Phosphatidylinositol- and phosphatidylcholine-transfer activity of PITPβ is essential for COPI-mediated retrograde transport from the Golgi to the endoplasmic reticulum

Nicolas Carvou[1], Roman Holic[1], Michelle Li[1], Clare Futter[2], Alison Skippen[1] and Shamshad Cockcroft[1,*]

[1]Lipid Signalling Group, Department of Neuroscience, Physiology and Pharmacology, University College London, Gower St, London, WC1E 6BT, UK

[2]Department of Cell Biology, Institute of Ophthalmology, University College London, Gower St, London, WC1E 6BT, UK

*Author for correspondence (s.cockcroft@ucl.ac.uk)

Accepted 14 January 2010
Journal of Cell Science 123, 1262-1273

doi:10.1242/jcs.061986

Summary

Vesicles formed by the COPI complex function in retrograde transport from the Golgi to the endoplasmic reticulum (ER). Phosphatidylinositol transfer protein β (PITPβ), an essential protein that possesses phosphatidylinositol (PtdIns) and phosphatidylcholine (PtdCho) lipid transfer activity is known to localise to the Golgi and ER but its role in these membrane systems is not clear. To examine the function of PITPβ at the Golgi-ER interface, RNA interference (RNAi) was used to knockdown PITPβ protein expression in HeLa cells. Depletion of PITPβ leads to a decrease in PtdIns(4)*P* levels, compaction of the Golgi complex and protection from brefeldin-A-mediated dispersal to the ER. Using specific transport assays, we show that anterograde traffic is unaffected but that KDEL-receptor-dependent retrograde traffic is inhibited. This phenotype can be rescued by expression of wild-type PITPβ but not by mutants defective in docking, PtdIns transfer and PtdCho transfer. These data demonstrate that the PtdIns and PtdCho exchange activity of PITPβ is essential for COPI-mediated retrograde transport from the Golgi to the ER.

Key words: Golgi, Phosphatidylinositol transport proteins, Retrograde transport

Introduction

Phosphatidylinositol transfer proteins (PITPα and β) are 35 kDa soluble lipid transfer proteins which can bind and exchange phosphatidylinositol (PtdIns) and phosphatidylcholine (PtdCho) between membranes in vitro (Cockcroft, 2007). Both PITPs are single domain proteins consisting of an eight-stranded β-sheet flanked by two long α helices that form a hydrophobic cavity capable of shielding a single lipid molecule. Access to the cavity is blocked by the C-terminal 11 amino acids that form a 'lid'; lipid release can only occur when the 'lid' is re-positioned when docked on the membrane (Shadan et al., 2008; Vordtriede et al., 2005; Tilley et al., 2004). Although PITPβ has 77% sequence identity and 94% similarity to PITPα, and has a similar three-dimensional structure, the two PITPs have non-redundant functions in vivo. A reduction of PITPα by 80% contributes to a neurodegenerative phenotype of the mouse vibrator mutation (Hamilton et al., 1997), whereas mice totally lacking in PITPα exhibit spinocerebellar degeneration, intestinal and hepatic steatosis and hypoglycaemia (Alb et al., 2003). By contrast, deletion of the PITPβ gene is embryonic lethal (Alb et al., 2002), emphasising that despite the many biochemical properties shared by these two PITPs, they have discrete roles in vivo. Clues to their distinct functions come from their different cellular localisation and expression. PITPα is abundantly expressed in the brain and is predominantly localised in the axons (Cosker et al., 2008), whereas PITPβ is highly expressed in the liver and is localised at the Golgi and endoplasmic reticulum (Morgan et al., 2006; Shadan et al., 2008). The cellular functions of the two PITPs remain ill-defined, but evidence has been presented that PITPα is required for maintaining dedicated pools of phosphoinositides utilised for phospholipase C and phosphoinositide 3-kinase signalling during neurite outgrowth (Thomas et al., 1993; Xie et al., 2005; Cosker et al., 2008).

Previous studies, all performed in vitro, have suggested that PITP (α or β) can reconstitute vesicle budding from the trans-Golgi network by maintaining a pool of phosphorylated PtdIns (Ohashi et al., 1995; Jones et al., 1998). In addition, PITPα was identified as a reconstitution factor in a cell-free assay designed to re-establish cis-to-medial intra-Golgi vesicular transport (Paul et al., 1998), and as a vesiculating factor for the scission of coatomer-coated vesicles (Simon et al., 1998). In all of these studies, either PITPα or PITPβ were functional in the reconstitution assays and moreover, the yeast PITP, Sec14p, which bears no structural or sequence similarity to mammalian PITPs, could also be used (Phillips et al., 2006b; Cockcroft and Carvou, 2007). In yeast, Sec14p is required for vesicular transport from the Golgi to the plasma membrane and it is thought that it controls diacylglycerol levels, which in turn regulate vesicular transport (Howe and McMaster, 2006). These findings would suggest that PITP function examined in in vitro systems in mammalian cells does not address the question of a specific function of PITPβ in vivo. We have therefore examined the role of PITPβ in intact cells by investigating the consequences for Golgi morphology and membrane traffic of depletion of PITPβ using RNA interference. The findings in this study show that PITPβ knockdown causes changes in Golgi and nuclear morphology, and that PITPβ is required for COPI-mediated retrograde transport from the Golgi to the endoplasmic reticulum.

Making use of PITPβ mutants that are selectively deficient in either PtdIns or PtdCho transfer, we conclude that PITPβ functions in retrograde transport by modulating PtdIns and PtdCho levels in a reciprocal fashion.

Results

PITPβ depletion causes compaction of the Golgi

We recently established that PITPβ localises to both the Golgi and the endoplasmic reticulum compartments and that the entire population of PITPβ cycles between a lipid-free open conformation and a lipid-loaded form on the membrane surface within 2 minutes (Shadan et al., 2008). To examine the function of PITPβ at the Golgi-ER interface, RNA interference (RNAi) was used to knockdown PITPβ protein expression in HeLa cells. For silencing PITPβ we used a combination of two siRNAs and the cells were transfected twice over a period of 6 days for optimal silencing. Western blot analysis confirmed that PITPβ expression was substantially and specifically reduced (Fig. 1A). (Quantification of a representative western blot can be found in supplementary material Fig. S1A.) Knockdown of PITPβ did not lead to any compensatory increases in PITPα levels (Fig. 1A). Likewise knockdown of PITPα was also unambiguously achieved using siRNA specific for PITPα with no changes in PITPβ expression. In cells treated with RNAi for PITPβ, the Golgi was rearranged to a more restricted juxtanuclear location with a more compact shape compared with the normal reticular and perinuclear Golgi morphology observed in control cells (Fig. 1B,D). Manual quantification showed that 70% of the PITPβ-knockdown cells exhibited a compacted Golgi phenotype compared to 30% in control cells (Fig. 1C). Such Golgi morphology alterations were also observed when the Golgi was stained with antibodies to ARF1, β-COP, ERGIC-53, giantin, GM130 and TGN38 (Figs 2, 3, 4 and supplementary material Fig. S2A).

The effects on Golgi structure represented specific effects of PITPβ silencing based on several criteria. First, the same phenotype was observed when a second set of two siRNAs was used (supplementary material Fig. S1). Second, when HeLa cells were challenged with *PITPα* siRNA the Golgi morphology was not affected despite PITPα expression being effectively silenced (Fig. 1A). Third, a double knockdown of PITPα and PITPβ gave a phenotype which was the same as seen for PITPβ knockdown alone (Fig. 1B). Fourth, a control siRNA which is non-silencing for any known protein was without effect on Golgi morphology. Fifth, the

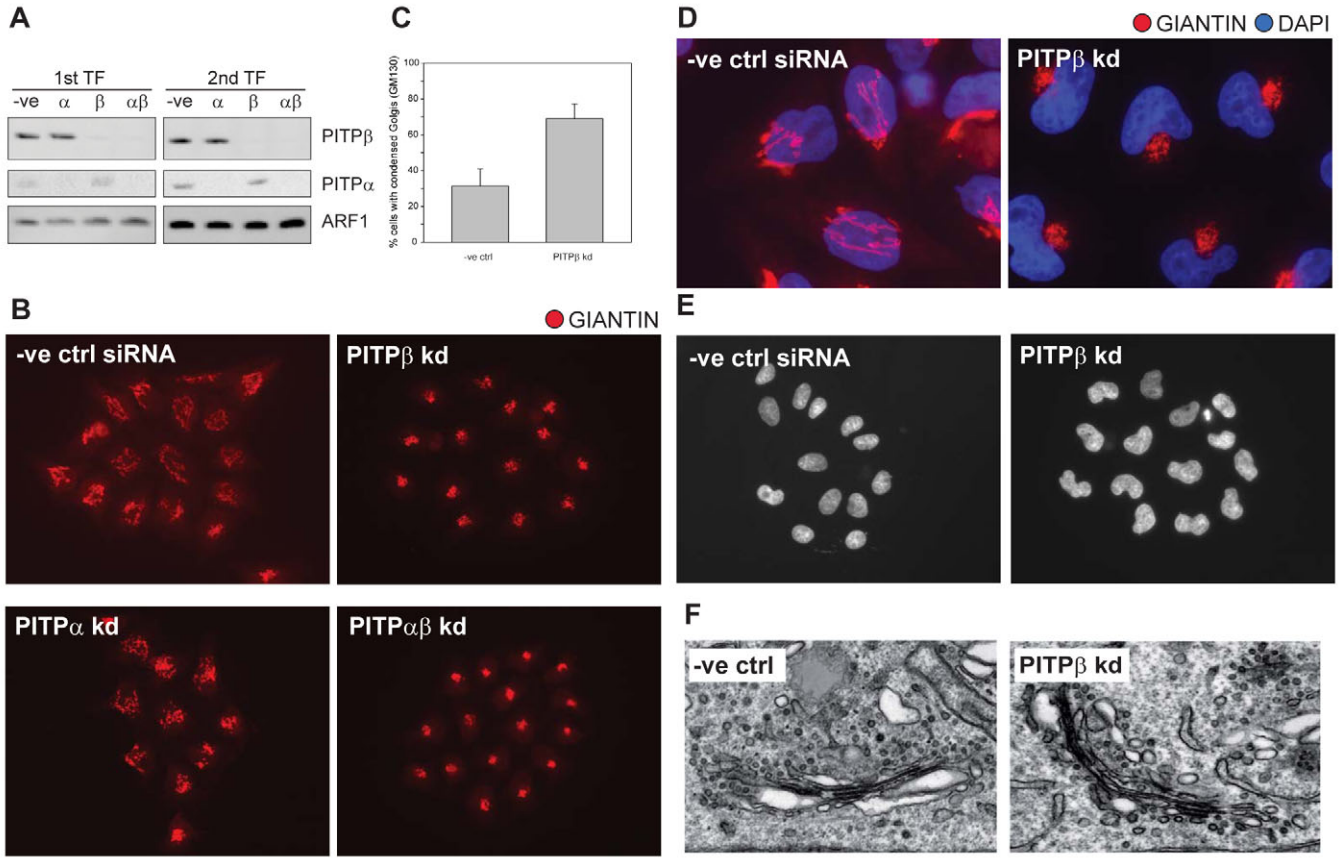

Fig. 1. Depletion of PITPβ by RNAi causes Golgi compaction and deforms the nucleus. (**A**) Western blot showing knockdown of PITPβ or PITPα by siRNA specific for each protein. ARF1 was used as a loading control. Quantification of numerous blots indicates that the efficiency of knockdown after the second round of transfection (TF) was always greater than 90 % (supplementary material Fig. S1). (**B**) PITPα and/or PITPβ siRNA-treated cells were fixed 6 days after transfection, immunostained with a Golgi marker (giantin antibody), treated with DAPI and examined by microscopy. Representative images of control and PITP siRNA-treated cells are shown (×40 objective). (**C**) Golgi condensation in control and PITPβ siRNA-treated cells was quantified following immunostaining with the Golgi marker, GM130. Values are the percentage of cells with condensed Golgi from three experiments. (**D**) High magnification images with nuclear staining (×100 objective). (**E**) DAPI-stained nuclei of HeLa cells after siRNA transfection (×40 objective). Compared with control cells, which have regular oval shaped nuclei, those of PITPβ knockdown cells have atypical 'kidney' or 'doughnut' shapes. (**F**) Transmission EM images of control and PITPβ knockdown cells (see supplementary material Fig. S4 for a low magnification view of the juxtanuclear area).

