** and **B**), the molecular mass in kDa is indicated on the left-hand side.

GBF1 *in vivo* (Figure 2D). To confirm that CDK1 can directly phosphorylate GBF1, GST–GBF1 was incubated with purified recombinant CDK1–cyclin B in the presence of [γ-^{32}P]ATP. As shown in Figure 2(E), GST–GBF1 was phosphorylated by CDK1-cyclin B to a level comparable with that seen with mitotic cytosol. We conclude from these experiments that GBF1 is a substrate for the mitotic kinase CDK1.

Dissociation of GBF1 from Golgi membranes in mitosis

We next wanted to address the significance of GBF1 phosphorylation. We noticed that the amount of membrane-associated GBF1 was reduced following incubation with mitotic cytosol (Figures 1A and 1B), suggesting that phosphorylation may promote membrane dissociation. We investigated this in more detail by performing a time-course experiment. GBF1 rapidly dissociated from Golgi membranes incubated with mitotic cytosol, but not from those incubated with interphase cytosol (Figure 3A). The kinetics of dissociation were similar to those of p115, a golgin that is released from membranes upon CDK1-mediated phosphorylation of GM130 [18,53]. Release of GBF1 was blocked by the general kinase inhibitor staurosporine or incubation with alkaline phosphatase, indicating that it is phosphorylation-dependent (Figure 3B). Analysis of subcellular fractions prepared from interphase and mitotic HeLa cells confirmed that membrane dissociation also occurs *in vivo* (Figure 3C; see also Figures 6B and 6C).

To further examine membrane dissociation of GBF1 *in vivo*, NRK cells were synchronized using an aphidicolin block/release protocol and analysed at various mitotic stages by immunofluorescence microscopy. The amount of GBF1 localized to the Golgi apparatus appeared reduced in late prophase and prometaphase cells compared with neighbouring interphase cells (Figure 4A, top panel). There was, however, residual Golgi staining evident at these mitotic stages, albeit with reduced intensity compared with the membrane-associated Golgi markers golgin-84 and GM130 (Figures 4A and Supplementary Figure S1A at http://www.BiochemJ.org/bj/427/bj4270401add.htm). In metaphase, when the Golgi is highly fragmented, GBF1 staining appeared mostly diffuse in the cytoplasm (Figure 4A, bottom panel). A diffuse haze is also seen with golgin-84, which probably reflects the conversion of Golgi cisternal rims into numerous dispersed vesicles [20,24,27,54]. The Golgi matrix protein GM130 remains associated with Golgi remnants that are readily observed at the light-microscope level in metaphase cells [52]. These fragments are largely devoid of GBF1, although a low

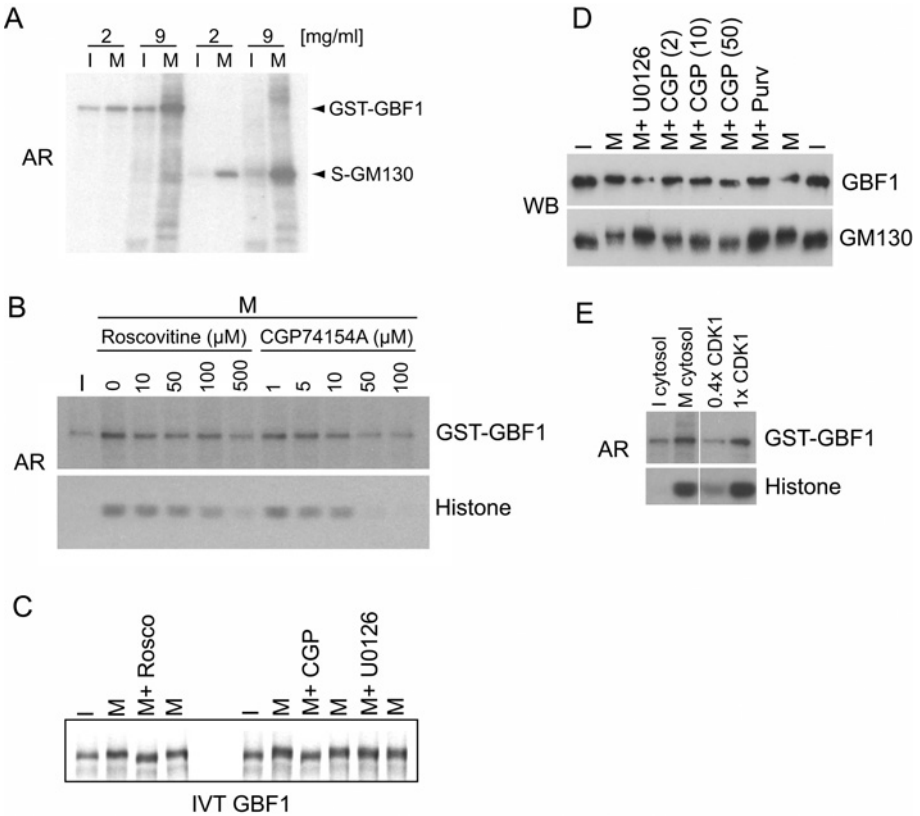

Figure 2 GBF1 is phosphorylated by CDK1

(**A**) Equal amounts of recombinant GST–GBF1 or Protein S-tagged GM130 were immobilized on beads and incubated with interphase (I) or mitotic (M) cytosol in the presence of [γ-^{32}P]ATP for 30 min at 30 °C. Bound proteins were analysed by SDS/PAGE and autoradiography (AR). (**B**) Immobilized GST–GBF1 or purified histone type III-S were incubated with interphase (I) or mitotic (M) cytosol containing [γ-^{32}P]ATP in the absence or presence of roscovitine or CGP74154A at the concentrations indicated. Phosphorylation was monitored by SDS/PAGE and autoradiography (AR). (**C**) ^{35}S-labelled *in-vitro*-translated GBF1 was incubated with interphase (I) or mitotic (M) cytosol in the absence or presence of 500 μM roscovitine (Rosco), 50 μM CGP74154A (CGP) or 500 μM U0126 as indicated for 30 min at 30 °C prior to SDS/PAGE and fluorography. (**D**) Interphase (I) or mitotic (M) sHeLa cells were treated with U0126 (10 μM), CGP74154A (CGP, 2, 10 or 50 μM) or purvalanol A (Purv, 10 μM) for 1 h at 37 °C prior to extraction and analysis by Western blotting (WB) with antibodies against GBF1 and GM130. (**E**) Immobilized GST–GBF1 or purified histones were incubated with interphase (I) or mitotic (M) cytosol or purified CDK1–cyclin B (added at 0.4× and 1× equivalents of the mitotic cytosol histone kinase activity) in the presence of [γ-^{32}P]ATP for 30 min at 30 °C. Phosphorylation was monitored by SDS/PAGE and autoradiography (AR).

