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Phosphatidylinositol 4 Phosphate Regulates Targeting of Clathrin Adaptor AP-1 Complexes to the Golgi

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Summary

Phosphatidylinositol 4 phosphate [PI(4)P] is essential for secretion in yeast, but its role in mammalian cells is unclear. Current paradigms propose that PI(4)P acts primarily as a precursor to phosphatidylinositol 4,5 bisphosphate (PIP2), an important plasma membrane regulator. We found that PI(4)P is enriched in the mammalian Golgi, and used RNA interference (RNAi) of PI4KIIα, a Golgi resident phosphatidylinositol 4 kinase, to determine whether PI(4)P directly regulates the Golgi. PI4KIIα RNAi decreases Golgi PI(4)P, blocks the recruitment of clathrin adaptor AP-1 complexes to the Golgi, and inhibits AP-1-dependent functions. This AP-1 binding defect is rescued by adding back PI(4)P. In addition, purified AP-1 binds PI(4)P, and anti-PI(4)P inhibits the in vitro recruitment of cytosolic AP-1 to normal cellular membranes. We propose that PI4KIIα establishes the Golgi's unique lipid-defined organelle identity by generating PI(4)P-rich domains that specify the docking of the AP-1 coat machinery.

Introduction

Phosphoinositides (PPIs) have been implicated in many membrane trafficking events. Phosphatidylinositol 4,5 bisphosphate [PI(4,5)P2 or PIP2] is now firmly established as an essential regulator of plasma membrane trafficking (Cremona and De Camilli, 2001), and is increasingly implicated in trafficking of other organelle membranes (Brown et al., 2001; Rozelle et al., 2000), including the Golgi (De Matteis et al., 2002; Cockcroft and De Matteis, 2001). Phosphatidylinositol 4 phosphate [PI(4)P] is the immediate precursor of PIP2, and it was until recently overshadowed by PIP2. However, PI(4)P is beginning to emerge as a Golgi regulator in its own right, since it was discovered that *S. cerevisiae* uses PI(4)P, but not PIP2, to regulate constitutive secretion from the late Golgi (Walch-Solimena and Novick, 1999; Hama et al., 1999; Audhya et al., 2000).

The yeast Golgi PI(4)P is generated by a Golgi-associated type III phosphatidylinositol 4 kinase (PI4K) called Pik1p (Walch-Solimena and Novick, 1999). The other major type III PI4K, Stt4p, has no known role in secretion (Audhya et al., 2000). Yeast also has a type II PI4K, called LSB6, which does not appear to have an essential role (Shelton et al., 2003; Han et al., 2002).

Mammals have the same two type III PI4K orthologs as yeast (called PI4KIIIα and PI4KIIIβ or PI4Kβ) and two type II PI4Ks called PI4KIIα and β (Minogue et al., 2001; Barylko et al., 2001; Wei et al., 2002; Balla et al., 2002). At least three of these kinases have been definitively localized to the Golgi (Wong et al., 1997; Godi et al., 1999; Wei et al., 2002), but little is known about their relative contributions. For example, biochemical studies showed that Golgi-enriched membranes synthesize PI(4)P constitutively, and Arf1 increases PI(4)P synthesis by recruiting the cytosolic PI4KIIIβ (Godi et al., 1999; Skippen et al., 2002). However, the identity of the "resident" Golgi PI4K is not known. Since Arf1 also recruits phosphatidylinositol 4 phosphate 5 kinases (PIP5Ks) to Golgi-enriched membranes (Godi et al., 1999; Skippen et al., 2002), PIP2 can potentially be an Arf1 effector for multiple Golgi functions (De Matteis et al., 2002). For example, it has been proposed that PIP2 may be involved in the recruitment of the AP-1 adaptor coat protein complexes to the trans Golgi network (TGN) (Crottet et al., 2002).

In this paradigm, PI(4)P is relegated to a secondary role as a substrate for Golgi PIP2 synthesis. Paradoxically, in spite of the overwhelming evidence for PIP2 regulation of plasma membrane trafficking, a direct role of PIP2 in Golgi membrane trafficking has not been established. Strikingly, all currently available evidence suggests that the Golgi, unlike the plasma membrane, has remarkably little PIP2 (Watt et al., 2002).

The low abundance of PIP2 argues against a pluripotent role in the Golgi, especially in the stoichiometric recruitment of abundant peripheral proteins. PI(4)P, however, could potentially fulfill this role because it may be more abundant than PIP2 in the Golgi, and several Golgi-associated proteins that bind PI(4)P have recently been identified. These include epsinR, an AP-1 accessory protein (Hirst et al., 2003; Mills et al., 2003), and oxysterol binding protein (OSBP) (Levine and Munro, 2002).

In this study, we show that the Golgi AP-1 adaptor complex directly binds PI(4)P. Furthermore, PI(4)P, but not PIP2, is required for AP-1 recruitment to the Golgi, and this PI(4)P is generated primarily by the Golgi resident PI4KIIα. Besides exerting a direct role in AP-1 recruitment through PI(4)P, PI4KIIα also supports the export of secretory proteins from the TGN in a PIP2-dependent manner. Our results suggest that the mammalian Golgi uses a more expanded repertoire of phosphoinositides than the yeast Golgi (Walch-Solimena and Novick, 1999; Hama et al., 1999; Audhya et al., 2000).