Golgi specificity of the *PITPβ* siRNA was also observed. PITPβ depletion had no obvious effects on ER architecture (supplementary material Fig. S2A).

Another prominent feature of cells treated with *PITPβ* siRNA is a change in the shape of the nucleus. The nucleus has a regular elongated shape in control cells which is malformed into a kidney shape in the PITPβ-depleted cells (Fig. 1E). The shape of the nucleus is maintained by a nuclear envelope and nesprins (alternative name: Syne), a family of spectrin repeat-containing proteins involved in the anchoring of the nucleus to the cytoskeleton (Warren et al., 2005).

The Golgi morphology described here is reminiscent of that caused by a number of agents that impact on cytoskeletal architecture including latrunculin B, cytochalasin D and disruption of the cytoskeletal anchor, Syne-1 (Valderrama et al., 1998; Valderrama et al., 2001; Gough and Beck, 2004; Lazaro-Dieguez et al., 2006). Syne-1 localises to the Golgi (Gough et al., 2003) and expression of fragments from Syne-1 alters the structure of the Golgi complex, which collapses into a compact juxtanuclear structure (Gough and Beck, 2004). A similar change in morphology has been described in cells treated with latrunculin B and cytochalasin D. We therefore treated HeLa cells with latrunculin B to disrupt the actin cytoskeleton and a compacted Golgi phenotype was also observed (supplementary material Fig. S2B). However, there are discrepancies between the phenotypes observed with latrunculin B treatment and cells knocked down for PITPβ. While the entire cytoskeleton is disturbed in latrunculin-B-treated cells and the cells shrink and their adhesion is often compromised, this is not observed in PITPβ-knockdown cells. In PITPβ-knockdown cells, no changes are seen in the actin organisation at the cell cortex or in the cell shape. However, we do see an accumulation of actin filaments at the Golgi (supplementary material Fig. S3). Furthermore, PITPβ knockdown also causes a malformation of the nucleus, whereas latrunculin B treatment does not (supplementary material Fig. S2B), suggesting that these two morphological changes are independent effects of PITPβ knockdown, and here we focus on the Golgi.

At the ultrastructural level, differences exist in the extent of Golgi disruption depending on the specific mode of action of the actin-disrupting agent, despite the fact that all anti-actin agents induce compactness of the Golgi (Lazaro-Dieguez et al., 2006). For example, in latrunculin-B-treated HeLa cells, significant swelling of stacked cisternae is observed as well as an increase in the number of associated vesicles, which accumulate in the lateral portions of the swollen cisternae. By contrast, jasplakinolide-treated cells have flattened cisternae with numerous perforations and vesicles are non-uniformly distributed, being mostly located in the lateral portions of stacked cisternae (Lazaro-Dieguez et al., 2006). When we examined the Golgi in PITPβ-knockdown cells by transmission EM, the Golgi stacks and individual cisternae remained unaffected (Fig. 1F). The Golgi was rearranged so that it occupied a restricted area, whereas in the control cells, the Golgi was spread along a wider perinuclear region (supplementary material Fig. S4). Following analysis of Golgi stacks in many cells in four independent experiments we have not observed a clear change in the number of vesicles in the Golgi region. Given the number of different types of vesicles budding from and being delivered to the Golgi apparatus a selective depletion of retrograde transport vesicles may not significantly alter the total number of vesicles in the Golgi region. The changes in PITPβ-knockdown cells are relatively subtle compared with the studies where actin-disrupting agents were used. In addition to the compact Golgi phenotype, both latrunculin B treatment and expression of fragments of Syne-1 cause defects in retrograde transport from the Golgi complex to the ER (Valderrama et al., 2001; Gough and Beck, 2004). We therefore analysed whether PITPβ deficiency also results in defects in retrograde transport from the Golgi to the ER.

PITPβ depletion leads to defects in Golgi to ER retrograde transport

Brefeldin A (BFA) prevents guanine nucleotide exchange factor activation of ARF1 and consequently the binding of COPI proteins onto Golgi membranes. This results in Golgi-membrane tubulation and redistribution into the ER (Sciaky et al., 1997). In latrunculin-B-treated cells, disassembly of the Golgi complex induced by BFA is delayed, suggesting that latrunculin B causes defects in retrograde traffic between the Golgi and ER (Valderrama et al., 2001). A similar delay in the disruption of the Golgi with BFA is observed in PITPβ-knockdown cells (Fig. 2A): 10 minutes after BFA treatment, 84% of the cells still had an intact Golgi compared with 9% in control cells. Upon BFA treatment, ARF1 was released from Golgi membranes to the cytosol in control cells whereas ARF1 remained in a perinuclear position in PITPβ-knockdown cells (Fig. 2B). Thus the Golgi complex is protected from disassembly induced by BFA in PITPβ-depleted cells, suggesting that PITPβ is required for retrograde flux from the Golgi to the ER.

Although the compact Golgi structure is resistant to BFA, it could still be fragmented when cells were treated with nocodazole, indicating that the microtubule network maintaining the Golgi is unaffected in PITPβ knockdown cells. Moreover, the reassembly of the Golgi after nocodazole treatment was also unaffected (data not shown).

To test the role of PITPβ in COPI-mediated transport more directly, we have examined the steady state distribution of ERGIC-53. The ER-Golgi intermediate compartment (ERGIC) consists of a constant number of tubulovesicular clusters predominantly localised near the cis-side of the Golgi stacks that stain positive for ERGIC-53 (Fig. 3A). Transport between the ERGIC and the ER is mediated by COPI-coated vesicles retrieving ERGIC-53. ERGIC-53 contains a C-terminal dilysine ER retrieval signal, KKXX, which can directly interact with the COPI coat proteins (Letourneur et al., 1994). ERGIC-53 binds COPI and, at steady state, ERGIC-53-stained vesicles in the peripheral region of the cells colocalise with COPI (Klumperman et al., 1998) (Fig. 3A). In addition, the intense perinuclear COPI staining colocalises with ERGIC-53. However, in PITPβ-knockdown cells, peripheral ERGIC-53- and COPI-stained vesicles diminished in number. On average we counted 145±10 ERGIC-53-stained vesicles and 134±11 β-COP-stained vesicles in control cells (n=22). By contrast, in PITPβ-knockdown cells ERGIC-53- and β-COP-stained vesicles decreased to 69±12 and 55±9, respectively (n=23). In addition, ERGIC-53 is present as a dense cluster close to the Golgi complex and is protected from distribution to the ER by BFA (supplementary material Fig. S5). The ERGIC phenotype was also observed with a different set of siRNA (supplementary material Fig. S6). This distribution is remarkably like that observed when the cells are cooled to 16°C from 37°C (Fig. 3B). At 16°C, protein exit from the ERGIC is arrested and leads to an accumulation of ERGIC-53 in the ERGIC at the expense of ERGIC-53 in the ER. Concomitantly, the ERGIC clusters move closer to the Golgi complex. Upon warming to 37°C for 10 minutes, ERGIC-53 tubules appear, indicating that the retrograde pathway from ERGIC to ER is now operational and the original distribution of ERGIC clusters is re-established (Ben Tekaya et al., 2005).