level of residual staining persists in some cells (Figure 4B). GBF1 remains diffuse in anaphase, and is largely absent from GM130-positive Golgi clusters (Figure 4B). During telophase, when the Golgi undergoes reassembly and secretory membrane traffic resumes [55], GBF1 is again abundant at the Golgi apparatus (Figure 4C). Similar results were seen in unsynchronized HeLa cells (Supplementary Figure S1B).

The GBF1 C-terminus regulates its phosphorylation and membrane association

GBF1 contains many potential CDK1 phosphorylation sites, several of which are clustered in its C-terminus. To determine whether these residues are mitotically phosphorylated, we generated a deletion mutant lacking the extreme C-terminal 59 residues and tested for phosphorylation by mitotic cytosol. As shown in Figure 5(A), deletion of the C-terminal 59 residues abolished the molecular-mass shift of *in-vitro*-translated GBF1 seen upon incubation with mitotic cytosol. Similarly, deletion of the C-terminus significantly reduced the amount of mitotic phosphorylation of GST–GBF1, but had little effect on the level of interphase phosphorylation (Figure 5B). Thus the C-terminus of GBF1 is required for its phosphorylation in mitosis. We decided to use this information to confirm that it is phosphorylation of GBF1 itself, rather than other proteins, that regulates its membrane association. Wild-type and C-terminally truncated GBF1 were expressed at low levels in interphase or mitotic HeLa cells by retroviral transduction, and the amount of membrane-associated GBF1 assessed by subcellular fractionation. Expression of wild-type GBF1 or the ΔC59 mutant had no effect upon mitotic entry, as revealed by FACS analysis (results not shown). Wild-type GBF1 dissociated from mitotic membranes as expected, whereas the ΔC59 mutant remained bound during both interphase and mitosis (Figure 5C). This suggests that the C-terminal 59 residues of GBF1 are required for regulation of its membrane binding through mitotic phosphorylation. Unfortunately further attempts to map the C-terminal residues important for regulation of membrane binding were unsuccessful. Mutagenesis of all eight potential phosphorylation sites to alanine failed to affect mitotic phosphorylation (results not shown), suggesting that the C-terminus is required for kinase docking, either directly as a binding site or through maintaining the correct three-dimensional structure of the protein.

Membrane association of ARF and COPI in mitosis

GBF1 appears to be the only ARF GEF that is responsible for membrane recruitment of COPI [35,36,38,39]. We therefore

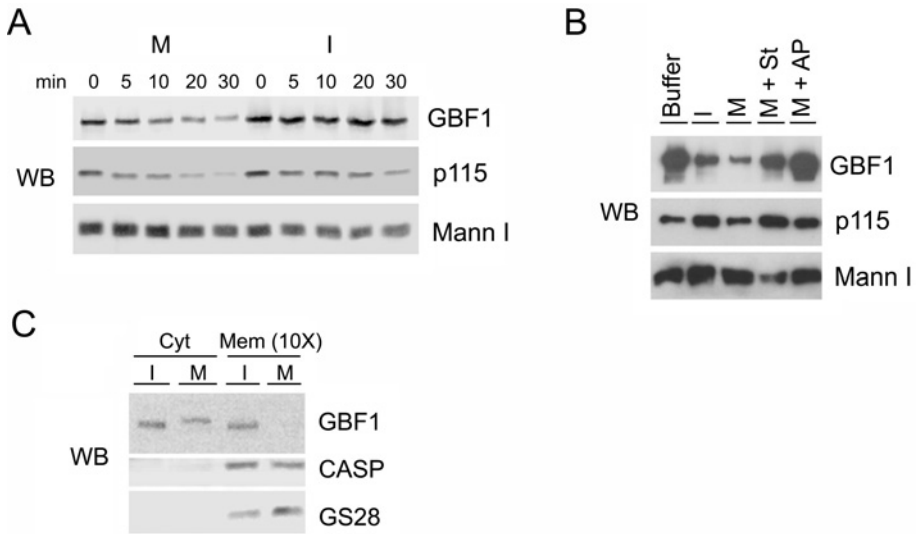

Figure 3 Dissociation of GBF1 from Golgi membranes in mitosis

(**A**) Rat liver Golgi membranes were incubated with interphase (I) or mitotic (M) cytosol for the times indicated, re-isolated by centrifugation and analysed by Western blotting (WB) with antibodies against GBF1, p115 and mannosidase I (Mann I). (**B**) Rat liver Golgi membranes were incubated with buffer, interphase (I) or mitotic (M) cytosol alone or in the presence of 10 μM staurosporine (St) or alkaline phosphatase (AP) for 30 min at 30 °C, re-isolated and analysed by Western blotting (WB). (**C**) Interphase (I) and mitotic (M) sHeLa cells were separated into cytosol (Cyt) and membrane (Mem) fractions and subjected to Western blotting (WB) with the antibodies indicated. Note that 10-fold more membrane fraction was loaded relative to cytosol.