Results

Effects of PI4KIIα Overexpression

PI4KIIα behaves as an integral membrane protein (Barylko et al., 2001) that is predominantly Golgi associated

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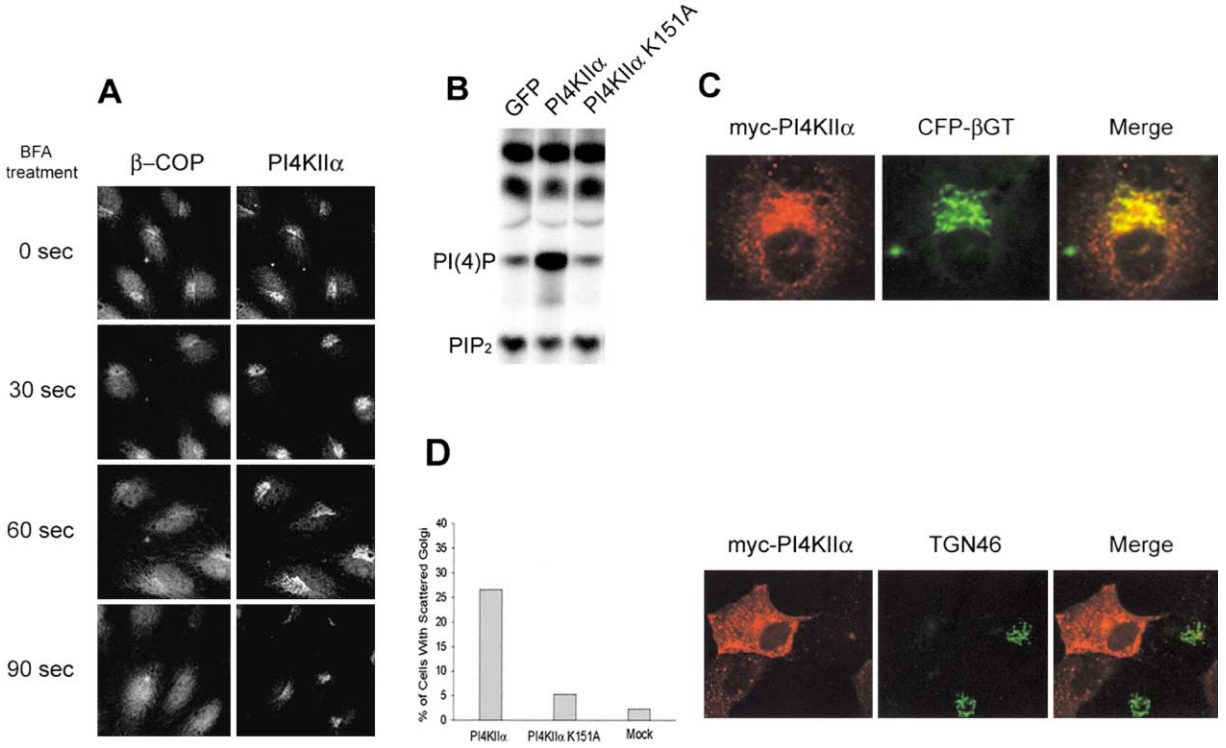

Figure 1. PI4KIIα Golgi Association and Effects of PI4KIIα Overexpression

(A) Brief BFA treatment had no effect on PI4KIIα Golgi association. CV1 cells were incubated with BFA (5 μg/ml) at 37°C for the intervals indicated. They were fixed, permeabilized, and double stained with anti-β-COP and anti-PI4KIIα.

(B) PI4KIIα overexpression increased PI(4)P synthesis. COS7 cells were infected with adenovirus expressing GFP, wild-type PI4KIIα, or kinase-dead PI4KIIαK151A. Cells were labeled with ^{32}P for 4 hr, and lipids were extracted and analyzed by TLC. The identity of PI(4)P and PIP2 was established by comigration with bona fide lipid standards.

(C) Colocalization of myc-PI4KIIα with CFP-βGT. CV1 cells were cotransfected with myc-PI4KIIα and CFP-βGT cDNA, and processed for immunofluorescence 12 hr after the start of the transfection.

(D) Effects of myc-PI4KIIα overexpression on Golgi morphology. CV1 cells transfected with wild-type or kinase dead (K151A) myc-PI4KIIα were fixed 18 hr after the start of transfection and stained with anti-myc and anti-TGN46. The two cells overexpressing wild-type myc-PI4KIIα shown in the field have scattered Golgi. Transfected cells with scattered Golgi were scored. More than 100 cells were counted per condition, and the result was representative of three similar experiments.

(Wei et al., 2002). PI4KIIα association with the Golgi was unaffected by brief brefeldin A (BFA) treatment (Figure 1A), establishing it as an Arf1-independent Golgi resident protein. The resistance to BFA distinguishes PI4KIIα from the peripherally associated PI4KIIβ (Wei et al., 2002) and PI4KIIIβ (Godi et al., 1999), which are readily dissociated from the Golgi by BFA.

Overexpressing PI4KIIα increased ^{32}P incorporation into PI(4)P (by 8.4-fold in the experiment shown in Figure 1B) but did not increase PIP2 synthesis. Thus, the supply of PI(4)P is not rate limiting for overall PIP2 synthesis under these conditions. PI4KIIα containing a mutation in its putative ATP binding site K151A (Barylko et al., 2002) had minimal effect on ^{32}P-PI(4)P or PIP2 accumulation at the whole cell level, confirming that it is kinase dead. However, it does not act as a dominant negative inhibitor.

At low-level overexpression, PI4KIIα was found predominantly in the Golgi and colocalized with cyan fluorescent protein that was targeted to the Golgi via the β-1,4 galactosyltransferase (CFP-βGT) targeting motif (Figure 1C). In addition, some PI4KIIα was found in vesicular structures surrounding the nucleus and Golgi. This is more obvious at high-level overexpression (Figure 1D). The vesicles have a range of sizes (data not shown), and are positive for many organelle markers, including LAMP-1 (lysosomes), EEA1 (early endosomes), or LABP (late endosomes) (Kobayashi et al., 1998) (data not shown). This pattern of staining suggests that PI4KIIα association with the Golgi may be saturable, and PI4KIIα spills over to a variety of intracellular vesicles after forced overexpression.