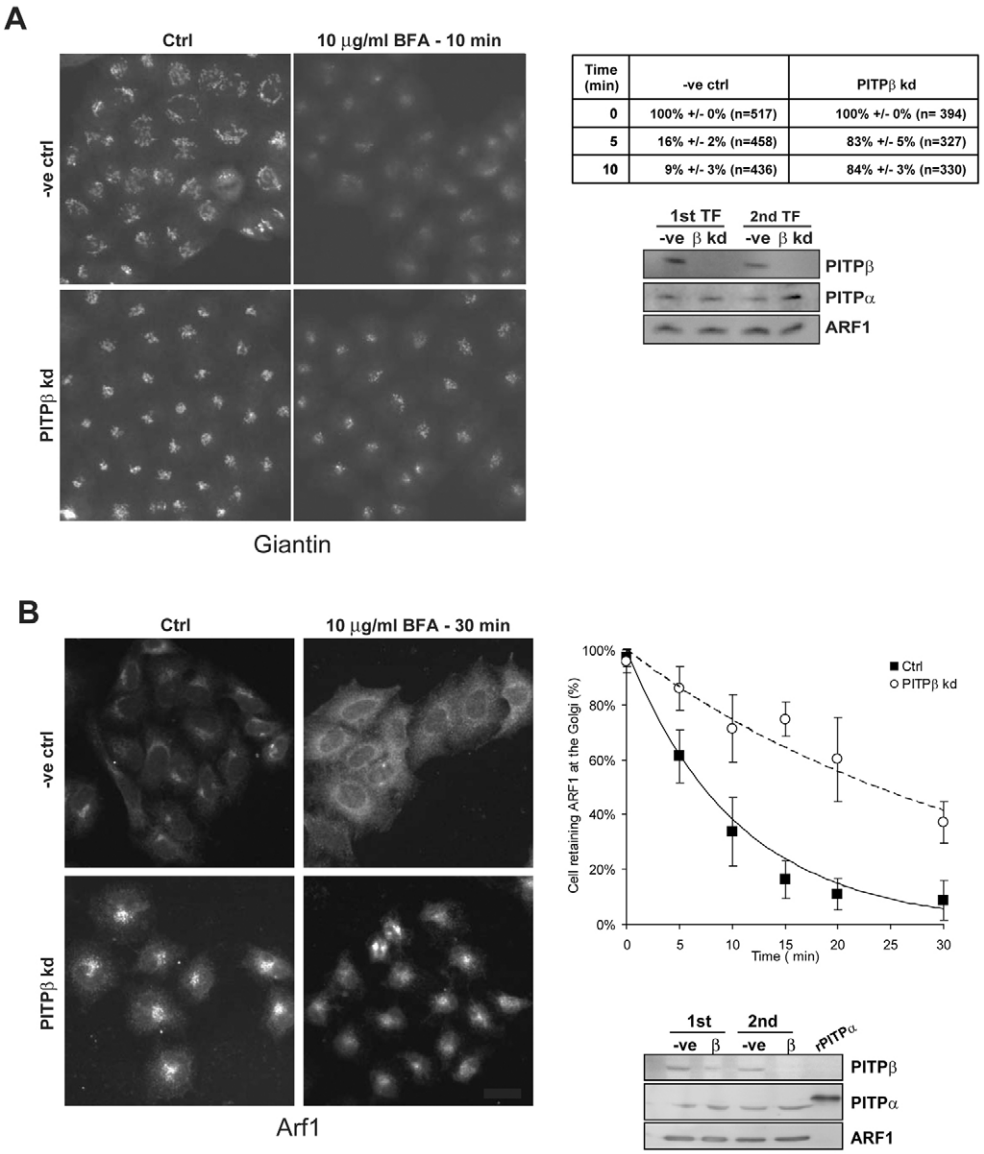

Fig. 2. Disassembly of the Golgi by BFA is delayed in cells depleted of PITPβ. HeLa cells transfected with control or PITPβ siRNA were treated with 10 μg/ml BFA at 37°C for the indicated times. (**A**) In PITPβ-knockdown cells, giantin staining shows a tight Golgi morphology and is protected from redistribution to the ER upon BFA treatment. Cells with conserved Golgi were quantified for both populations (right). (**B**) In the knockdown cells, ARF1 shows tight perinuclear staining and upon BFA treatment, ARF1 dissociation from the Golgi into the cytosol is retarded. The percentage of cells with ARF1 remaining at the Golgi was quantified in control and knockdown cells after BFA treatment (right). Ten random fields per time point were analysed (×40 objective). TF, transfection.

The strikingly similar accumulation of ERGIC-53 in the ERGIC at 16°C and in PITPβ-depleted cells suggests that PITPβ is required for exit out of the ERGIC. To examine this, we incubated control cells and PITPβ-depleted cells at 16°C to accumulate ERGIC-53 in the ERGIC clusters close to the Golgi complex (Fig. 3B). The cells were then shifted to 37°C and ERGIC-53 was allowed to re-establish to its steady state. In control cells, ERGIC-53 had established its steady state by 40 minutes. By contrast, in the PITPβ-depleted cells, ERGIC-53 remained in a tight perinuclear cluster that did not redistribute to the ER (Fig. 3B).

An alternative approach to block ERGIC-53 transport is the kinase inhibitor H89, which blocks protein export from the ER, and because ERGIC-53 constantly shuttles between the ER and the ERGIC, H89 causes redistribution of ERGIC-53 to the ER (Ben Tekaya et al., 2005; Aridor and Balch, 2000; Lee and Linstedt, 2000) (Fig. 4A, top panel). We anticipated that interfering with the retrograde transport of ERGIC-53 would prevent its accumulation at the ER upon H89 treatment. ERGIC-53 did not redistribute to the ER in PITPβ-depleted cells (Fig. 4A, bottom panel). The Golgi, identified by the marker GM130, did not redistribute to the ER in the presence of H89 (Fig. 4B). However, we note that treatment with H89 resulted in Golgi compaction compared with non-treated control cells. This was not analysed further, although it has been reported that H89 traps ARF1 in the GTP bound form at the Golgi and also protects the cells from BFA-induced Golgi disassembly (Lee and Linstedt, 2000; Altan-Bonnet et al., 2003). In the PITPβ-knockdown cells, ERGIC-53 and GM130 staining significantly colocalised and showed the compact Golgi phenotype both in the absence and presence of H89 (Fig. 4A,B).

The results described so far suggest that PITPβ is required for retrograde traffic from the Golgi and the ERGIC to the ER, mediated by COPI-coated vesicles. To confirm that COPI-mediated traffic from the Golgi to ER is disrupted in PITPβ knockdown cells, we took advantage of a previously established in vivo assay for COPI-dependent transport, which tracks the redistribution of a chimaeric VSVGts045-KDEL-R construct [the fusion protein of KDEL receptor with the thermo-reversible folding mutant of vesicular stomatitis virus (VSV) G protein (VSVGts045), that misfolds at 40°C and is therefore retained within the ER]. At the permissive temperature (32°C) it refolds and can exit the ER, and redistribute

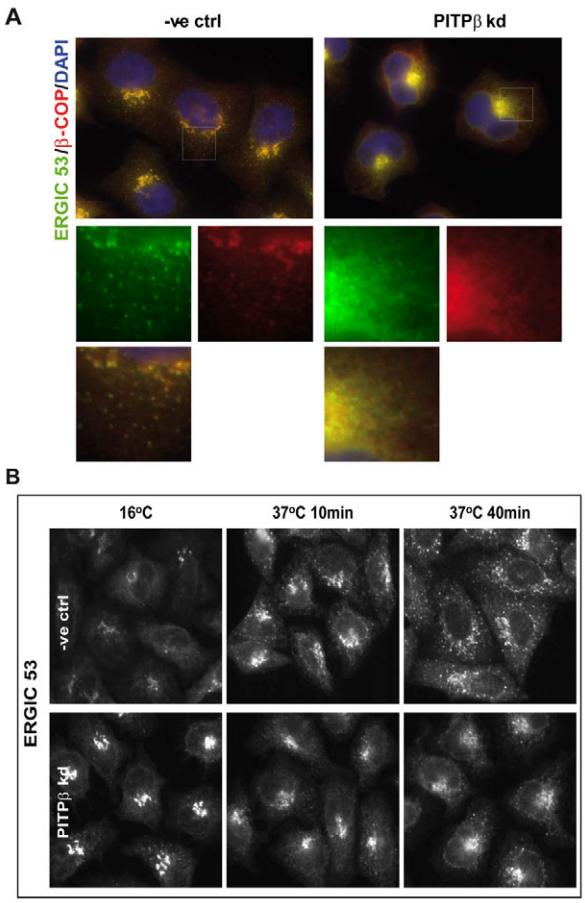

Fig. 3. Depletion of PITPβ affects ERGIC-53 and β-COP localisation and inhibits the recycling pathway of ERGIC-53. (**A**) Control and PITPβ-knockdown cells were stained for ERGIC-53 and β-COPI. These proteins colocalise and in the PITPβ-knockdown cells the peripheral localisation of both ERGIC-53 and β-COPI is lost (see boxed area, and enlarged panels below). Instead both proteins show tight perinuclear staining. (**B**) HeLa cells transfected with control or PITPβ siRNA were incubated at 16°C for 3 hours to accumulate ERGIC-53 in the perinuclear Golgi region. The cells were then transferred to 37°C for the indicated times and redistribution of ERGIC-53 was monitored by immunostaining with an anti-ERGIC-53 antibody. ERGIC-53 is retained in the perinuclear region in PITPβ-knockdown cells after the temperature shift, whereas in control cells ERGIC-53 redistributes to the periphery of the cells.

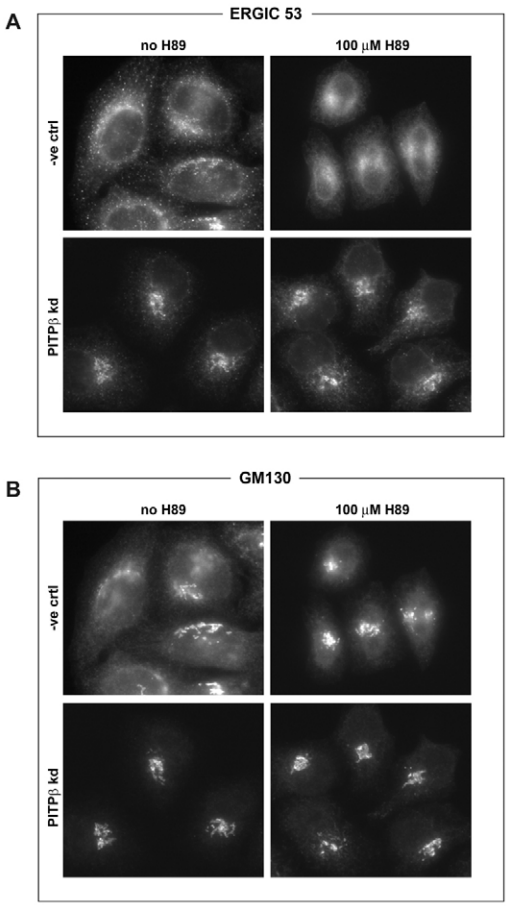

Fig. 4. H89-induced relocalisation of ERGIC-53 to the ER is prevented in cells depleted of PITPβ. Control and *PITPβ* siRNA-treated cells were treated with 100 μM H89 for 30 minutes at 37°C and immunostained for (**A**) ERGIC-53 and (**B**) GM130.

to the Golgi (Fig. 5C). Cells transfected with VSVGts045-KDEL-R, when grown at the permissive temperature, show a predominant localisation of VSVGts045-KDEL-R to the Golgi complex (Fig. 5A). However, when the cells are shifted to 40°C, VSVGts045-KDEL-R accumulates in the ER because of its thermo-sensitivity (Cole et al., 1998). We therefore examined the retrograde transport of VSVGts045-KDEL-R in control and PITPβ-knockdown cells. The cells were initially maintained at the permissive temperature to accumulate the chimaeric KDEL receptor at the Golgi. To monitor the retrograde transport, the cells were shifted to 40°C and the disappearance of the chimaeric KDEL receptor from the Golgi was observed over time in control cells (Fig. 5A,B). After 1 hour, only 23% of the cells showed staining at the Golgi. By contrast, in the PITPβ-depleted samples 72% of the cells retain Golgi staining after 1 hour (Fig. 5B).

DAG has been recently reported to be required for COPI-mediated retrograde transport (Fernandez-Ulibarri et al., 2007; Asp et al., 2009). ARF-GAP1 is required for COPI-coated vesicle formation and is recruited and subsequently activated by DAG (Bigay et al., 2003; Bigay et al., 2005). We therefore investigated whether the distribution of ARF-GAP1 was disrupted in PITPβ-knockdown cells as an explanation for the blockade in retrograde traffic. We examined the localisation of ARF-GAP1 in control and PITPβ-knockdown cells (supplementary material Fig. S7A). As a control we used propranolol, which depletes DAG by inhibiting phosphatidate phosphohydrolase. As reported, propranolol treatment caused the release of ARF-GAP1 from the Golgi to the cytosol (Fernandez-Ulibarri et al., 2007) (supplementary material Fig. S7B). However, in PITPβ-knockdown cells, ARF-GAP1 localisation is unaffected.