predicted that reduced levels of GBF1 at the Golgi in mitosis would affect membrane binding of both ARF and COPI. This was first analysed using the *in vitro* Golgi assay. To our surprise, both ARF and COPI remained membrane-associated for the duration of the incubation, even though GBF1 was rapidly dissociated from the membrane (Figure 6A). We next investigated whether this was also true *in vivo*. Subcellular fractions were prepared from HeLa cells and analysed by Western blotting. As expected, both GBF1 and p115 were dissociated from the membrane in mitosis (Figure 6B). As seen *in vitro*, COPI remained membrane-bound in mitotic cells (Figures 6B and 6C). We could also show the membrane association of COPI in early mitosis by immunofluorescence microscopy (Figure 6D). In contrast with what was observed *in vitro*, ARF appeared to partially dissociate from the membrane in mitotic cells (Figures 6B and 6C). Fluorescence microscopy of transiently expressed ARF1–GFP also indicated a reduced level of membrane association in mitosis (Supplementary Figure S2 at http://www.BiochemJ.org/bj/427/bj4270401add.htm). These results suggest that levels of ARF–GTP may be reduced in mitosis, which would be consistent with a reduced amount of ARF GEF activity in mitotic cells. To test this possibility extracts were prepared from interphase and mitotic cells, and binding to the ARF effector GGA3 was used to monitor ARF–GTP levels. As shown in Figures 6(E) and 6(F), there was a reduction in the levels of ARF–GTP in mitotic extracts. This result is consistent with a loss of GBF1 from the membrane resulting in reduced nucleotide-exchange on ARF, which in turn causes reduced membrane binding. In both interphase and mitotic conditions BFA, which inhibits several ARF GEFs [33], causes a large decrease in ARF–GTP levels, suggesting that there remains significant total ARF GEF activity in mitotic cells.

Since reduced GBF1 activity would be predicted to result in dissociation of COPI from the membrane, our results suggested that COPI may be uncoupled from GBF1 in mitosis. To investigate this possibility, mitotic cells were treated with BFA for 5 min and COPI localization was studied. As observed previously, BFA treatment prevented Golgi dispersion in prometaphase [56], resulting in distinct golgin-84-positive foci in metaphase cells (Figure 7A). The Golgi fragments were devoid of COPI, indicating that COPI recruitment remains BFA-sensitive in mitosis. A similar effect was observed with transiently expressed ARF–GFP, indicating that the low level of ARF recruitment in mitosis remains BFA-sensitive (Supplementary Figure S2). The simplest interpretation is that COPI still requires GBF1 for its recruitment to the membrane in mitosis, and that BFA inhibits GBF1 in mitotic cells. If this were the case we might expect BFA to lock GBF1 on to the membrane in an inactive complex with ARF–GDP, as is observed in interphase [32,34,35]. Note that the bulk of ARF is cytosolic under these conditions [32]. Analysis of GBF1 in BFA-treated cells confirmed that BFA did indeed lock GBF1 on to the membrane in mitosis, giving a clear labelling of Golgi fragments by microscopy (Figure 7A) and a redistribution to the membrane fraction upon subcellular fractionation (Figure 7B). We observed similar effects upon COPI (and GBF1) distribution with the more specific GBF1 inhibitor golgicide A [39], confirming that these effects are not due to inhibition of other ARF GEFs in mitotic cells (results not shown). These results suggest that, even though GBF1 distribution shifts to the soluble pool in mitosis, it continues to cycle on and off Golgi membranes such that it can be trapped in an inactive complex with ARF–GDP on membranes upon BFA or golgicide A treatment. This complex would contain phosphorylated GBF1, since the level of GBF1 phosphorylation in mitotic cells is unaffected by treatment with BFA or golgicide A (results not shown). Taken together our results suggest that, despite its reduced membrane binding, GBF1 provides sufficient ARF exchange activity to allow continued recruitment of COPI on to Golgi membranes in mitotic cells. In line with this, FRAP analysis of GFP-tagged COPI indicates that its membrane recruitment occurs at a similar rate in mitosis to that in interphase, consistent with a similar mode of recruitment during both cell-cycle stages (Supplementary Figure S3 at http://www.BiochemJ.org/bj/427/bj4270401add.htm).