Some cells with high PI4KIIα overexpression had scattered or no perinuclear Golgi at all (Figure 1D). Direct counting showed that 27% of cells overexpressing wild-type PI4KIIα had no perinuclear Golgi, compared with 2% and 5% in control and PI4KIIαK151A transfected cells, respectively (Figure 1D). Since the actin cytoskeleton and microtubules were not obviously affected (data not shown), Golgi disruption was not simply due to cytoskeletal perturbations. A more likely possibility is that too much PI(4)P scatters the Golgi, perhaps by upsetting the balance in membrane trafficking that normally maintains Golgi integrity.

siRNA Knocks Down PI4KIIα Expression

We used RNA interference (RNAi) (Elbashir et al., 2001) to examine PI4KIIα's role in the mammalian Golgi. West-

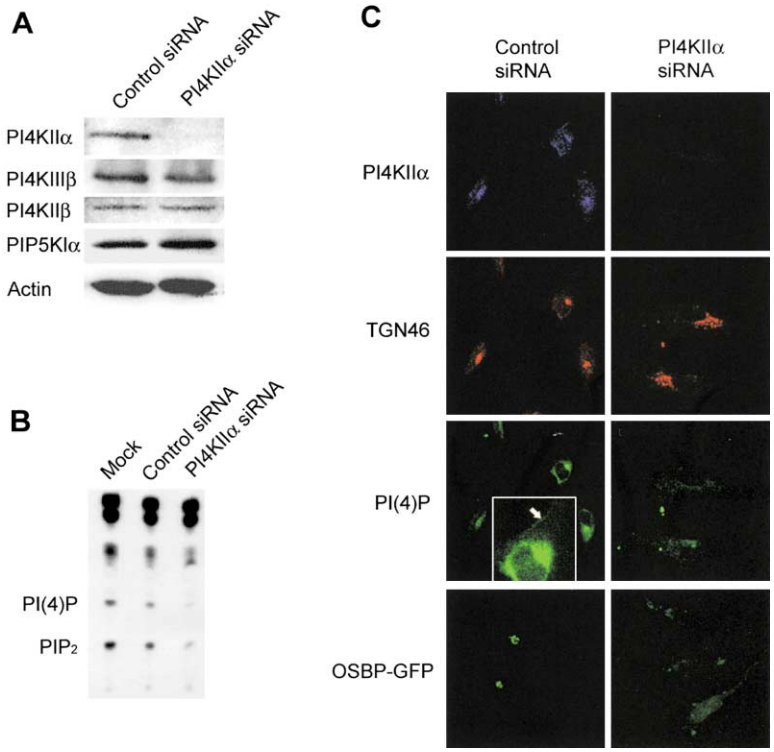

Figure 2. Effects of PI4KIIα RNAi on Golgi Morphology and Phosphoinositide Content

HeLa cells were harvested or fixed 72 hr after the start of siRNA transfection.

(A) Western blotting of PI4KIIα, other phosphoinositide kinases, and actin. 10 μg of total cell proteins was loaded per lane.

(B) ^{32}P incorporation into lipids, analyzed by TLC and autoradiography. Mock refers to treatment with oligofectAMINE in the absence of added siRNA. Data is representative of more than 3 independent experiments.

(C) Immunolocalization of PI(4)P. Cells were fixed with formaldehyde and cracked open by freeze/thawing. Top three rows, control and PI4KIIα RNAi cells were triple stained. Inset shows an enlarged image of a control cell which was image-enhanced to highlight the small amount of anti-PI(4)P staining at the plasma membrane (indicated by arrow). Bottom row shows control and PI4KIIα RNAi cells that were transfected with OSBP-PH-GFP cDNA 18 hr prior to fixation.

ern blotting showed that PI4KIIα protein expression was reduced to 76% of control level at 72 hr after exposure to siRNA (small interfering RNA) (Figure 2A). The decrease was specific for PI4KIIα; PI4KIIIβ, PI4KIIβ, human type I phosphatidylinositol phosphate 5 kinase α (PIP5KIα), and actin levels were not significantly changed.

Immunofluorescence staining confirmed that approximately 80% of PI4KIIα siRNA-treated cells had no or reduced PI4KIIα staining (Figure 2C). Nevertheless, most of these cells had perinuclear TGN46 staining, at intensities that were comparable to that of control cells (Table 1). Therefore, PI4KIIα RNAi did not disrupt the Golgi under these conditions. The TGN46 staining pattern was however altered in appearance. Its mean area increased by 3.1-fold (n = 10) and staining was more punctate and/or tubular compared with control cells. The majority of cells with decreased PI4KIIα expression exhibited the expanded Golgi phenotype.

PI4KIIα RNAi Inhibits Phosphoinositide Synthesis and Selectively Decreases Golgi PI(4)P

PI4KIIα siRNA profoundly inhibited ^{32}P incorporation into PI(4)P and PIP2 (to 48.9 ± 6.3% and 58.9 ± 14.4% of control, respectively [n = 5]) (Figure 2B). PI4KIIα is therefore a major PI4K and a significant contributor to overall PI(4)P and PIP2 synthesis.

We used anti-PI(4)P and a GFP-PH reporter to visualize PI(4)P pools in cells. We avoided using detergents in order to maximize the preservation of membrane lipids. Cells were fixed with formaldehyde and then subjected to one controlled freeze-thaw cycle in the presence of 1 M sucrose (Tran et al., 1999). In control cells, PI(4)P immunofluorescence was enriched in the Golgi and in a band of cytoplasm surrounding the nucleus (Figure 2C). The Golgi PI(4)P fluorescence accounts for 46.5% of total PI(4)P (Table 1). Surprisingly, the plasma membrane had very little anti-PI(4)P staining (Figure 2C). PI4KIIα

Table 1. Effect of PI4KIIα RNAi and Shuttle PPIs on Intact Cells

	% Intensity in Golgi[1]		Golgi γ-adaptin	
	PI(4)P	TGN46	Intensity (A.U.)[2]	% cells with Golgi staining[3]
Control	46.5 ± 1.7	82.1 ± 1.2	12.1 ± 1.4	89
PI4KIIα RNAi				
Carrier[4]	16.7 ± 1.4	76.6 ± 1.6	3.6 ± 0.5	29
Carrier + PI(4)P	ND	85.9 ± 1.8	7.0 ± 0.5	79
Carrier + PIP2	ND	92.6 ± 0.8	4.2 ± 0.9	6

[1] Values are mean ± SEM of 8–10 randomly chosen cells per condition.
[2] Values are mean ± SEM of 8–10 cells with Golgi γ-adaptin staining.
[3] Approximately 200 randomly chosen cells were scored per condition.
[4] Carrier is polyamine shuttling reagent.
A.U., arbitrary units.

RNAi dramatically reduced the intensity of Golgi PI(4)P fluorescence (Figure 2C), which now accounts for only 16.7% of total PI(4)P, instead of 46.5% in control cells (Table 1). Based on these values, we estimate that roughly 36% (16.7 divided by 46.5) of PI(4)P remains in the Golgi after RNAi. This is likely to underestimate, because we did not take into account the PI(4)P decrease in other regions of the cell.