PITPβ-deficient cells remain competent for anterograde transport

To examine whether the effect of PITPβ knockdown was specific for a particular transport step, we looked at anterograde transport. It is noted that latrunculin B treatment or interference with Syne-1 does not disrupt anterograde transport (Valderrama et al., 2001;

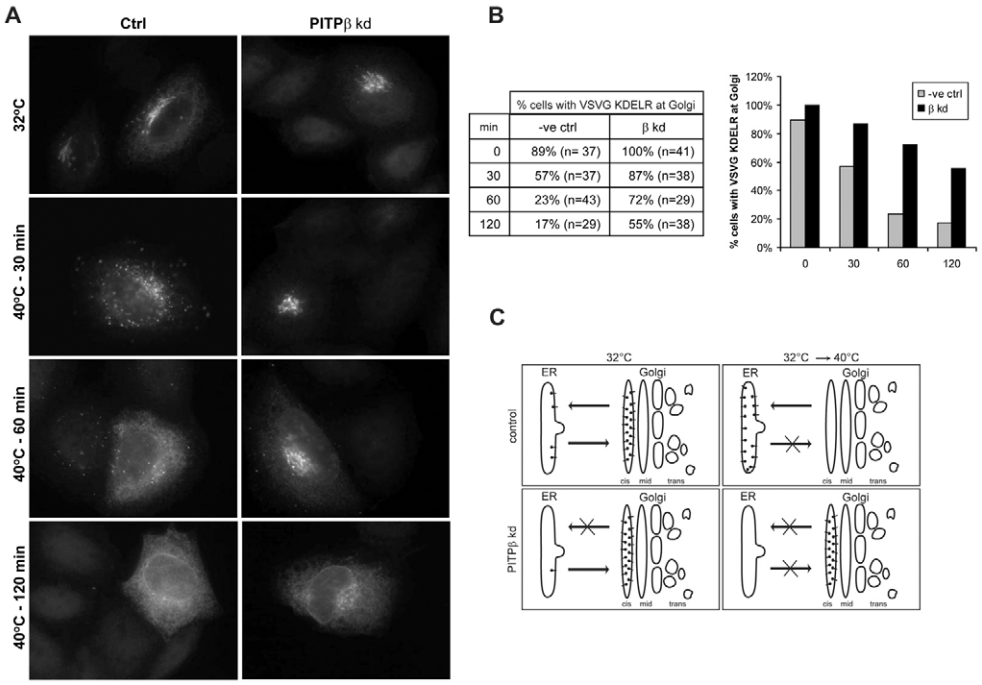

Fig. 5. Knockdown of PITPβ inhibits COPI-mediated transport. (**A**) Retrograde transport of the chimaeric KDEL receptor (VSVGts045-KDEL-R) from the Golgi to the ER is delayed in PITPβ-knockdown cells. At the permissive temperature (32°C), the VSVGts045-KDEL-R is localised at the Golgi and after shifting to the non-permissive temperature (40°C), the receptor moves to the ER where it gets trapped. (**B**) The percentage of cells with VSVGts045-KDEL-R at the Golgi is quantified for control and PITPβ-knockdown cells. (**C**) Model demonstrating the trafficking step that is inhibited in PITPβ-knockdown cells. In control cells (top two panels), the construct cycles freely between the ER and the Golgi at the permissive temperature (32°C) and at steady state it is mainly at the cis-Golgi. Upon shift to the non-permissive temperature (40°C), the construct can move to the ER but gets trapped upon arrival at the ER. In PITPβ-knockdown cells (bottom two panels), the construct remains at the cis-Golgi even at the non-permissive temperature because of a blockade of retrograde trafficking.

Gough and Beck, 2004). The transport efficiency was monitored for a synchronised wave of ts045-VSV-G trafficking from the ER to the Golgi complex, and from the Golgi to the plasma membrane. No significant differences were observed between PITPβ-depleted cells and control cells in either transport stage (supplementary material Fig. S8A). Anterograde transport was also monitored by measuring the release of newly synthesised ^{35}S-labelled proteins into the extracellular medium in pulse-chase experiments. Control and PITPβ-knockdown cells were labelled with [^{35}S]methionine for 30 minutes at 37°C. The cells were then washed and chased for various times (5, 10, 15, 40, 45 minutes) and the percentage of ^{35}S-labelled protein secreted in the external medium was monitored. No difference was observed between control and PITPβ-knockdown cells (supplementary material Fig. S8B).

Depletion of PITPβ causes a decrease in PtdIns(4)*P* levels with no effect on sphingolipid and glycosphingolipid synthesis

To monitor whether PITPβ knockdown leads to changes in phosphoinositide levels, we incubated HeLa cells with [^{3}H]inositol for 3 days to achieve steady state labelling of the inositol lipids. Lipids were extracted from control and PITPβ-knockdown cells and the phosphoinositides were resolved by thin layer chromatography (Fig. 6A). In PITPβ-knockdown cells the level of phosphatidylinositol 4-phosphate [PtdIns(4)*P*] was diminished by as much as ~45% whereas phosphatidylinositol (4,5)-bisphosphate [PtdIns(4,5)P_2] levels did not change significantly (P=0.10; Fig. 6B).

It has been reported that PITPβ is localised to the trans-Golgi network (TGN) (Phillips et al., 2006a). One possibility is that PITPβ may function in this compartment to maintain PtdIns(4)*P* levels which are required for both sphingomyelin and glycosphingolipid (GSL) synthesis (D'Angelo et al., 2007). We examined whether PITPβ deficiency, which does cause a reduction in PtdIns(4)*P* levels, consequently leads to defects in sphingomyelin and GSL synthesis. CERT and FAPP2 both localise to the TGN because of a PH domain that binds PtdIns(4)*P* and ARF1 (Hanada et al., 2003; D'Angelo et al., 2007). CERT transfers ceramide from the ER to the TGN and FAPP2 transfers glucosylceramide (GlcCer) from the cis-Golgi to the TGN. Ceramide is converted into sphingomyelin and GlcCer is used for GSL synthesis (D'Angelo et al., 2007). To examine whether PITPβ was responsible for the provision of PtdIns for PtdIns(4)*P* synthesis at the TGN required for the localisation of CERT or FAPP2, we analyzed the synthesis of sphingomyelin and GSL in control and PITPβ-knockdown cells. Synthesis of sphingomyelin or GSLs was unaffected in the knockdown cells (supplementary material Fig. S9A). These data rule out a role for PITPβ in maintaining phosphoinositide levels at the TGN. We also used the PH domain of OSBP, the localisation of which is dependent on PtdIns(4)*P* and ARF1 (Levine and Munro, 2002). The targeting of the OSBP-PH domain to the Golgi compartment was maintained in PITPβ-depleted cells (supplementary material Fig. S9B). The compact nature of the Golgi was evident nonetheless. From these data, we would suggest that PITPβ specifically affects a pool of PtdIns(4)*P*, which is separate from the pool at the TGN.

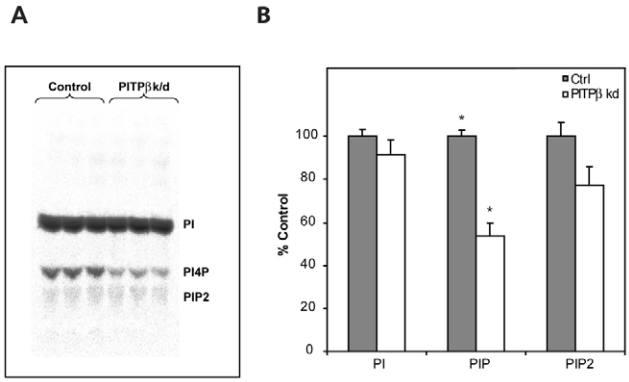

Fig. 6. PtdIns(4)*P* (PI4P) levels are reduced in PITPβ-knockdown cells. (**A**) HeLa cells treated with control and *PITPβ*-specific siRNA were grown in the presence of [^{3}H]inositol for 3 days, and the lipids were extracted and analysed by TLC and phosphorimaged. (**B**) Quantification of the phosphoinositide levels from three independent experiments each carried out in triplicate. **P*<0.05. PI, PtdIns; PIP, PtdIns(4)*P*; PIP2, PtdIns(4,5)P_2.

Both PtdIns and PtdCho transfer activity of PITPβ is required for COPI-mediated retrograde transport

Next we set out to examine whether the PtdIns transfer properties were necessary for Golgi-ER retrograde traffic. *In vivo* and *in vitro* PITPβ contains a single phospholipid molecule in its hydrophobic cavity, which can be either PtdIns or PtdCho (Morgan et al., 2006). From the crystal structure of PtdIns-liganded PITPα, four amino acid residues, T59, K61, E86 and N90 interact specifically with the inositol headgroup of PtdIns; mutation of any one of these residues leads to loss of PtdIns binding and transfer without affecting PtdCho binding or transfer (Tilley et al., 2004). PITPβ shares 77% identity with PITPα and the residues that co-ordinate the inositol headgroup identified in PITPα are conserved in PITPβ. We mutated the corresponding residues in PITPβ (K60 and N89) and found that transfer of PtdIns was diminished in both mutants whereas PtdCho transfer remained unaffected (Fig. 7A).

Although we have identified PITPβ mutants that are deficient in PtdIns transfer, no mutants have yet been identified that are deficient in PtdCho transfer for either PITP. Examination of the binding site for PtdCho in the crystal structure of PtdCho-PITPβ does not provide any obvious candidate residues that could influence PtdCho binding without affecting PtdIns binding and transfer (Vordtriede et al., 2005). One residue that is in close proximity to the headgroup binding site for both PtdCho and PtdIns is cysteine 95, and mutation of this residue to C95T or to C95A does not affect PtdIns transfer (Shadan et al., 2008). We analysed these mutants and found that the PtdCho transfer activity of C95T and C95A is lost whereas PtdIns transfer activity is unaffected (Fig. 7B).

In order to deposit its lipid cargo to a membrane, PITPβ has to dock to the membrane surface. This is dependent on two tryptophan residues, W203 and W204; mutation of these residues to alanine causes loss of transfer activity (Shadan et al., 2008). We used three classes of mutants with deficiencies in each of PtdIns transfer, PtdCho transfer and in docking to the membrane surface. We first established that wild-type PITPβ was able to rescue the retrograde transport defect in *PITPβ* siRNA-treated cells. Control and siRNA-treated cells were first maintained at 32°C for 3 hours and were shifted to 40°C and the disappearance of the chimaeric KDEL receptor from the Golgi was monitored after 1 hour (Fig. 7C). In the vector control, only ~30% of the cells showed staining at the Golgi compared to ~70% in the PITPβ-depleted cells. Upon expression of wild-type PITPβ, the amount of the chimaeric KDEL receptor at the Golgi was reduced to 30%. None of the mutants were able to rescue the retrograde transport of the chimaeric KDEL receptor as evidenced by its retention at the Golgi (Fig. 7C).