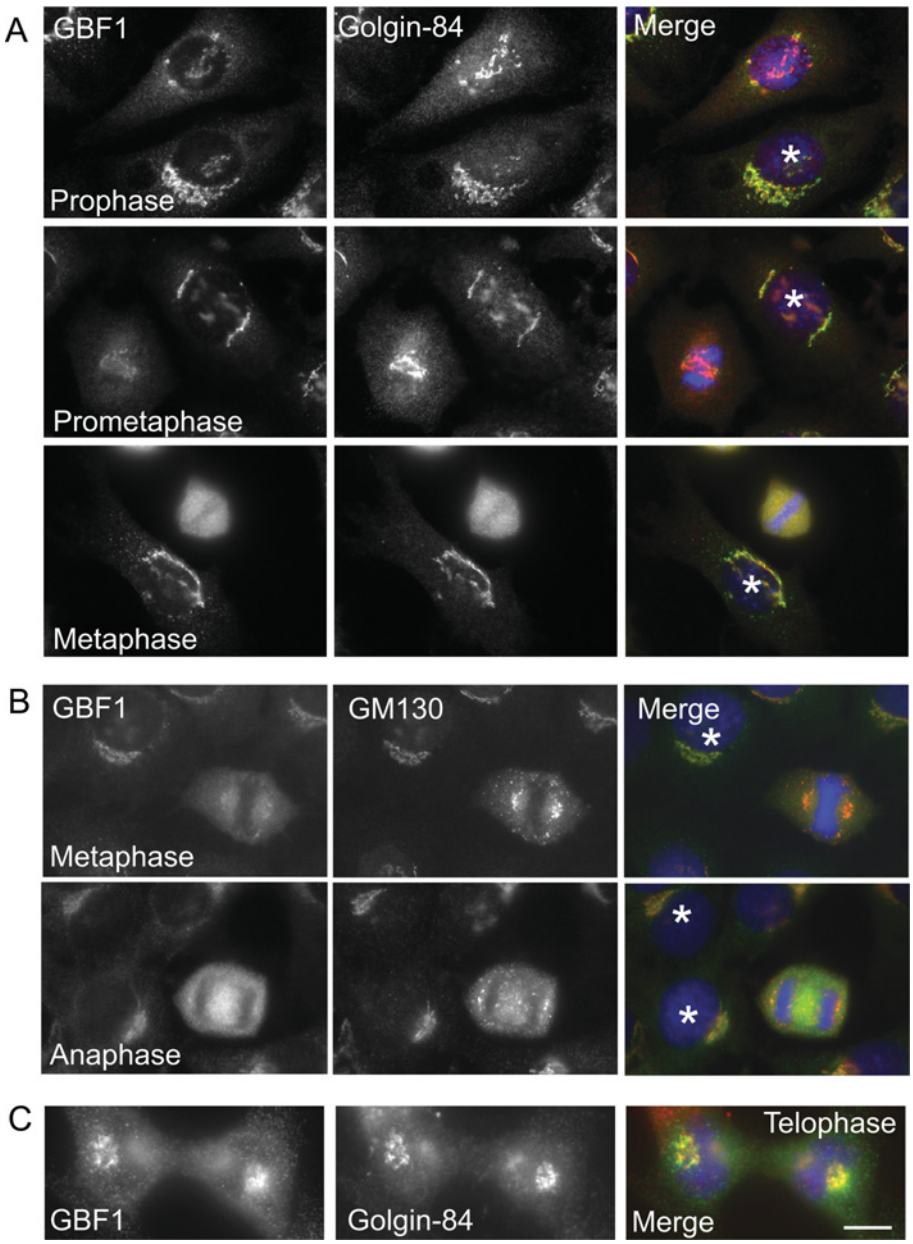

Figure 4 Localization of GBF1 during stages of mitosis

(**A**) NRK cells were synchronized to enrich for mitotic cells and analysed at the mitotic stages indicated by immunofluorescence microscopy with antibodies against GBF1 (green) and the *cis*-Golgi marker golgin-84 (red). (**B**) Synchronized NRK cells were double-labelled with antibodies against GBF1 (green) and the *cis*-Golgi matrix protein GM130 (**B**) or golgin-84 (**C**) (red). DNA is in blue. Asterisks indicate interphase cells. Scale bar, 10 μm.

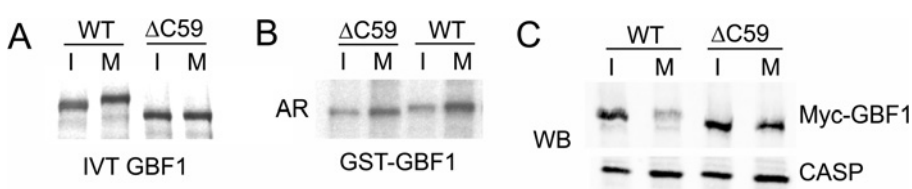

Figure 5 Phosphorylation of the GBF1 C-terminus regulates membrane binding

(**A**) *In-vitro*-translated (IVT) ^{35}S-labelled wild-type (WT) full-length GBF1 or a mutant lacking the C-terminal 59 amino acids (ΔC59) was incubated with interphase (I) or mitotic (M) cytosol for 30 min at 30 °C and analysed by fluorography. (**B**) GST-tagged wild-type (WT) full-length or the ΔC59 mutant was incubated with interphase (I) or mitotic (M) cytosol in the presence of [γ-^{32}P]ATP for 30 min at 30 °C and analysed by autoradiography (AR). (**C**) Myc-tagged wild-type (WT) or the ΔC59 mutant GBF1 was expressed in HeLa cells by retroviral infection and membrane fractions were recovered from the cells analysed by Western blotting (WB) with antibodies against the Myc tag or CASP.