The decrease in Golgi PI(4)P was confirmed by using OSBP-PH as a PI(4)P reporter (Levine and Munro, 2002) (Figure 2C). Although GFP-OSBP-PH was expressed in PI4KIIα RNAi cells, it was no longer concentrated in the perinuclear Golgi.

PI4KIIα RNAi Blocks AP-1 Association with the TGN

PI4KIIα RNAi dramatically decreased the association of γ-adaptin (a subunit of the AP-1 complex) with the Golgi, both in terms of the percentage of cells with strong perinuclear γ-adaptin (89% versus 29%) and their intensity (12.1 A.U. to 3.6 A.U.) (Figure 3A, Table 1). In contrast, β-COP (a component of the COP1 coatomer complex) remained on the Golgi, although it was also expanded because the Golgi is enlarged (Figure 3B).

The Golgi has two other PI4Ks. We therefore used RNAi to determine if they are also required for AP-1 recruitment. PI4KIIβ RNAi had no obvious effect on either γ-adaptin or TGN46 perinuclear staining (Figure 3A). PI4KIIIβ decreased Golgi γ-adaptin and TGN46 intensities in parallel in most cells, suggesting that the decrease may be secondary to the loss of Golgi membranes. Additional studies will be required to determine if PI4KIIIβ RNAi disrupts the Golgi. From the results presented here, it is clear that PI4KIIα RNAi uniquely blocks AP-1 association with the Golgi.

AP-1 regulates clathrin-coated vesicle (CCV) trafficking between the TGN and endosome/lysosme system (Bonifacino and Lippincott-Schwartz, 2003). As expected from the profound loss of AP-1 in PI4KIIα RNAi cells, Golgi clathrin is also greatly reduced (Figure 3B). In addition, mannose 6 phosphate receptors (MPR300) are scattered throughout the cytoplasm, instead of clustering normally around the TGN (Figure 3B). This recapitulates the MPR mislocalization phenotype observed in $\mu 1^{-/-}$ fibroblasts that do not have functional AP-1 complexes (Meyer et al., 2000, 2001).

AP-1 Directly Binds PI(4)P

PI4KIIα synthesizes PI(4)P, and PI(4)P is the immediate precursor of PIP2. Therefore, the decrease in Golgi AP-1 after PI4KIIα RNAi is likely to be a direct consequence of the loss of either Golgi PI(4)P or PIP2. We used a number of approaches to determine whether PI(4)P or PIP2 is the regulator of AP-1 Golgi recruitment.

As a first step, we examined the possibility that AP-1 directly binds phosphoinositides. Using a solid phase lipid binding assay, we found that purified AP-1 binds PI(4)P slightly better than PI(5)P, and significantly less well to PI(3,5)P2. Since there is much less PI(5)P than PI(4)P in cells (49 times less according to one estimate [Rameh et al., 1997]), and PI(4)P is most concentrated in the Golgi (Figure 2C), PI(4)P is likely to be the primary lipid for AP-1 recruitment.

In addition, AP-1 bound phosphatidic acid (PA) (Figure 4A). We do not know if this interaction is physiologically relevant, because AP-1 did not bind PA coupled beads (Krugmann et al., 2002).

Most significantly, AP-1 did not bind PI(4,5)P2 and PI(3,4,5)P3 (Figure 4), establishing that it has a different lipid specificity than the plasma membrane-associated AP-2 adaptor protein (Gaidarov and Keen, 1999; Rhode et al., 2002). AP-1's preference for less highly charged phosphoinositides (such as PI4P instead of PIP2) was in fact predicted based on the fact that AP-1's putative phosphoinositide binding subunits (γ and μ1) lack a few of the basic residues that contact PIP2 in AP-2 (Collins et al., 2002).

AP-1 Association with the Golgi Is Regulated by PI(4)P

Having demonstrated that AP-1 has the potential to bind PI(4)P, we wanted to determine if PI(4)P is required for AP-1 recruitment to the Golgi. Three approaches were used. First, we attempted an in vivo PI4KIIα RNAi rescue experiment. PI(4)P or PIP2 was shuttled into live cells with membrane permeant polyamine carriers (Ozaki et al., 2000). After incubation with shuttle PI(4)P for 30 min at 37°C, at least 79% of PI4KIIα RNAi cells had perinuclear γ-adaptin staining compared with 29% of cells incubated with carrier alone (Figure 5A, Table 1). The average perinuclear γ-adaptin intensity increased from 3.6 A.U. to 7.0 A.U., which is 29.8% and 57.9%, respectively, of control cells without PI4KIIα RNAi (Table 1). Carrier alone or lipid without carrier had no effect, establishing that PI(4)P rescued AP-1 recruitment after it entered cells.

The brief diC16-PI(4)P treatment did not reverse the expanded Golgi morphology (Figure 5A), perhaps because Golgi reorganization requires more time than simple restoration of AP-1 binding. In contrast, shuttled PIP2 did not rescue AP-1 binding (Figure 5A and Table 1). In fact, it consistently decreased the percent of cells with Golgi AP-1 (Table 1). The basis for this was not explored further, but it is clear that PIP2 does not promote AP-1 recruitment.

In a second series of experiments, we studied AP-1 recruitment in semi-intact cells. As expected, cytosolic γ-adaptin was recruited to the Golgi of control cells, resulting in a 2.5-fold increase in γ-adaptin intensity (Figure 5B, Table 2). In contrast, the Golgi of PI4KIIα RNAi cells, which had less Golgi γ-adaptin staining initially, were still not able to recruit AP-1 from the normal control cytosol (Table 2). These results showed that the primary defect is inherent in the PI4KIIα RNAi Golgi and not due to depletion of a cytosolic recruiting factor by RNAi. This defect was overcome by adding exogenous PI(4)P, resulting in a 2.3-fold increase in bound γ-adaptin (Figure 5B, Table 2). PIP2 again had no effect. Therefore, defective AP-1 binding is due to a lack of Golgi PI(4)P but not PIP2.