PITPβ-mediated transfer of both PtdIns and PtdCho is essential for retrograde transport as well as docking to membranes. This implies that exchange of one lipid for another is necessary. Phosphoinositides are required for membrane traffic at many membrane compartments and we examined whether phosphorylated PIs are also required for retrograde traffic from the cis-Golgi to the ER. We used wortmannin (10 μM) and quercetin (30 μM) which can inhibit type III PI 4-kinases (α and β), to examine their effects on Golgi to ER traffic. PI4KIIIα is localised at the ER and the PI4KIIIβ is localised at the Golgi (Wong et al., 1997; Balla et al., 2008). Both wortmannin and quercetin were found to inhibit Golgi to ER retrograde transport only when used at concentrations that inhibit type III PI 4-kinases (Fig. 8). Cells treated with wortmannin at 100 nM, a concentration that only inhibits PI 3-kinases, were unaffected. From this we conclude that phosphoinositide metabolism, most probably via PI4KIIIβ, is required for retrograde traffic and PITPβ is a probable candidate for PtdIns delivery from its site of synthesis, the ER, and deliver it to the Golgi in exchange for PtdCho.

Discussion

We provide compelling evidence that PITPβ is required for retrograde traffic from the Golgi and the ERGIC to the ER, mediated by COPI-coated vesicles in vivo. This has been demonstrated using three different methods. Firstly, ERGIC-53 is trapped at the ERGIC and does not relocalise to the ER upon release from a 16°C temperature block in PITPβ-depleted cells. Secondly, treatment with H89, which causes the accumulation of ERGIC-53 in the ER, does not occur in PITPβ-knockdown cells. Thirdly, trafficking of the KDEL receptor from the Golgi to the ER is arrested in PITPβ-knockdown cells. The retrograde trafficking defect could be rescued with wild-type PITPβ but not with mutant proteins deficient in PtdIns or PtdCho transfer activity, or with a mutant that is unable to dock on membranes. Retrograde traffic is also inhibited when PI 4-kinase inhibitors are used, supporting the possibility that PITPβ functions by regulating phosphoinositide levels at the Golgi.

The Golgi is the major site for PtdIns(4)*P* generation (Weixel et al., 2005) and in the PITPβ knockdown cells, we observed a substantial depletion of PtdIns(4)*P*. However, anterograde traffic from the TGN is unchanged suggesting that the TGN pool of PtdIns(4)*P* is unaffected in PITPβ-knockdown cells. In addition we do not see any defects in sphingomyelin or GSL synthesis, both steps being dependent on the presence of PtdIns(4)*P* at the TGN (Mayinger, 2009).

We would suggest that the pool of PtdIns(4)*P* that is disrupted in PITPβ-knockdown cells is localised at the cis-side of the Golgi where COPI-mediated transport vesicles emerge.

In principle, PITPβ may also control COPI-mediated retrograde transport by regulation of DAG levels. Phosphoinositides together with ARF1 regulate phospholipase D activity and the products of phospholipase D activity, phosphatidic acid (PA) and its metabolite, diacylglycerol (DAG) are thought to be required for COPI vesicle biogenesis (Fernandez-Ulibarri et al., 2007; Yang et al., 2008; Asp et al., 2009). However, PITPβ does not affect the recruitment of ARF1-GAP, which is dependent on DAG for its localisation at the

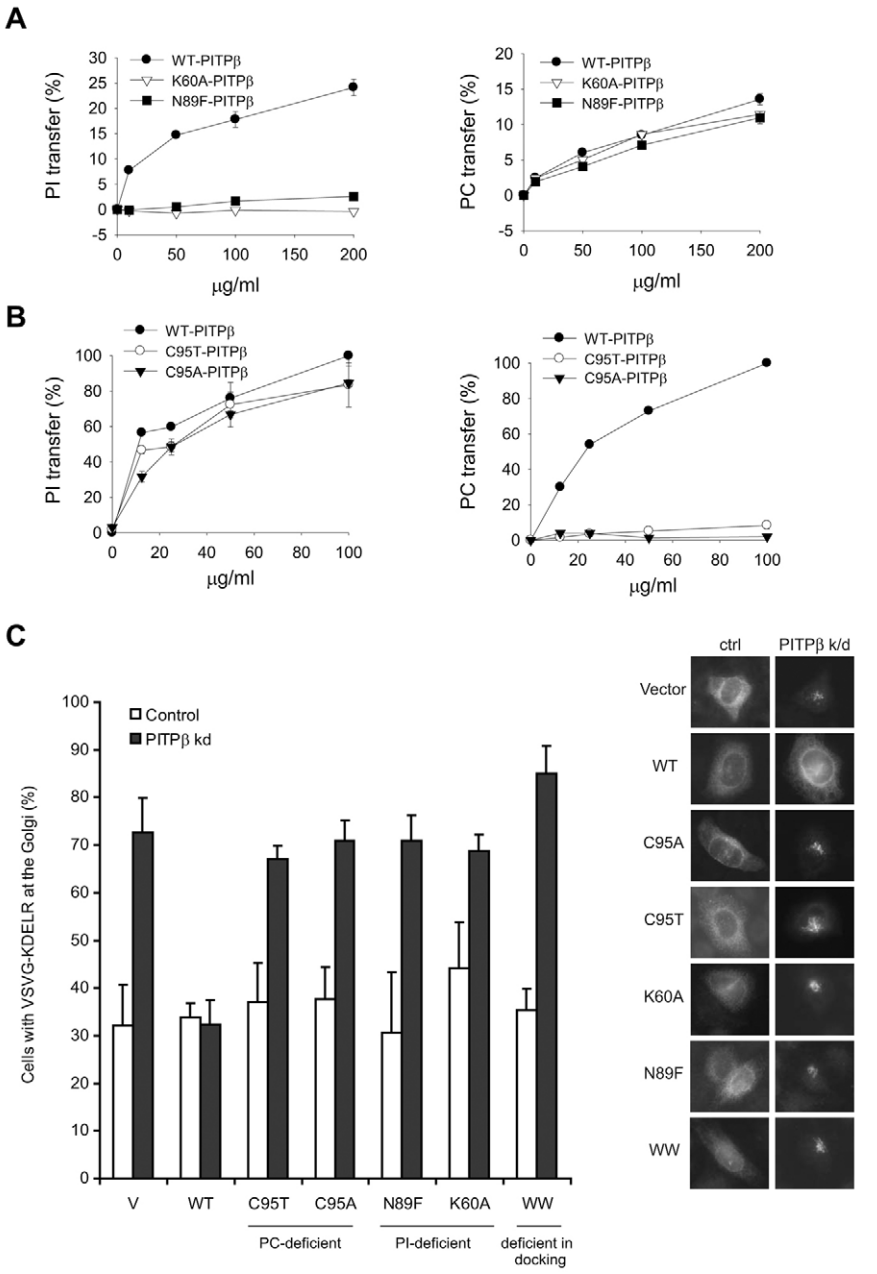

Fig. 7. PtdIns and PtdCho transfer activity of PITPβ is required for rescue of retrograde transport from the Golgi to ER. (**A**) Mutation K60A and N89F causes loss of PtdIns (PI) but not PtdCho (PC) transfer. (**B**) Mutation of cysteine 95 to alanine or to threonine causes loss of PtdCho transfer but not PtdIns transfer. PtdIns and PtdCho transfer activity is expressed as a percentage of the maximum transfer observed with wild-type PITPβ. (**C**) Rescue of the retrograde traffic defect of VSVGts045-KDEL-R by wild-type PITPβ but not mutants deficient in PtdCho transfer (C95A and C95T), PtdIns transfer (N89F and K60A) and a mutant incapable of docking to the Golgi and ER membranes (WW203/204AA, respectively). Results were combined from three independent experiments (± s.d.). In each experiment, an average of 50 cells were counted per condition. (Right) Representative immunofluorescence images of VSVGts045-KDEL-R in control and PITPβ-knockdown cells rescued with either empty vector, wild-type PITPβ or PITPβ mutants.

Golgi. In contrast to PITPβ, depletion of another PtdIns transfer protein, Nir2 (also known as RdgBαI), which regulates DAG levels at the TGN via the CDP-choline pathway results in defects in anterograde transport and dispersion of the Golgi (Litvak et al., 2005). None of these phenotypes are observed in PITPβ-knockdown cells and indeed opposite effect are observed with regards to Golgi shape. These observations would suggest that PITPβ does not regulate a pool of DAG at the Golgi, unlike Nir2 in mammalian cells and Sec14p in yeast.

Depletion of PITPβ leads to protection against BFA-induced release of ARF1 in the cytosol (Fig. 2) suggesting that ARF1 is maintained in the GTP form at the Golgi. It is interesting to note that expression of ARF1.Q71L results in a similar phenotype to that observed in PITPβ-knockdown cells (supplementary material Table S1). Previous studies, all done in vitro, have reported that activated ARF1 can recruit actin at the Golgi (Godi et al., 1998; Fucini et al., 2000; Heuvingh et al., 2007). Our results show an accumulation of actin at the Golgi in PITPβ-knockdown cells, which may be a consequence of an increase in ARF1.GTP (supplementary material Fig. S3). We speculate that PtdIns(4)*P* can regulate the ARF GTPase cycle since depletion of PITPβ leads to a decrease in PtdIns(4)*P*. We considered the possibility that PITPβ could potentially affect the ARF GTPase cycle via DAG regulation of ARF.GAP1 (Bigay et al., 2005; Asp et al., 2009) but our results clearly indicate that in PITPβ-knockdown cells, ARF.GAP1 localisation to the Golgi is unaffected (supplementary material Fig. S7). How a decrease in PtdIns(4)*P* can lead to an increase in ARF1.GTP is not clear but there are two possibilities – either loss of PtdIns(4)*P* decreases ARF1.GAP activity or increases ARF1.GEF activity.

In Fig. 9, we provide a scheme that integrates PITPβ with other components of the COPI-mediated retrograde transport machinery.

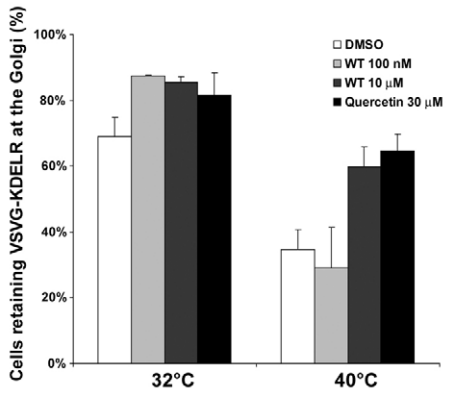

Fig. 8. Retrograde transport of VSVGts045-KDEL-R is blocked by inhibitors of type III PI 4-kinases. Cells were treated with the indicated concentrations of inhibitors for 2 hour and the percentage of cells retaining the chimaera at the Golgi was monitored. The number of cells counted per condition were 70-100 cells in each individual experiment and the results were compiled from three independent experiments (± s.e.m.).

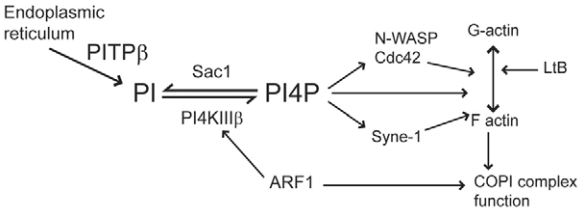

Fig. 9. Scheme illustrating how PITPβ participates in the retrograde Golgi to ER transport. PITPβ delivers PtdIns (PI) from the ER to the cis-Golgi, where it is phosphorylated to PtdIns(4)*P* (PI4P) by PI4KIIIβ. PtdIns(4)*P* directly or indirectly, via N-WASP and Cdc42 or via Syne-1 (nesprin1), regulates the balance of G- to F-actin; actin dynamics is required for COP1-complex function.