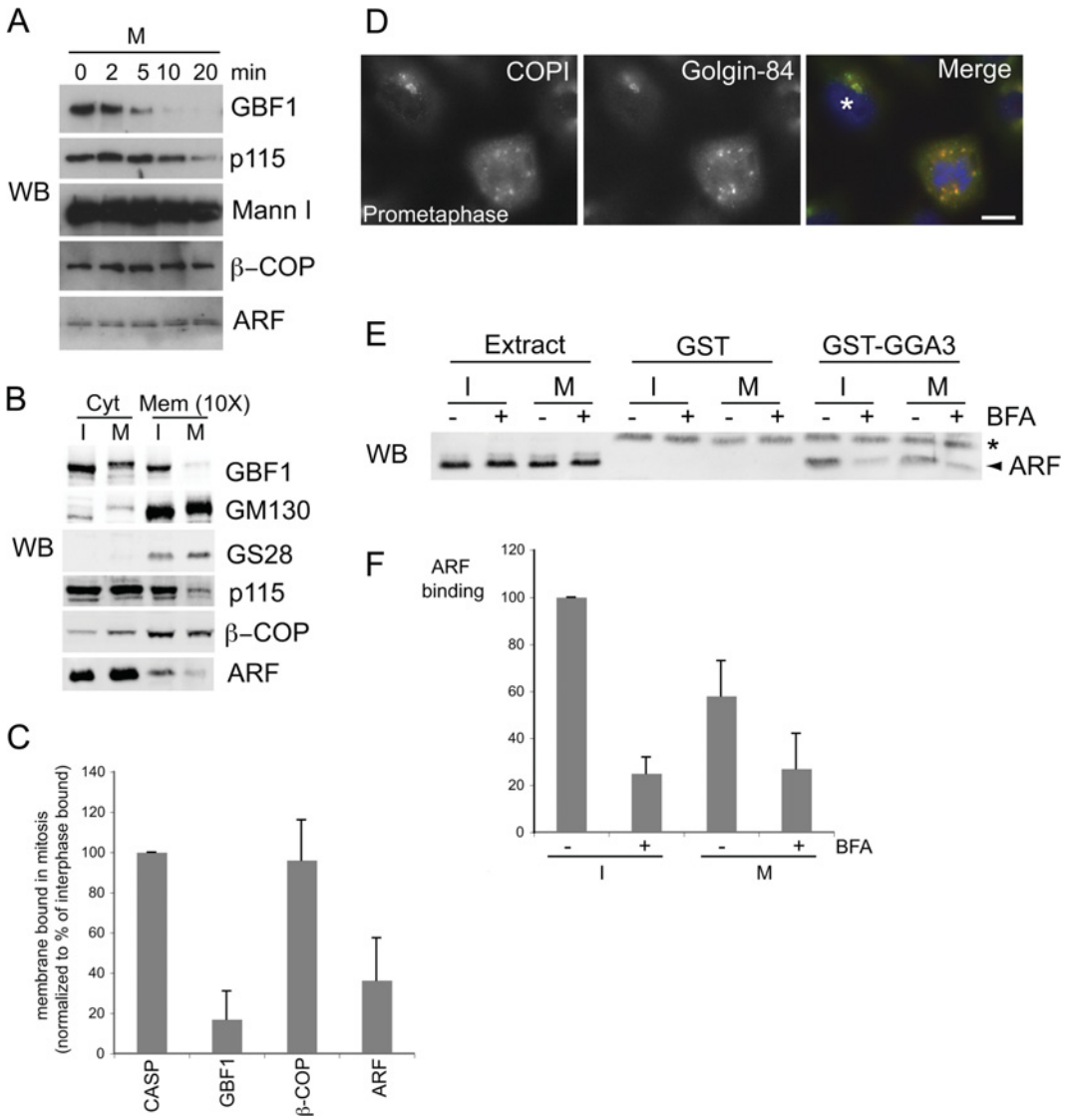

Figure 6 Membrane association of ARF and COPI in mitosis

(**A**) Rat liver Golgi membranes were incubated with mitotic cytosol (M) for the times indicated at 30 °C, re-isolated and analysed by Western blotting (WB) with the antibodies indicated. (**B**) Interphase (I) and mitotic (M) HeLa cells were fractionated into cytosol (Cyt) and membrane (Mem) fractions and subjected to Western blotting (WB) with the antibodies indicated. Note that 10-fold more membrane fraction was loaded relative to cytosol. (**C**) Quantification of membrane association of the indicated proteins. Results show the amount of protein bound to mitotic membranes expressed as a percentage of that bound in interphase, normalized to the recovery of membranes as monitored by CASP. Values are means + S.D. ($n = 4$–7). (**D**) NRK cells were synchronized and analysed by immunofluorescence microscopy with antibodies against COPI (green) and golgin-84 (red). DNA is in blue. The asterisk marks an interphase cell. Scale bar, 10 μm. (**E**) Extracts prepared from interphase (I) or mitotic (M) sHeLa cells were incubated with beads containing GST or GST–GGA3, and bound ARF was detected by Western blotting (WB). The asterisk indicates a cross-reacting protein bound to the beads. (**F**) Quantification of ARF bound to GST–GGA3, indicating ARF–GTP levels. Values are means+S.D. ($n = 4$). I, interphase; M, mitotic.

DISCUSSION

In the present study we have identified GBF1 as a novel mitotic phosphoprotein. Phosphorylation is quantitative; that is, the entire pool of cellular GBF1 is phosphorylated in mitotic cells. Phosphorylation causes membrane dissociation of GBF1, resulting in a redistribution to the cytoplasm. Since GBF1 is thought to catalyse nucleotide-exchange on ARF at the membrane, this would be predicted to reduce cellular ARF–GTP levels, which is what we observed in our experiments. However, membrane-bound levels of COPI appeared unaffected, suggesting that low levels of GBF1 activity persist in mitosis that are sufficient to recruit COPI to the membrane. In line with this, the dynamics of COPI membrane association are similar in both interphase and mitotic cells. Thus the pool of ARF1 responsible for COPI recruitment appears unaffected by membrane dissociation of GBF1 in mitosis. This pool of ARF is probably important for driving the COPI-mediated vesiculation of the Golgi as it undergoes mitotic disassembly [14,57]. Interestingly, COPI subunits can undergo phosphorylation in mitosis [58,59] and, consistent with this, the COPI subunit β-COP undergoes a molecular-mass shift under mitotic conditions, although this could correspond to another post-translational modification, such as ubiquitylation [57]. The significance of these modifications is so far unclear, but our results suggest they do not have a major effect on COPI membrane recruitment.

Two models have been proposed to explain the Golgi fragmentation that occurs in mitosis. These are the COPI budding