In the third series of experiments, we examined the impact of reducing PI(4)P accessibility on AP-1 recruitment to normal membranes. Anti-PI(4)P pretreatment of microsome membranes blocked AP-1 binding, while anti-PIP2 and anti-talin (used as a control here) did not (Figure 5C). Thus, anti-PI(4)P selectively inhibits AP-1

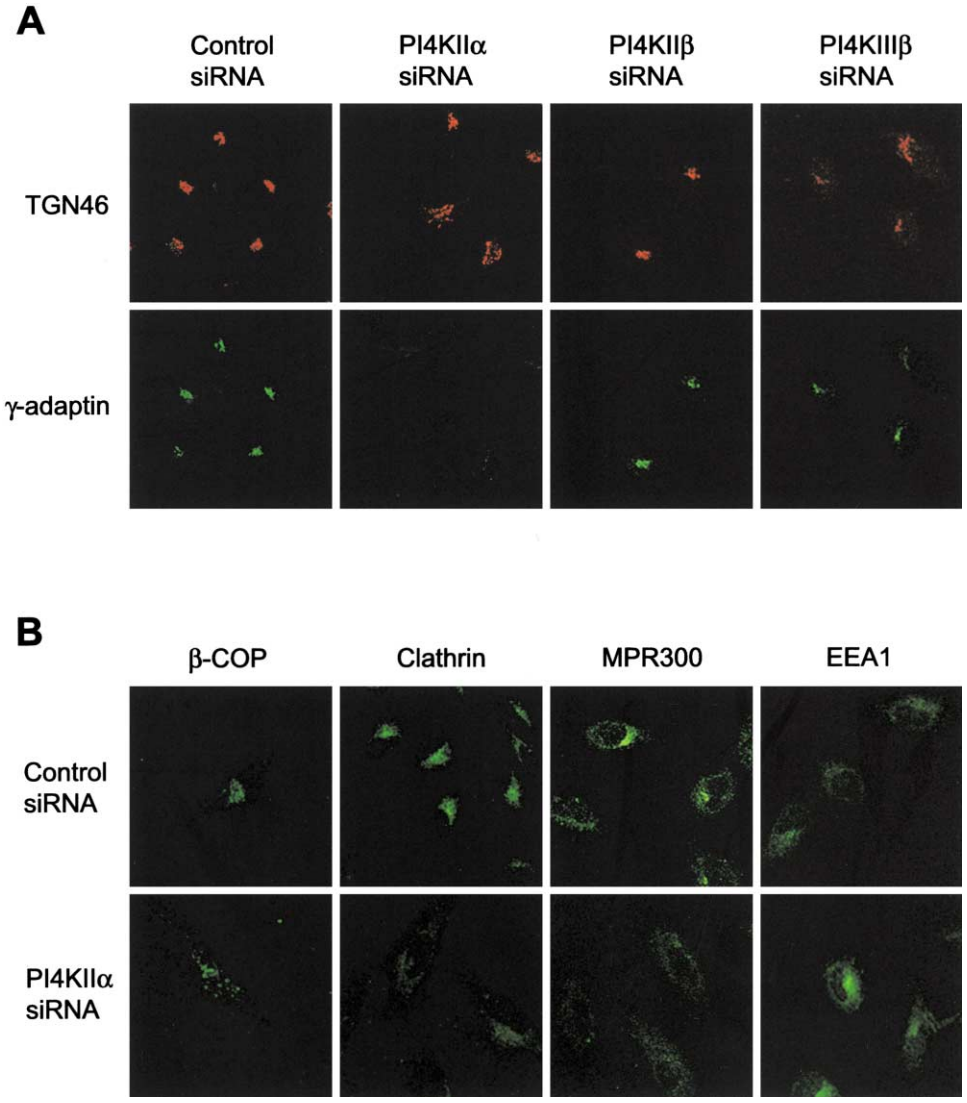

Figure 3. Effects of PI4KIIα RNAi on AP-1 Recruitment

(A) Comparing the effects of knocking down each of the three Golgi-associated PI4Ks. Cells were triple labeled, and TGN46 and γ-adaptin images in the same field are shown. Isoform specific anti-PI4K antibodies confirmed that there was knockdown of each kinase.

(B) Control and PI4KIIα RNAi cells were stained with the antibodies to the proteins indicated. Although not shown, PI4KIIα knockdown and Golgi localization of these markers were confirmed by anti-PI4KIIα and anti-TGN46 staining in all cases. Images were collected at optical sections at the middle of the cell.

recruitment, establishing that PI(4)P is critically important and that it acts independently of PIP2.

PI4KIIα Regulates Late Secretory Functions in a PIP2-Dependent Manner

Since PI4KIIα RNAi has such a dramatic effect on AP-1 coat recruitment, we wanted to determine if it alters Golgi functions that are not dependent on AP-1 as well. To this end, we examined the effect of PI4KIIα RNAi on constitutive secretion of HA (influenza virus hemaglutinnin protein) and VSVG (vesicular stomatitis virus G protein). HA secretion was followed by conventional biochemical transport assays (Lin et al., 1998) that measure the rate of acquisition of endo H resistance as HA becomes glycosylated in the early Golgi and the increase in accessibility of HA to extracellular trypsin after insertion into the plasma membrane. We found that PI4KIIα RNAi had little effect on intra-Golgi trafficking (Figure 6A), but it inhibited TGN-to-PM export by 35% (Figure 6B).