Many of these components when mutated or knocked down present a similar phenotype, the most prominent being Golgi compaction, with defects in Golgi to ER retrograde traffic (supplementary material Table S1 provides complete information on phenotypes seen, and these are compared with what is observed in PITPβ-knockdown cells). We envisage that PITPβ delivers PtdIns from its site of synthesis, the ER, to the cis-Golgi where it can be phosphorylated to PtdIns(4)*P* by PI 4-kinaseIIIβ. ARF1 would co-ordinate the synthesis of PtdIns(4)*P* and COPI recruitment both temporally and spatially and PtdIns(4)*P* would thus play a key role in retrograde trafficking from the cis-Golgi and/or ERGIC to the ER by regulating cytoskeletal dynamics. Previous studies have established that ARF1 recruits and stimulates the activity of PI 4-kinaseIIIβ at the Golgi (Godi et al., 1999; Jones et al., 2000). Inhibition of PI 4-kinases with wortmannin or quercetin results in the same defects in COPI-mediated retrograde transport as observed with PITPβ silencing.

Golgi-localised cdc42 activates N-WASP, which in turn activates the Arp2/3 complex to initiate actin branching (Luna et al., 2002; Heuvingh et al., 2007; Dubois et al., 2005). Overexpression of the constitutively active mutant of cdc42 also induces defects in retrograde transport and Golgi compaction (Luna et al., 2002). Disruption of the actin cytoskeleton with latrunculin results in Golgi compaction (Valderrama et al., 2001) (supplementary material Fig. S2B) and defects in retrograde trafficking (Valderrama et al., 2001) suggesting that it is the polymerisation-depolymerisation cycle of actin that is required for retrograde transport. At the Golgi, actin and actin binding proteins are found and several of the actin binding proteins are present as Golgi-specific isoforms but the effects of phosphoinositides, in particular PtdIns(4)*P* on these actin binding proteins have not been described (Egea et al., 2006).

Syne-1 plays a key role in the maintenance of Golgi structure and over-expression of Syne-1 peptides, which act in a dominant-negative manner, disturbs retrograde transport (Gough and Beck, 2004). Interestingly, Syne-1 is also an important component of the nuclear envelope where it maintains nuclear shape by interacting with SUN (Sad1 and UNC-84 homology) proteins to form a bridge between intra-nuclear lamins and extra-nuclear actin filaments. Our results suggest that PITPβ could function by trafficking PtdIns from the endoplasmic reticulum to the Golgi and nuclear envelope to maintain the actin and spectrin dynamics at these sites.

In PITPβ knockdown cells, in addition to defects in retrograde traffic, alterations in nuclear morphology are also observed. Although, retrograde traffic could be restored upon expression of wild-type PITPβ, the nuclear morphology could not. PITPβ is present as two splice variants, and in all of our rescue experiments we have used only one splice variant (PITPβ-sp1) (Morgan et al., 2006). If the two splice variants have distinct functions, one operating at the Golgi and the other at the nuclear envelope, this would account for our inability to rescue the nuclear phenotype. We are currently investigating this possibility.

Some of the morphological changes described here have been observed when the actin cytoskeleton is disrupted with latrunculin, with the difference that it does not affect nuclear morphology (supplementary material Fig. S2B). The distortion in nuclear morphology observed in PITPβ-knockdown cells is similar to that observed when Syne-1 is disrupted. The spectrin repeats of Syne-1 are thought to be important in maintaining nuclear shape (Luke et al., 2008). PITPβ may be independently required for localisation of the spectrin network at the Golgi and the nuclear envelope and loss of PITPβ expression would result in altered Golgi morphology and also nuclear shape.

Is there a relationship between the alterations of the Golgi morphology and inhibition of COPI-mediated traffic or is transport independent of Golgi structure? We suggest that the two could be intimately linked. Golgi compaction and defects in COPI-retrograde traffic are often simultaneously observed. This includes expression of Syne-1 fragments, ARF1Q71L, active cdc42, latrunculin B treatment and PITPβ (supplementary material Table S1). From our findings, we propose that PITPβ regulates the levels of PtdIns(4)*P* by delivery of PtdIns at the site of COPI recruitment and thus modulate the cytoskeletal dynamics required for COPI-mediated retrograde transport.

Materials and Methods

Chemicals

Latrunculin B, H89, TRITC-phalloidin and DAPI (4′,6-diamidino-2-phenylindole dihydrochloride) were obtained from Sigma-Aldrich. Hiperfect was obtained from Qiagen, Fugene HD from Roche, and brefeldin A (BFA) from Calbiochem.

Plasmids

OSBP-PH-GFP and ts045-VSVG-GFP were a gift from Tim Levine. VSVGts045-KDEL-R construct was a gift from Victor Hsu. Untagged wild-type PITPβ and mutants were cloned in pcDNA3.1 vector as described elsewhere (Shadan et al., 2008).

Antibodies

For immunofluorescence the following antibodies were used: giantin (rabbit polyclonal) 1:5000 (a gift from Antonella de Matteis); GM130 (MLO7, rabbit polyclonal) 1:5000 (a gift from Martin Lowe); TGN38 (GB1, primate-specific rabbit polyclonal from George Banting) 1:500; ARF1 679 (affinity purified rabbit polyclonal) 1:100 (made in-house); ERGIC-53 (mouse monoclonal) 1:2000 (a gift from Hans Peter Hauri, Switzerland) and VSVG antibody (Bw8G65 mouse monoclonal supernatant) 1:4 (a gift from Victor Hsu). β-COP (Ab 2899, rabbit polyclonal) 1:1000 was purchased from Abcam. For western blots the following antibodies were used: PITPβ (mAb: 1C1) mouse monoclonal (1:1000) (Morgan et al., 2006), PITPα (Ab: 103) rabbit antiserum (1:1000) (Cosker et al., 2008) and ARF1 (Ab: 678) rabbit antiserum (1/2000) (Skippen et al., 2002). These antibodies were all prepared in-house and have been described previously. The antibody to PITPβ (mAb: 1C1) recognises both splice variants of PITPβ (Morgan et al., 2006).

Cell culture

HeLa cell cultures were maintained in DMEM (Sigma) supplemented with 10% heat inactivated foetal bovine serum (SLI), 4 mM glutamine (Sigma), 50 IU/ml penicillin and 50 μg/ml streptomycin (Sigma).

PITPβ and PITPα silencing by RNA interference

The siRNA sequences against human *PITPβ* used were: oligonucleotide no. 1: 5′-ACGGATATTTACAAACTTCCA-3′; oligonucleotide no. 2: 5′-CAAGCTGTGGA-CCAACATATA-3′; oligonucleotide no. 3: 5′-AACAATTGTAACGAATGAATA-3′ and oligonucleotide no. 4: 5′-CTGCATTGCAATACCCTGTTA-3′ and were obtained from Qiagen. Cells were transfected with a mixture of two siRNA (oligonucleotides 1 and 2, Set 1) or (oligonucleotides 3 and 4, Set 2). Both sets were equally efficient for silencing PITPβ in HeLa cells and gave the same results. For PITPα knockdown a set of two siRNA duplexes with the following target sequences was used: 5′-AACCGTTATTACAAATGAGTA-3′ and 5′-CTCAGAGGGACTTATGATAAA-3′. For the experiments where the cells were rescued with wild-type PITPβ and the mutants, oligonucleotides 2 and 4 (Set 3) were used as the sequences are not in the coding region, and therefore the human *PITPβ* plasmids used for rescue were RNAi resistant. Negative control non-silencing siRNA was purchased from Qiagen and the siRNA sequence was: 5′-AATTCTCCGAACGTGTCACGT-3′. Transfections were performed using Hiperfect (Qiagen) according to the manufacturer's instructions. Briefly, the cells were seeded at a density of 0.4×10^5 cell/ml in 6-well or 24-well tissue culture plates (33.7 mm and 15.7 mm diameter/well, respectively). Immediately after seeding the cells were transfected with a mixture of two siRNAs (Set 1 or Set 2) at a final concentration of 6 nM for each individual duplex. Non-silencing siRNA was used for control cells at a final concentration of 12 nM. 72 hours after transfection, control and PITPβ knockdown cells were detached with trypsin, re-seeded at a density of 0.4×10^5 cell/ml in 6-well or 24-well tissue culture plates and transfected a second time as above. 72 hours following the second transfection, cells were collected for western blot and fixed for immunofluorescence analysis. For immunofluorescence, the cells were grown on glass coverslips placed in the tissue culture dishes before transfection.

Immunofluorescence

HeLa cells transfected with siRNA were grown for 72 hours after the second transfection on glass coverslips (13 mm, thickness 0). Cells were washed in phosphate-buffered saline (PBS) and subsequently fixed with 4% formaldehyde in PBS, pH 7.4 with 100 μM $MgCl_2$ and 100 μM $CaCl_2$ for 10 minutes at room temperature. Following a further three washes in PBS, cells were permeabilised with PBS containing 0.2% Triton X-100 and 0.1 M glycine for 10 minutes at room temperature. Cells were then washed again three times, before blocking with 0.1% BSA in PBS with 0.1 M glycine, for 10 minutes at room temperature. The cells were then incubated with primary antibodies diluted in the same blocking buffer. Incubations with specified antibodies were carried out for 1 hour at room temperature in a humidified box, in order to reduce the volume of antibody solution required (50-100 μl is sufficient under these conditions in a 24-well plate). In order to stain for nuclei, 49,6-diamidino-2-phenylindole (DAPI) was added to the primary antibody solution at a final concentration of 400 μg/ml. Following incubation with primary antibodies, cells were washed three times with blocking solution. Cells were then incubated with Alexa Fluor 488- or 546-conjugated secondary antibodies (Molecular Probes) at a dilution of 1:1000 in blocking solution for 30 minutes at room temperature. For actin staining, the cells were treated with blocking solution containing TRITC-phalloidin (10 nM) for 20 min. Subsequently cells were washed three times in PBS and finally rinsed in water prior to mounting on a microscope slide with mowiol [13% (w/v) in 0.1 M Tris pH 8.5 with 33% glycerol (w/v)].

Quantification of fields of cells

To quantify the effect of PITPβ silencing, ten random fields per experiment of PITPβ-knockdown and control HeLa cells were taken with a ×20 objective following fluorescence immunostaining for GM130. Using the imaging software Cell-F (Olympus), Golgi were individually selected with the *Magic wand* selection tool and their area and sphericity were measured. Golgi with an area ≤65 μm^2 and a sphericity ≥0.4 were scored as 'condensed'. Alternatively, the random fields were analysed using ImageJ. For each field, the Golgi were automatically selected according to a set threshold using the *Analyse particles* tool. Golgi with a relative area of ≤0.05 and a circularity of ≥0.7 were scored as 'condensed'.