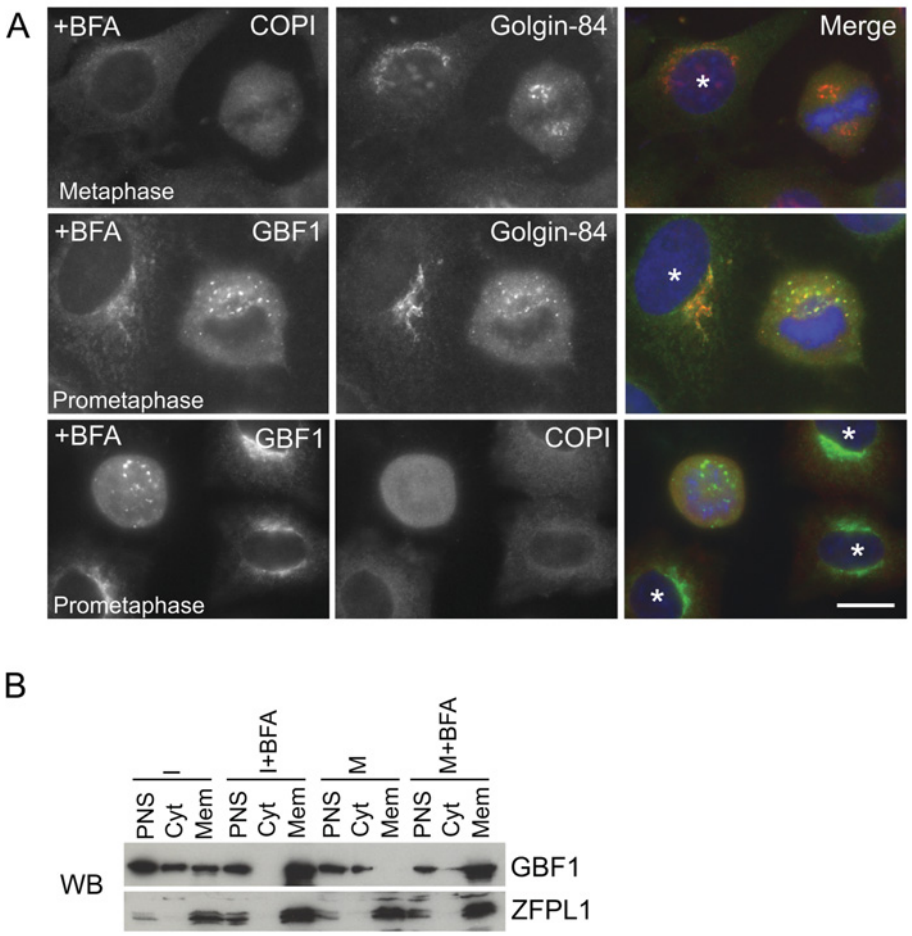

Figure 7 Effects of BFA on GBF1 and COPI in mitosis

(**A**) NRK cells were synchronized to enrich for mitotic cells and incubated with 5 μg/ml BFA for 5 min at 37 °C prior to fixation and labelling with the antibodies indicated. Left-hand panels are green, middle panels are red; in the merge, DNA is in blue. Asterisks indicate interphase cells. Scale bar, 10 μm. (**B**) Interphase (I) or nocodazole-arrested mitotic (M) sHeLa cells were treated with or without 5 μg/ml BFA for 10 min at 37 °C, lysed and post-nuclear supernatants (PNS) fractionated into post-nuclear cytosol and membrane fractions prior to analysis by Western blotting (WB) with the antibodies indicated. ZFPL1 is an integral Golgi membrane protein and is used as a membrane marker. Note that 10-fold more membrane fraction was loaded relative to PNS (post-nuclear supernatant) and cytosol.

model, in which the cisternae are converted into vesicles by continual COPI budding [13], or the BFA-like fragmentation model, in which loss of ARF and COPI from the membrane cause extensive tubulation and eventually Golgi dispersion, which may be followed by fusion with the ER [21,22]. The loss of GBF1 from the membrane would appear to support the latter model. However, as described above, we do not see a loss of COPI from the membrane, in contrast with Altan-Bonnet et al. [21], who used fluorescence imaging techniques to study COPI membrane association [21]. We did see a reduction in ARF binding to the membrane, but there remained a pool of membrane-associated GTP-bound ARF in mitotic cells. Thus our findings do not support a BFA-type Golgi dispersion in mitosis, and are more consistent with a COPI-mediated fragmentation mechanism, as described by others [14,57]. If phosphorylation and membrane dissociation of GBF1 does not affect membrane-bound levels of COPI, what is its significance? ARF is responsible for the recruitment of numerous effectors to the Golgi membrane [60,61], including some proteins that appear to have non-Golgi roles in mitosis [21]. Thus, as originally suggested by Altan-Bonnet et al. [21], the regulation of ARF in mitosis, which we have shown occurs through GBF1 phosphorylation, may be important for mitotic events that are distinct from those occurring at the Golgi apparatus. A reduced pool of GBF1-catalysed active ARF at the Golgi would allow release of factors from the membrane and their recruitment to distal sites where they carry out their mitosis-specific functions, that in turn are required for cell-cycle progression. It is also possible that reduced levels of ARF–GTP may influence Golgi events other than COPI-mediated vesicle budding.

We found that GBF1 is phosphorylated by CDK1–cyclin B. Inhibition of MEK1 failed to affect phosphorylation arguing against downstream MAPKs as relevant GBF1 kinases. However, we cannot exclude a role for other kinases in GBF1 phosphorylation. We identified GBF1 as a Plk1 PBD-binding partner, suggesting that GBF1 can bind to Plk1 in mitosis. Thus it is possible that Plk1 can phosphorylate GBF1, which may occur after initial priming phosphorylation by CDK1 on Plk1 PBD recognition sites. Alternatively, binding of Plk1 to GBF1 in mitosis may simply target the kinase to regions of the Golgi involved in COPI vesicle budding, at least at early stages of mitosis when the majority of GBF1 has yet to dissociate from the membrane. This would suggest that Plk1 can be recruited to the Golgi through binding to several Golgi proteins, including GRASP65 and Rab1 [51]. The large number of Golgi phosphoproteins isolated in our Plk1 PBD-binding experiments supports this possibility. This may ensure that relevant targets

on distinct regions and functional domains of the Golgi are phosphorylated in an appropriate and timely manner.

Despite several attempts we were unable to identify the mitosis-specific phosphorylation sites in GBF1. Deletion of the C-terminal 59 residues of GBF1 abrogated mitotic phosphorylation, suggesting that sites within this region are phosphorylated. However, mutation of all eight potential sites in this region failed to prevent GBF1 phosphorylation, suggesting that this region is required for recognition by CDK1 and possibly other kinases. Interestingly there are another six potential CDK1 sites immediately upstream of this region, some of which may correspond to the GBF1 phosphorylation sites, although it is equally possible that CDK1 sites in other parts of the protein are the ones that are phosphorylated. Using MS we could identify several GBF1 phosphorylation sites, including Ser^{1298}, Ser^{1318}, Ser^{1320}, Ser^{1335} and Thr^{1337}, but all of these sites were phosphorylated to the same degree in both interphase and mitosis (results not shown). Thr^{1337} was previously shown to be phosphorylated by AMPK (AMP-activated protein kinase) when cellular AMP levels are elevated, and to trigger Golgi disassembly under these conditions [62]. Our results suggest that Thr^{1337} is also phosphorylated constitutively in interphase cells, and that the steady-state levels of phosphorylation at this site do not disassemble the Golgi. Several large-scale proteomics studies have identified additional GBF1 phosphorylation sites, of which only four (Ser^{314}, Ser^{599}, Tyr^{1316} and Ser^{1773}) appear to be mitosis-specific and conserved between mammals [58,59,63]. Of these, two are potential CDK1 sites (Ser^{314} and Ser^{1773}), although both lack the basic residue found in the CDK1 consensus site S/T-P-X-R/K. Additional studies will be required to identify the mitosis-specific phosphorylation sites in GBF1 that regulate membrane binding.