VSVG secretion from the TGN was followed by fluorescence microscopy. When TGN export is blocked by incubating cells at 19°C, VSVG accumulates at the Golgi (Figure 7A). When the temperature is shifted to 32°C, VSVG moves out of the TGN, as evidenced by the decrease in perinuclear VSVG fluorescence in control cells. In PI4KIIα RNAi cells, VSVG export was delayed and occurred at a slower rate (Figures 6C and 7A). For example, 40 min after shifting from 19°C to 32°C, the TGN of

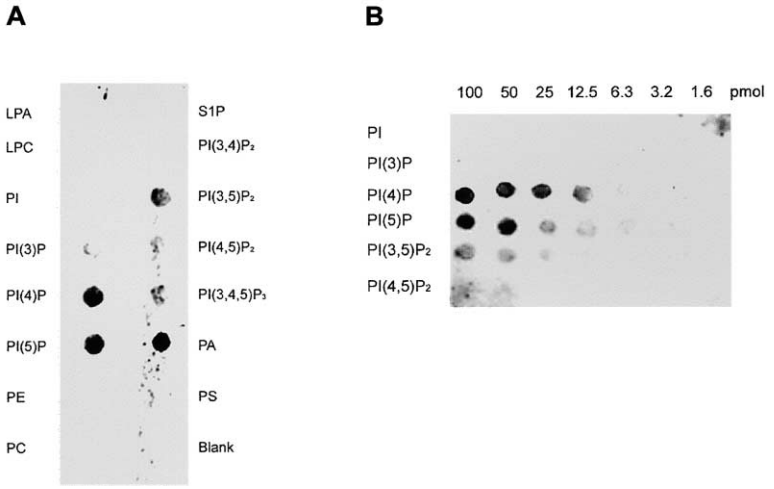

Figure 4. AP-1 Binding in a Protein:Lipid Overlay Assay

Lipids dotted strips (Echelon Biosciences) were incubated with purified AP-1, and bound γ-adaptin was detected with antibody.

(A) The PIP-Strip was dotted with 100 pmol of each of the lipids indicated.

(B) The PIP-Array was dotted with different amounts of selected lipids. LPA, lysophosphatidic acid; LPC, lysophosphatidylcholine; PI, phosphatidylinositol; S1P, sphinogosine 1-phosphate; PA, phosphatidic acid.

control and RNAi cells had lost 85% and 25% of the initial VSVG (at 0 time), respectively. The maximal rate of VSVG exit in RNAi cells was 38% of control.

Unlike the AP-1 block, the block in VSVG export can be rescued by shuttling either PI(4)P or PIP2 into PI4KIIα RNAi cells during the cold block (Figure 7B). In fact, PI(4)P or PIP2 shuttling accelerates VSVG exit from the PI4KIIα RNAi Golgi to a higher level than in control cells not treated with phosphoinositides (Figures 7B and 7A). The simplest explanation is that PIP2 is rate limiting in the TGN and it is required for VSVG export. Shuttled PI(4)P rescues VSVG export after it is converted to PIP2.

Discussion

We have identified PI4KIIα as the major Golgi resident PI4K that generates a large portion of total cellular PI(4)P and PIP2, and particularly Golgi PI(4)P. We achieved significant PI4KIIα RNAi knockdown without affecting the expression of several other closely related lipid kinases. The observed phenotypes were therefore due to a bona fide loss-of-function.

PI(4)P-Dependent AP-1 Recruitment to the Golgi

PI4KIIα RNAi decreased the amount of Golgi PI(4)P. It also decreased the association of AP-1 with the Golgi, while PI4KIIβ or PI4KIIIβ RNAi did not. Therefore, PI4KIIα has a unique role in the Golgi.

We used a variety of methods to establish that PI(4)P is required for AP-1 recruitment in vivo. We find that shuttling PI(4)P into live cells or adding PI(4)P to semi-intact PI4KIIα RNAi cells restores AP-1 recruitment. Furthermore, blocking access to PI(4)P by anti-PI(4)P decreases AP-1 recruitment to normal membranes. Our results establish the following: (1) there is a cause-and-effect relation between the change in Golgi PI(4)P and AP-1; (2) the interaction of AP-1 with the Golgi is absolutely dependent on PI(4)P; (3) PIP2, which is so important for plasma membrane recruitment of AP-2, is not involved in AP-1 recruitment; and (4) AP-1 directly binds PI(4)P but not PIP2.

Taken together, our results can be most simply explained by postulating that AP-1 binds the Golgi by docking on Golgi membrane PI(4)Ps.

The Relation Between Arf and PI4KIIα in AP-1 Recruitment

Besides a requirement for PI(4)P shown in this paper, others have shown that AP-1 recruitment is also absolutely dependent on Arf1 (Bonifacino and Lippincott-Schwartz, 2003; Zhu et al., 1998, 1999). Arf1 can potentially promote AP-1 binding to PI(4)P by recruiting/activating PI4Ks to increase the amount of available PI(4)P (Godi et al., 1999) or by generating a second docking site, presumably a protein (Zhu et al., 1998, 1999), that binds AP-1 synergistically with PI(4)P.

The first possibility is unlikely because there is currently no evidence to suggest that Arf1 regulates PI4KIIα. PI4KIIα association with the Golgi is resistant to brefeldin A (Figure 1A), and the kinase activity of the resident type II PI4K (almost certainly PI4KIIα) in the Golgi and in immature secretory granules is not regulated by Arf1 (Godi et al., 1999; Panaretou and Tooze, 2002). We therefore favor the second possibility that PI4KIIα and Arf1 generate two independent signals; each is necessary, but individually insufficient, for stable AP-1 association with Golgi membranes.

The proposed two-component docking mechanism (PI4KIIα-generated docking lipids and Arf-generated docking proteins) is similar to those used to anchor many low affinity phosphoinositide binding modules tightly to membranes (Yin and Janmey, 2003; McLaughlin et al., 2002). In most cases, the initial docking mediated by the phosphoinositides increases the probability of binding to the truly specific binding site elsewhere in the protein and the membrane. The situation for AP-1 is likely to be considerably more complex, because AP-1 binds many other ligands, and these combinatorial and cooperative binding events may be necessary to anchor AP-1 firmly to the Golgi. It is particularly intriguing that epsinR, an AP-1 accessory protein that is recruited to the Golgi by Arf1, also binds PI(4)P (Mills et al., 2003; Hirst et al., 2003). Thus, the recruitment of epsinR and AP-1 to a common PI(4)P-rich membrane patch may