Analysis of PITPβ mutants for lipid transfer and binding

Recombinant His-tagged wild-type PITPβ and mutants were expressed in *Escherichia coli*, purified using Ni-NTA(Ni^{2+}-nitrilotriacetate) resin (Qiagen), and lipid transfer was assayed as previously described (Tilley et al., 2004).

Treatment with latrunculin B, brefeldin A or H89

Prior to treatment with latrunculin B or BFA, the HeLa cells, grown for 48 hours on glass coverslips, were washed twice with HEPES buffer (20 mM HEPES, 137 mM NaCl, 3 mM KCl, 1 mM $CaCl_2$, 2 mM $MgCl_2$, 1 mg/ml glucose, 0.1 mg/ml BSA). The cells were treated with 500 nM latrunculin B or DMSO (solvent control) for 15 minutes at 37°C, or with 10 μg/ml BFA or methanol (solvent control) for 5, 10, 15, 20 and 30 minutes at 37°C. Following treatment, the cells were fixed with formaldehyde and processed for immunofluorescence as described above. For quantitative analysis of GM130 and ARF1 redistribution, images from 10 randomly selected fields (×40 objective) were obtained and at least 100 cells were counted per experimental condition. 72 hours after the second siRNA transfection, control or PITPβ-knockdown cells were incubated with 100 μM H89 in growth medium for 30 minutes prior to fixation in paraformaldehyde and immunostaining as above.

Temperature treatment

72 hours after the second siRNA transfection, control or PITPβ-knockdown cells were incubated for 3 hours at 16°C and shifted to 37°C for 10 and 40 minutes. Cells were then processed for immunostaining with antibodies against ERGIC-53.

DNA transfections in PITPβ-knockdown cells

HeLa cells were transfected with control or PITPβ siRNA (Set 3) as described above. 4 hours after the second siRNA transfection, the Hiperfect-containing medium was replaced with fresh growth medium. The next day the cells were transfected with the appropriate plasmid DNA using Fugene HD (Roche) following the manufacturer's instructions and kept at 37°C. This included wild-type PITPβ and mutants, VSVGts045-KDEL-R, GFP-VSV-G and GFP-OSBP-PH domain. Expression of proteins was monitored by western blot or by fluorescence microscopy 48 hours post-DNA transfection.

Monitoring retrograde transport using VSVGts045-KDEL receptor

Control and PITPβ-knockdown cells were transfected with VSVGts045-KDEL-R as described above. The cells were equilibrated at the permissive temperature of 32°C for 3 hours and subsequently misfolding of the chimaera was induced by shifting the cells to the non-permissive temperature of 40°C for the indicated times. The cells were immmunostained with anti-VSVG antibody and the percentage of the cells in which the construct was at the Golgi was measured. For the rescue experiments, wild-type and PITPβ mutants were co-expressed with VSVGts045-KDEL-R and the cells, initially kept at 32°C for 3 hours, were shifted to 40°C for 1 hour. The location of VSVGts045-KDEL-R was examined by immunofluorescence. To examine the effect of PI 4-kinase inhibitors on retrograde transport, wortmannin and quercetin were added 1 hour prior to shifting the cells to 40°C and they were then kept at 40°C for 1 hour.

Electron microscopy

HeLa cells were transfected with siRNA as described above and cultured on thermanox plastic coverslips (Nunc). 72 hours after the second transfection the cells were fixed in 0.1 M cacodylate, 2% paraformaldehyde and 2% glutaraldehyde for 30 minutes at room temperature and embedded on Epon stubs as described previously (Tomas et al., 2004). Enface 70 nm sections were viewed on a JEOL 1010 transmission electron microscope, and images gathered with a Gatan OriusSC100B CCD camera. Montages were assembled using Gatan Digital Micrograph.

Measurement of inositol lipids in control and knockdown cells

After one round of transfection with siRNA, HeLa cells were transferred to Medium199 containing 5 μCi/ml [^{3}H]inositol and subsequently transfected with siRNA and after 3 days, the cellular lipids were extracted and analysed by thin layer chromatography (Cunningham et al., 1995).

We thank Victor Hsu for suggesting the use of the VSVG-KDEL-R construct and for providing it together with the VSVG antibodies. We thank Tommy Nilsson for providing the ARF1-GAP antibody, Antonella De Matteis for the giantin antibody, Martin Lowe for the GM130 antibody and Hans-Peter Hauri for the ERGIC-53 antibody. We acknowledge the early contributions of both Victoria Allen-Baume and Clive Morgan. We would like to thank Antonella De Matteis, Martin Lowe, Sandip Patel and John Carroll for discussions. This work was funded by grants from the Wellcome Trust and Cancer Research UK. Deposited in PMC for release after 6 months.

Supplementary material available online at
http://jcs.biologists.org/cgi/content/full/123/8/1262/DC1

References

Alb, J. G., Jr, Phillips, S. E., Rostand, K., Cui, X., Pinxteren, J., Cotlin, L., Manning, T. G. S., York, J. D., Sontheimer, J. F., Collawn, J. F. et al. (2002). Genetic ablation of phosphatidylinositol transfer protein function in murine embryonic stem cells. *Mol. Biol. Cell* **13**, 739-754.

Alb, J. G., Jr, Cortese, J. D., Phillips, S. E., Albin, R. L., Nagy, T. R., Hamilton, B. A. and Bankaitis, V. A. (2003). Mice lacking phosphatidylinositol transfer protein alpha exhibit spinocerebellar degeneration, intestinal and hepatic steatosis, and hypoglycemia. *J. Biol. Chem.* **278**, 33501-33518.

Altan-Bonnet, N., Phair, R. D., Polishchuk, R. S., Weigert, R. and Lippincott-Schwartz, J. (2003). A role for Arf1 in mitotic Golgi disassembly, chromosome segregation, and cytokinesis. *Proc. Natl. Acad. Sci. USA* **100**, 13314-13319.

Aridor, M. and Balch, W. E. (2000). Kinase signaling initiates coat complex II (COPII) recruitment and export from the mammalian endoplasmic reticulum. *J. Biol. Chem.* **275**, 35673-35676.

Asp, L., Kartberg, F., Fernandez-Rodriguez, J., Smedh, M., Elsner, M., Laporte, F., Barcena, M., Jansen, K. A., Valentijn, J. A., Koster, A. J., et al. (2009). Early stages of Golgi vesicle and tubule formation require diacylglycerol. *Mol. Biol. Cell* **20**, 780-790.

Balla, A., Kim, Y. J., Varnai, P., Szentpetery, Z., Knight, Z., Shokat, K. M. and Balla, T. (2008). Maintenance of hormone-sensitive phosphoinositide pools in the plasma membrane requires phosphatidylinositol 4-kinase III{alpha}. *Mol. Biol. Cell* **19**, 711-721.

Ben Tekaya, H., Miura, K., Pepperkok, R. and Hauri, H. P. (2005). Live imaging of bidirectional traffic from the ERGIC. *J. Cell Sci.* **118**, 357-367.

Bigay, J., Casella, J. F., Drin, G., Mesmin, B. and Antonny, B. (2005). ArfGAP1 responds to membrane curvature through the folding of a lipid packing sensor motif. *EMBO J.* **24**, 2244-2253.

Bigay, J., Gounon, P., Robineau, S. and Antonny, B. (2003). Lipid packing sensed by ArfGAP1 couples COPI coat disassembly to membrane bilayer curvature. *Nature* **426**, 563-566.

Cockcroft, S. (2007). Trafficking of phosphatidylinositol by phosphatidylinositol transfer proteins. *Biochem. Soc. Symp.* **74**, 259-271.

Cockcroft, S. and Carvou, N. (2007). Biochemical and biological functions of class I phosphatidylinositol transfer proteins. *Biochim. Biophys. Acta* **1771**, 677-691.

Cole, N. B., Ellenberg, J., Song, J., DiEuliis, D. and Lippincott-Schwartz, J. (1998). Retrograde transport of Golgi-localized proteins to the ER. *J. Cell Biol.* **140**, 1-15.

Cosker, K. E., Shadan, S., van Diepen, M., Morgan, C., Li, M., Allen-Baume, V., Hobbs, C., Doherty, P., Cockcroft, S. and Eickholt, B. J. (2008). Regulation of PI3K signalling by the phosphatidylinositol transfer protein PITP{alpha} during axonal extension in hippocampal neurons. *J. Cell Sci.* **121**, 796-803.

Cunningham, E., Thomas, G. M. H., Ball, A., Hiles, I. and Cockcroft, S. (1995). Phosphatidylinositol transfer protein dictates the rate of inositol trisphosphate production by promoting the synthesis of PIP_2. *Curr. Biol.* **5**, 775-783.

D'Angelo, G., Polishchuk, E., Di Tullio, G., Santoro, M., Di Campli, A., Godi, A., West, G., Bielawski, J., Chuang, C. C., van der Spoel, A. C. et al. (2007). Glycosphingolipid synthesis requires FAPP2 transfer of glucosylceramide. *Nature* **449**, 62-67.

Dawe, H. R., Adams, M., Wheway, G., Szymanska, K., Logan, C. V., Noegel, A. A., Gull, K. and Johnson, C. A. (2009). Nesprin-2 interacts with meckelin and mediates ciliogenesis via remodelling of the actin cytoskeleton. *J. Cell Sci.* **122**, 2716-2726.

Dubois, T., Paleotti, O., Mironov, A. A., Fraisier, V., Stradal, T. E., De Matteis, M. A., Franco, M. and Chavrier, P. (2005). Golgi-localized GAP for Cdc42 functions downstream of ARF1 to control Arp2/3 complex and F-actin dynamics. *Nat. Cell Biol.* **7**, 353-364.

Egea, G., Lazaro-Dieguez, F. and Vilella, M. (2006). Actin dynamics at the Golgi complex in mammalian cells. *Curr. Opin. Cell Biol.* **18**, 168-178.

Fernandez-Ulibarri, I., Vilella, M., Lazaro-Dieguez, F., Sarri, E., Martinez, S. E., Jimenez, N., Claro, E., Merida, I., Burger, K. N. and Egea, G. (2007). Diacylglycerol is required for the formation of COPI vesicles in the Golgi-to-ER transport pathway. *Mol. Biol. Cell* **18**, 3250-3263.

Fucini, R. V., Navarrete, A., Vadakkan, C., Lacomis, L., Erdjument-Bromage, H., Tempst, P. and Stamnes, M. (2000). Activated ADP-ribosylation factor assembles distinct pools of actin on golgi membranes. *J. Biol. Chem.* **275**, 18824-18829.

Godi, A., Santone, I., Pertile, P., Devarajan, P., Stabach, P. R., Morrow, J. S., Di Tullio, G., Polishchuk, R., Petrucci, T. C., Luini, A. et al. (1998). ADP ribosylation factor regulates spectrin binding to the Golgi complex. *Proc. Natl. Acad. Sci. USA* **95**, 8607-8612.

Godi, A., Pertile, P., Di Tullio, G., Iurisco, C., Luini, A., Corda, D. and De Matteis, M. A. (1999). ARF recruits PI 4-kinase type β and a PIP 5-kinase and stimulates PIP_2 synthesis at the Golgi. *Nature Cell Biol.* **1**, 280-287.