How might phosphorylation of GBF1 cause its release from the membrane? GBF1 binds to several proteins, including p115, Rab1b and γ-COP [40–42]. It has been proposed that direct binding to Rab1b is required for membrane recruitment of GBF1. However, despite numerous attempts, we were unable to detect binding of GBF1 to Rab1b, or indeed Rab1a (Y. Morohashi, Z. Balklava and M. Lowe, unpublished work). It therefore possible that Rab1b acts indirectly to recruit GBF1 to the membrane, possibly through recruiting effectors to which GBF1 can bind. One such effector is p115, but previous work has shown that p115 is not required for targeting GBF1 to the membrane [41] suggesting that an as yet unidentified Rab1 effector is involved. Further investigation of how GBF1 binds to the membrane will be required to determine how it is recruited in interphase, and in turn how this is regulated by mitotic phosphorylation.

AUTHOR CONTRIBUTION

Yuichi Morohashi identified GBF1 as a mitotic phosphoprotein, performed the characterization of GBF1 phosphorylation, investigated its effects on membrane binding, and analysed ARF and COPI in mitosis. Zita Balklava carried out *in vitro* phosphorylation and *in vivo* membrane-association experiments. Matthew Ball prepared recombinant insect cell-expressed GBF1. Helen Hughes carried out the FRAP experiments. Martin Lowe was involved in experimental design, and performed *in vitro* phosphorylation and, *in vivo* localization experiments, and wrote the manuscript.

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online data

SUPPLEMENTARY ONLINE DATA

Phosphorylation and membrane dissociation of the ARF exchange factor GBF1 in mitosis

Yuichi MOROHASHI[1], Zita BALKLAVA[2], Matthew BALL, Helen HUGHES[3] and Martin LOWE[4]

Faculty of Life Sciences, University of Manchester, The Michael Smith Building, Oxford Road, Manchester M13 9PT, U.K.

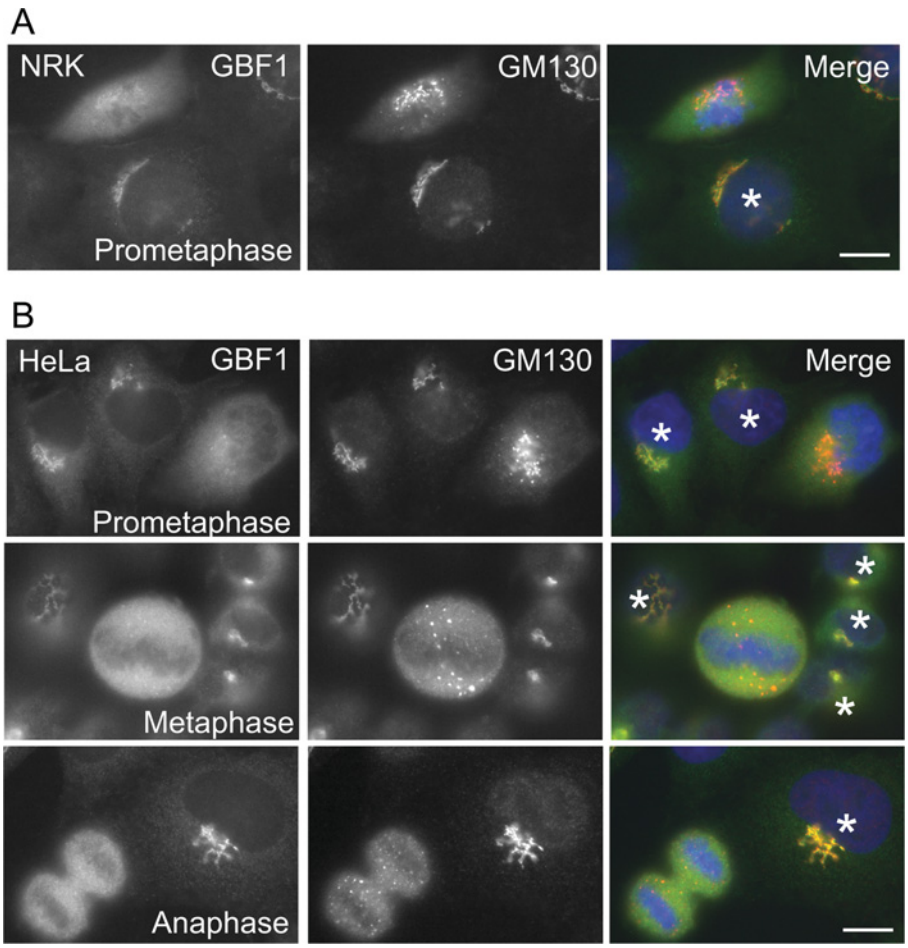

Figure S1 Localization of GBF1 in mitotic cells

(**A**) NRK cells were synchronized to enrich for mitotic cells and analysed by immunofluorescence microscopy with antibodies against GBF1 (green) and GM130 (red). (**B**) Unsynchronized HeLa cells were analysed by immunofluorescence microscopy with antibodies against GBF1 (green) and GM130 (red). DNA is blue in the merged images. Asterisks indicate interphase cells. Scale bars, 10 μm.

[1] Present address: Department of Neuropathology and Neuroscience, The Graduate School of Pharmaceutical Sciences, The University of Tokyo, 7-3-1 Hongo, Bunkyo-ku, Tokyo, Japan

[2] Present address: School of Life and Health Sciences, Aston University, Aston Triangle, Birmingham B4 7ET, U.K.

[3] Present address: Department of Biochemistry, University of Bristol, School of Medical Sciences, University Walk, Bristol BS8 1TD, U.K.

[4] To whom correspondence should be addressed (email martin.lowe@manchester.ac.uk).