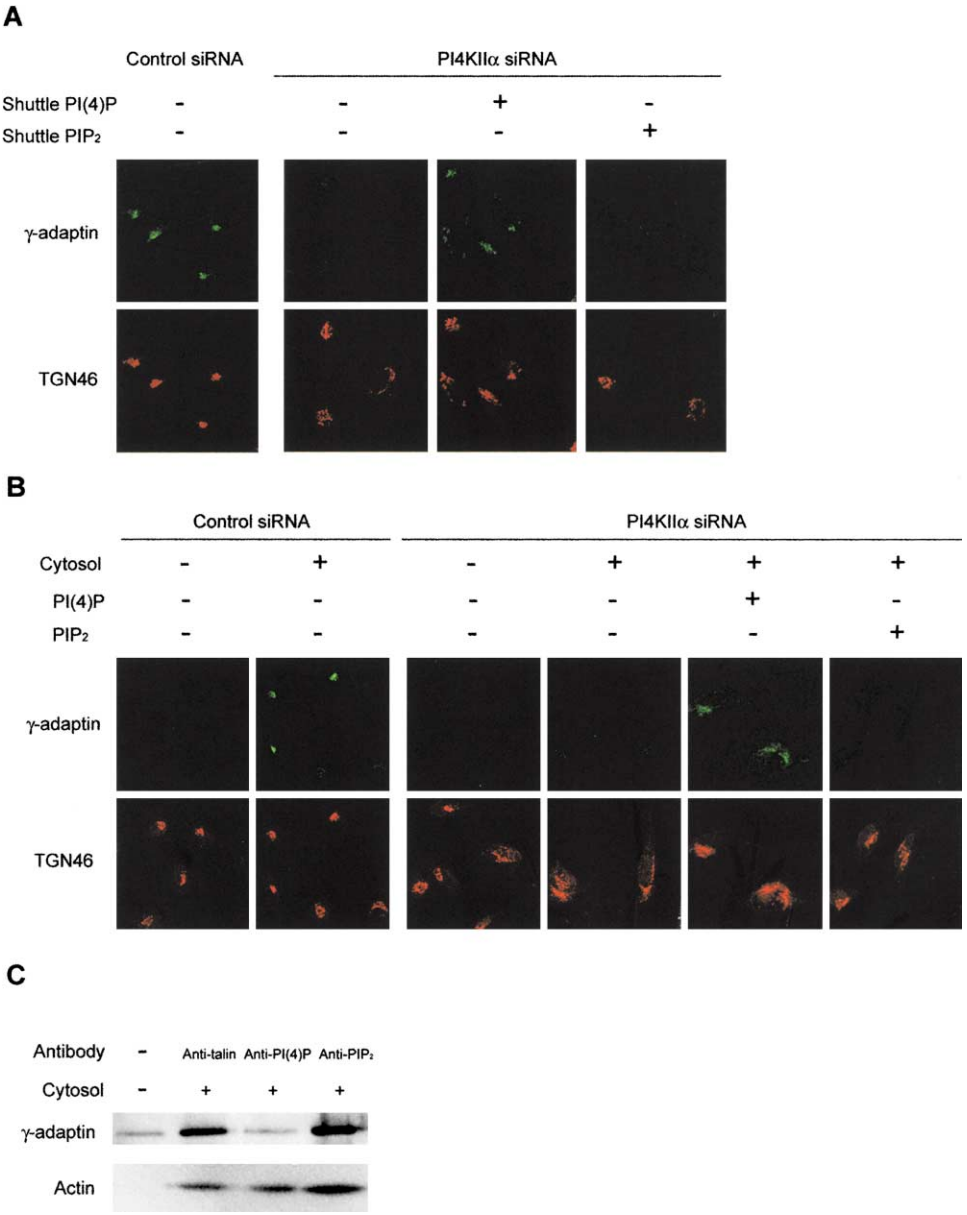

Figure 5. PI(4)P Is Required for AP-1 Association with the Golgi

(A) In vivo rescue of γ-adaptin (AP-1) binding by shuttle PI(4)P. PI4KIIα RNAi cells were incubated with polyamine shuttle carriers in the presence or absence of 20 μM diC16-PI(4)P or diC16-PIP2 for 30 min at 37°C. Cells were fixed, permeabilized with Triton X-100, and triple stained with anti-γ-adaptin, anti-TGN46, and anti-PI4KIIα. γ-adaptin and TGN46 images are shown.

(B) PI(4)P addback restores AP-1 binding to the Golgi of semi-intact cells. Control and PI4KIIα RNAi HeLa cells that were permeabilized with digitonin and salt stripped were incubated with concentrated HeLa cytosol in the presence or absence of 20 μM PI(4)P or PIP2 for 15 min at room temperature. Cells were triple labeled with anti-PI4KIIα (not shown), anti-γ-adaptin, and anti-TGN46.

(C) Anti-PI(4)P blocks AP-1 binding to normal membranes in vitro. HeLa microsome membranes (not treated with siRNA) that were first stripped with high salt to remove bound AP-1 were treated with anti-PI(4)P, anti-PIP2, or anti-talin. They were then exposed to HeLa cytosol. γ-adaptin and actin (as a control) were detected by Western blotting.

synergistically increase their affinity for each other and for PI(4)P. In this regard, the dramatic effect of PI4KIIα RNAi on AP-1 association points to an apical role of PI(4)P in the generation of this cascade.

PIP2-Dependent Export of Constitutively Secreted Proteins from the TGN

PI4KIIα RNAi inhibits VSVG and HA export from the TGN, but has no apparent effect on early Golgi functions. These results suggest that PI4KIIα acts primarily in the TGN, which would be consistent with its role in AP-1 recruitment to the TGN. However, unlike AP-1 recruitment, this function appears to be mediated through PIP2, because shuttled PI(4)P or PIP2 rescues the VSVG defect equally well. Although PIP2 has been implicated in regulated exocytosis (Siddhanta et al., 2000; Guo et al., 2003; Cremona and De Camilli, 2001), our results demonstrate that PIP2 promotes constitutive secretion

Table 2. Effects of PI(4)P and PIP2 on AP-1 Recruitment to the Golgi of Permeabilized Cells

	Golgi γ-adaptin intensity[1]	% cells with Golgi γ-adaptin staining[2]
Control w/o cytosol	5.2 ± 0.2	7
Control with cytosol	13.0 ± 0.7	57
PI4KIIα RNAi		
w/o cytosol	3.0 ± 0.3	5
with cytosol	3.4 ± 0.2	4
with cytosol + PI(4)P	6.9 ± 0.2	79
with cytosol + PIP2	3.2 ± 0.3	6

[1] Arbitrary units, obtained using Metamorph software. Values are mean ± SEM of 10 cells.
[2] 30–50 randomly chosen cells were scored per condition.

in living cells, and that at least some of this PIP2 is derived from PI4KIIα's PI(4)P pool.