Gough, L. L. and Beck, K. A. (2004). The spectrin family member Syne-1 functions in retrograde transport from Golgi to ER. *Biochim. Biophys. Acta* **1693**, 29-36.

Gough, L. L., Fan, J., Chu, S., Winnick, S. and Beck, K. A. (2003). Golgi localization of Syne-1. *Mol. Biol. Cell* **14**, 2410-2424.

Hamilton, B. A., Smith, D. J., Mueller, K. L., Kerrebrock, A. W., Bronson, R. T., Berkel, V. v., Daly, M. J., Kroglyak, L., Reeve, M. P., Nernhauser, J. L., et al. (1997). The *vibrator* mutation causes neurogeneration via reduced expression of PITPα: Positional complementation cloning and extragenic suppression. *Neuron* **18**, 711-722.

Hanada, K., Kumagai, K., Yasuda, S., Miura, Y., Kawano, M., Fukasawa, M. and Nishijima, M. (2003). Molecular machinery for non-vesicular trafficking of ceramide. *Nature* **426**, 803-809.

Heuvingh, J., Franco, M., Chavrier, P. and Sykes, C. (2007). ARF1-mediated actin polymerization produces movement of artificial vesicles. *Proc. Natl. Acad. Sci. USA* **104**, 16928-16933.

Howe, A. G. and McMaster, C. R. (2006). Regulation of phosphatidylcholine homeostasis by Sec14. *Can. J. Physiol. Pharmacol.* **84**, 29-38.

Jones, D. H., Morris, J. B., Morgan, C. P., Kondo, H., Irvine, R. F. and Cockcroft, S. (2000). Type I PIP 5-kinase directly interacts with ARF1 and is responsible for $PI(4,5)P_2$ synthesis in the Golgi compartment. *J. Biol. Chem.* **275**, 13962-13966.

Jones, S. M., Alb, J. G., Jr, Phillips, S. E., Bankaitis, V. A. and Howell, K. E. (1998). A phosphatidylinositol 3-kinase and phosphatidylinositol transfer protein act synergistically in formation of constitutive transport vesicles from the trans-golgi network. *J. Biol. Chem.* **273**, 10349-10354.

Klumperman, J., Schweizer, A., Clausen, H., Tang, B. L., Hong, W., Oorschot, V. and Hauri, H. P. (1998). The recycling pathway of protein ERGIC-53 and dynamics of the ER-Golgi intermediate compartment. *J. Cell Sci.* **111**, 3411-3425.

Lazaro-Dieguez, F., Jimenez, N., Barth, H., Koster, A. J., Renau-Piqueras, J., Llopis, J. L., Burger, K. N. and Egea, G. (2006). Actin filaments are involved in the maintenance of Golgi cisternae morphology and intra-Golgi pH. *Cell Motil. Cytoskel.* **63**, 778-791.

Lee, T. H. and Linstedt, A. D. (2000). Potential role for protein kinases in regulation of bidirectional endoplasmic reticulum-to-Golgi transport revealed by protein kinase inhibitor H89. *Mol. Biol. Cell* **11**, 2577-2590.

Letourneur, F., Gaynor, E. C., Hennecke, S., Demolliere, C., Duden, R., Emr, S. D., Riezman, H. and Cosson, P. (1994). Coatomer is essential for retrieval of dilysine-tagged proteins to the endoplasmic reticulum. *Cell* **79**, 1199-1207.

Levine, T. P. and Munro, S. (2002). Targeting of Golgi-specific pleckstrin homology domains involves both PtdIns 4-kinase-dependent and -independent components. *Curr. Biol.* **12**, 695-704.

Litvak, V., Dahan, N., Ramachandran, S., Sabanay, H. and Lev, S. (2005). Maintenance of the diacylglycerol level in the Golgi apparatus by the Nir2 protein is critical for Golgi secretory function. *Nat. Cell Biol.* **7**, 225-234.

Luke, Y., Zaim, H., Karakesisoglou, I., Jaeger, V. M., Sellin, L., Lu, W., Schneider, M., Neumann, S., Beijer, A., Munck, M., et al. (2008). Nesprin-2 Giant (NUANCE) maintains nuclear envelope architecture and composition in skin. *J. Cell Sci.* **121**, 1887-1898.

Luna, A., Matas, O. B., Martinez-Menarguez, J. A., Mato, E., Duran, J. M., Ballesta, J., Way, M. and Egea, G. (2002). Regulation of protein transport from the Golgi complex to the endoplasmic reticulum by CDC42 and N-WASP. *Mol. Biol. Cell* **13**, 866-879.

Mayinger, P. (2009). Regulation of Golgi function via phosphoinositide lipids. *Semin. Cell Dev. Biol.* **20**, 793-800.

Morgan, C. P., Allen-Baume, V., Radulovic, M., Li, M., Skippen, A. J. and Cockcroft, S. (2006). Differential expression of a C-terminal splice variant of PITPβ lacking the constitutive-phosphorylated Ser262 that localises to the Golgi Compartment. *Biochem. J.* **398**, 411-421.

Ohashi, M., Jan de Vries, K., Frank, R., Snoek, G., Bankaitis, V., Wirtz, K. and Huttner, W. B. (1995). A role for phosphatidylinositol transfer protein in secretory vesicle formation. *Nature* **377**, 544-547.

Paul, K. S., Bogan, A. A. and Waters, M. G. (1998). Phosphatidylinositol transfer protein (PITPα) stimulates *in vitro* intra-golgi transport. *FEBS Lett.* **431**, 91-96.

Phillips, S. E., Ile, K. E., Boukhelifa, M., Huijbregts, R. P. and Bankaitis, V. A. (2006a). Specific and nonspecific membrane-binding determinants cooperate in targeting phosphatidylinositol transfer protein beta-isoform to the mammalian trans-Golgi network. *Mol. Biol. Cell* **17**, 2498-2512.

Phillips, S. E., Vincent, P., Rizzieri, K. E., Schaaf, G., Bankaitis, V. A. and Gaucher, E. A. (2006b). The diverse biological functions of phosphatidylinositol transfer proteins in eukaryotes. *Crit. Rev. Biochem. Mol. Biol.* **41**, 21-49.

Sciaky, N., Presley, J., Smith, C., Zaal, K. J., Cole, N., Moreira, J. E., Terasaki, M., Siggia, E. and Lippincott-Schwartz, J. (1997). Golgi tubule traffic and the effects of brefeldin A visualized in living cells. *J. Cell Biol.* **139**, 1137-1155.

Shadan, S., Holic, R., Carvou, N., Ee, P., Li, M., Murray-Rust, J. and Cockcroft, S. (2008). Dynamics of lipid transfer by phosphatidylinositol transfer proteins in cells. *Traffic* **9**, 1743-1756.

Simon, J.-P., Morimoto, T., Bankaitis, V. A., Gottlieb, T. A., Ivanov, I. E., Adesnik, M. and Sabatini, D. D. (1998). An essential role for the phosphatidylinositol transfer protein in the scission of coatomer-coated vesicles from the trans-Golgi network. *Proc. Natl. Acad. Sci. USA* **95**, 11181-11186.

Skippen, A., Jones, D. H., Morgan, C. P., Li, M. and Cockcroft, S. (2002). Mechanism of ADP-ribosylation factor-stimulated phosphatidylinositol 4,5-bisphosphate synthesis in HL60 cells. *J. Biol. Chem.* **277**, 5823-5831.

Thomas, G. M. H., Cunningham, E., Fensome, A., Ball, A., Totty, N. F., Troung, O., Hsuan, J. J. and Cockcroft, S. (1993). An essential role for phosphatidylinositol transfer protein in phospholipase C-mediated inositol lipid signalling. *Cell* **74**, 919-928.

Tilley, S. J., Skippen, A., Murray-Rust, J., Swigart, P., Stewart, A., Morgan, C. P., Cockcroft, S. and McDonald, N. Q. (2004). Structure-function analysis of human phosphatidylinositol transfer protein alpha bound to phosphatidylinositol. *Structure* **12**, 317-326.

Tomas, A., Futter, C. and Moss, S. E. (2004). Annexin 11 is required for midbody formation and completion of the terminal phase of cytokinesis. *J. Cell Biol.* **165**, 813-822.

Valderrama, F., Babia, T., Ayala, I., Kok, J. W., Renau-Piqueras, J. and Egea, G. (1998). Actin microfilaments are essential for the cytological positioning and morphology of the Golgi complex. *Eur. J. Cell Biol.* **76**, 9-17.

Valderrama, F., Duran, J. M., Babia, T., Barth, H., Renau-Piqueras, J. and Egea, G. (2001). Actin microfilaments facilitate the retrograde transport from the Golgi complex to the endoplasmic reticulum in mammalian cells. *Traffic.* **2**, 717-726.

Vordtriede, P. B., Doan, C. N., Tremblay, J. M., Helmkamp, Jr, G. M. and Yoder, M. D. (2005). Structure of PITPβ in complex with phosphatidylcholine: Comparison of structure and lipid transfer to other PITP isoforms. *Biochemistry* **44**, 14760-14771.

Warren, D. T., Zhang, Q., Weissberg, P. L. and Shanahan, C. M. (2005). Nesprins: intracellular scaffolds that maintain cell architecture and coordinate cell function? *Expert. Rev. Mol. Med.* **7**, 1-15.

Weixel, K. M., Blumental-Perry, A., Watkins, S. C., Aridor, M. and Weisz, O. A. (2005). Distinct Golgi populations of phosphatidylinositol 4-phosphate regulated by phosphatidylinositol 4-kinases. *J. Biol. Chem.* **280**, 10501-10508.

Williams, C., Choudhury, R., McKenzie, E. and Lowe, M. (2007). Targeting of the type II inositol polyphosphate 5-phosphatase INPP5B to the early secretory pathway. *J. Cell Sci.* **120**, 3941-3951.

Wong, K., Meyers, R. and Cantley, L. C. (1997). Subcellular locations of phosphatidylinositol 4-kinase isoforms. *J. Biol. Chem.* **272**, 13236-13241.

Xie, Y., Ding, Y.-Q., Hong, Y., Feng, Z., Navarre, S., Xi, C.-X., Wang, C.-L., Zhu, X.-J., Ackerman. S. L., Kozlowski, D. et al. (2005). Role of phosphatidylinositol transfer protein α in netrin-1-induced PLC signalling and neurite outgrowth. *Nat. Cell Biol.* **7**, 1124-1132.

Yang, J. S., Gad, H., Lee, S. Y., Mironov, A., Zhang, L., Beznoussenko, G. V., Valente, C., Turacchio, G., Bonsra, A. N., Du, G. et al. (2008). A role for phosphatidic acid in COPI vesicle fission yields insights into Golgi maintenance. *Nat. Cell Biol.* **10**, 1146-1153.

Zhang, C.-J., Rosenwald, A. G., Willingham, M. C., Skuntz, S., Clark, J. and Kahn, R. A. (1994). Expression of a dominant allele of human ARF1 inhibits membrane traffic in vivo. *J. Cell Biol.* **124**, 289-300.

Journal of Cell Science