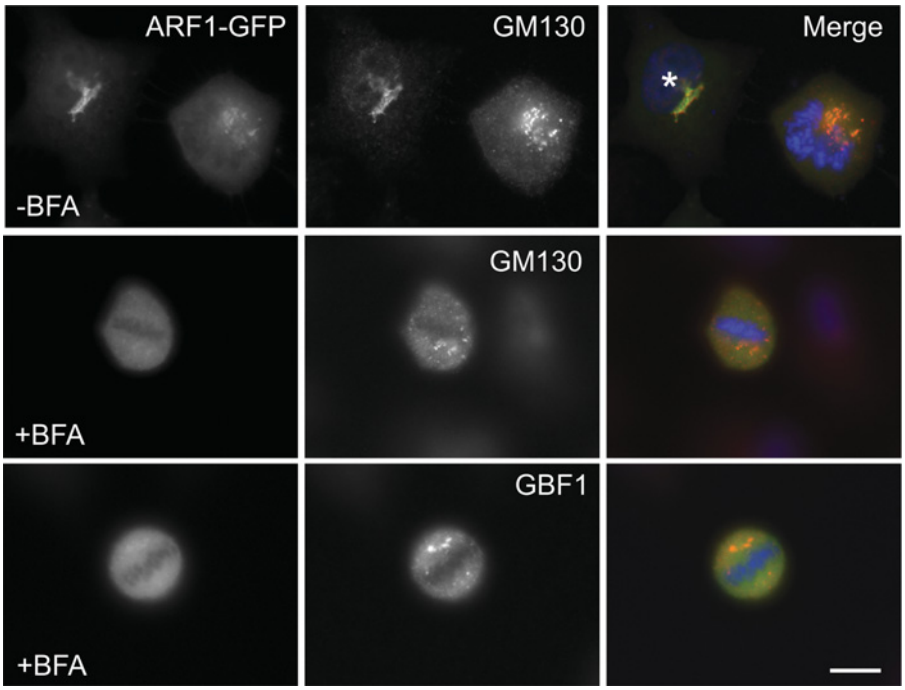

Figure S2 Localization of ARF1–GFP in interphase and mitotic cells

HeLa cells transiently expressing ARF1–GFP (green) were left untreated or treated for 5 min with 5 μg/ml BFA, fixed and labelled with antibodies against GBF1 or GM130 (red). DNA is blue in the merged images. The asterisk indicates an interphase cell. Scale bar, 10 μm.

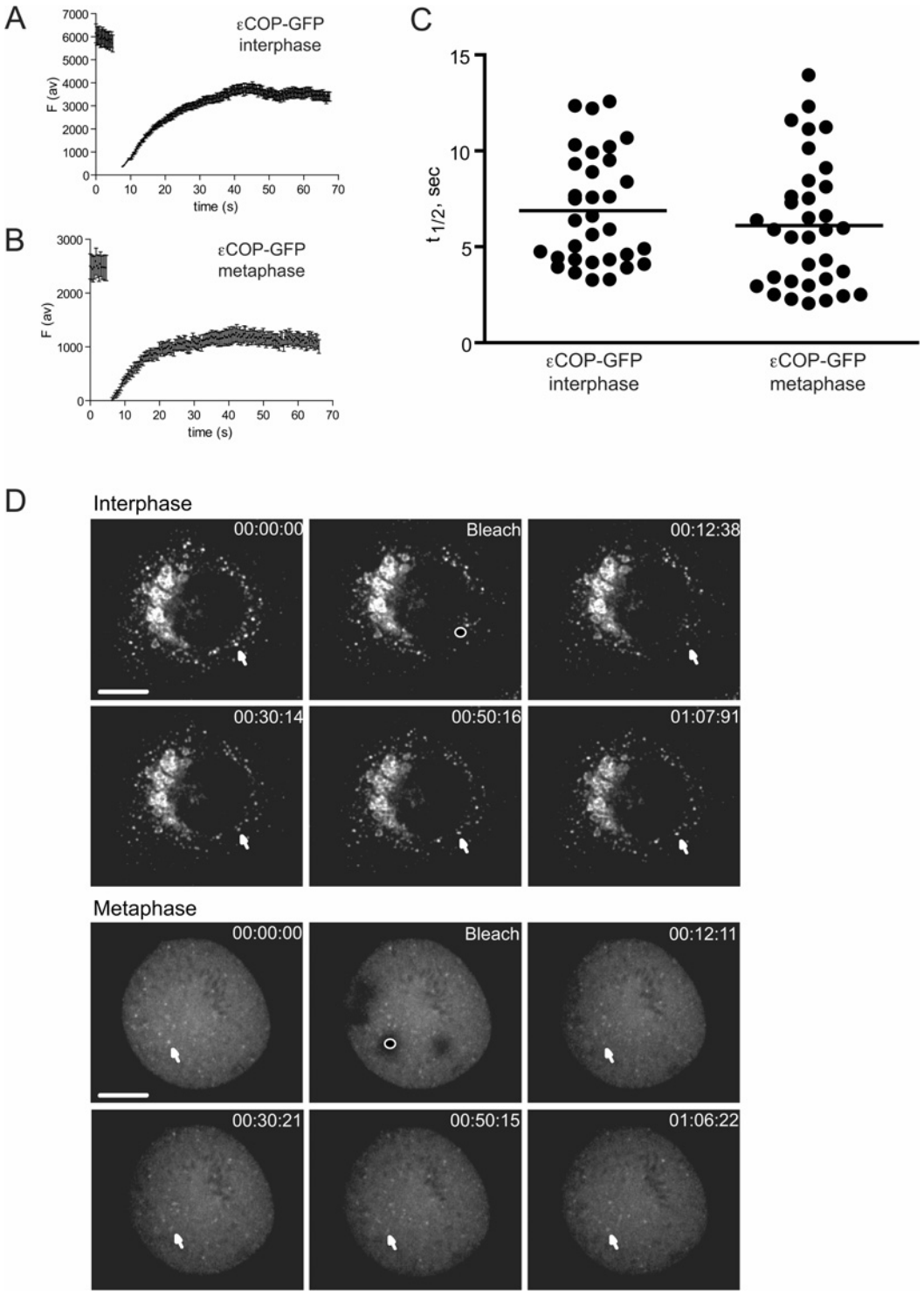

Figure S3 COPI membrane association kinetics in interphase and mitosis

NRK cells stably expressing GFP-tagged ε-COP were analysed by FRAP and the fluorescence recovery rate under interphase (**A**) or mitotic (**B**) conditions was analysed. (**C**) Plot of fluorescence recovery rates for individual interphase and mitotic cells. The average recovery rate is indicated by a horizontal line. (**D**) Example of a single FRAP experiment performed on an interphase (top panels) and mitotic (bottom panels) cell. Scale bar, 4 μm.

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