Since PI4KIIα supports PIP2 synthesis during secretion, the mammalian Golgi is different from the yeast Golgi in this respect (Walch-Solimena and Novick, 1999; Hama et al., 1999; Audhya et al., 2000). Furthermore, unlike its yeast counterpart, which has no apparent function (Han et al., 2002; Shelton et al., 2003), the mammalian PI4KIIα has several essential roles in the Golgi. However, in spite of these differences, the important point is that PI(4)P has a direct role in some aspects of Golgi functions in both yeast and mammalian cells that is not contingent on the subsequent generation of PIP2.

Phosphoinositide Compartmentalization to Specify Organelle Identity

Unexpectedly, the plasma membrane has very little PI(4)P. We and others had previously assumed that the bulk of PI(4)P synthesized by the Golgi is exported to the plasma membrane constitutively (Cremona and De Camilli, 2001), where it awaits to be converted to PIP2 on demand. Our current result suggests that this is unlikely to be the case, at least in cells that are not specialized for regulated exocytosis (such as HeLa). Instead, PI(4)P generated in the Golgi and Golgi-derived carriers is probably converted to PIP2 either en route to the plasma membrane or immediately after it reaches the plasma membrane.

The compartmentalization of PI(4)P and PIP2 between the Golgi and the plasma membrane raises the possibility that they may specify the differential recruitment of closely related effector proteins (Munro, 2002). PI(4)P-dependent recruitment of AP-1 to the Golgi distinguishes it from PIP2/PIP3-dependent recruitment of AP-2 to the plasma membrane (Gaidarov and Keen, 1999; Rhode et al., 2002; Collins et al., 2002). Likewise, epsin1 is recruited to the plasma membrane via PIP2 (Ford et al., 2002), and epsinR may be recruited to the Golgi via PI(4)P (Mills et al., 2003; Hirst et al., 2003).

The concept of lipid-defined organelle identity originated from the finding that endosomes are enriched in PI(3)P (Gillooly et al., 2000), and gained momentum when it was discovered that the plasma membrane is particu-

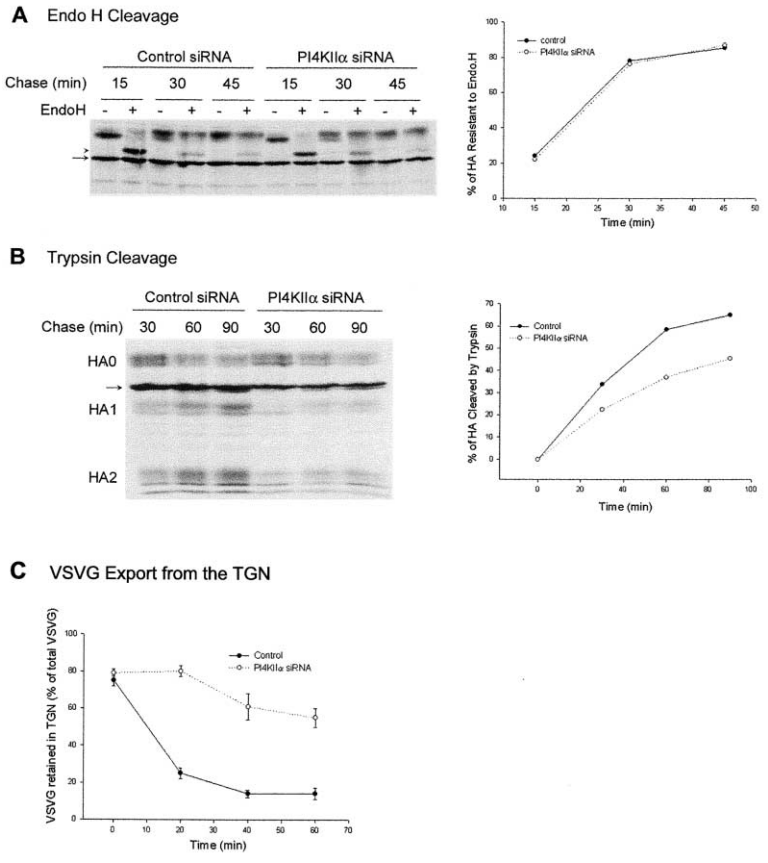

Figure 6. PI4KIIα RNAi Selectively Blocks Cargo Exit from the TGN but Has Little Effect on Early Secretory Steps

(A) Sensitivity of HA to endo H cleavage, as an indicator of intra-Golgi transit. Left, phosphorimage of labeled bands. Nonglycosylated HA was cleaved by endo H to generate a faster migrating band (arrowhead). HA that is glycosylated in the Golgi (e.g., after a 30 min chase) migrates slower on SDS-polyacrylamide gels and is endo H resistant. The radioactive band indicated by an arrow is an unidentified viral protein recognized nonspecifically by the anti-HA antibody. Data shown is representative of three independent experiments. Right, time course of the acquisition of endo H resistance.

(B) Sensitivity of HA to trypsin cleavage as an indicator of TGN to plasma membrane export. Left, phosphorimage of labeled bands. HA0, intact HA; HA1 and HA2, trypsin-cleaved HA polypeptides. Arrow indicates a protein that was immunoprecipitated nonspecifically. Right, time course of accessibility to trypsin. The amount of HA that was cleaved by extracellular trypsin (i.e., inserted into the plasma membrane) was expressed as percent of total HA (i.e., [(HA1+HA2)/(HA0+HA1+HA2)] × 100). Data shown is representative of 3 independent experiments.

(C) Inhibition of VSVG exit from the TGN. Cells infected with ts045 VSVG virus were held at 19°C for 2 hr to block TGN export and shifted to 32°C at time 0. Cells were fixed at time intervals, and triple labeled with anti-VSVG, anti-TGN46, and anti-PI4KIIα. The amount of VSVG fluorescence in the Golgi was expressed as a percent of that in the entire cell (see Figure 7A for images). 9–24 control or PI4KIIα RNAi cells were analyzed per time point per condition, and values shown are mean ± S.E.

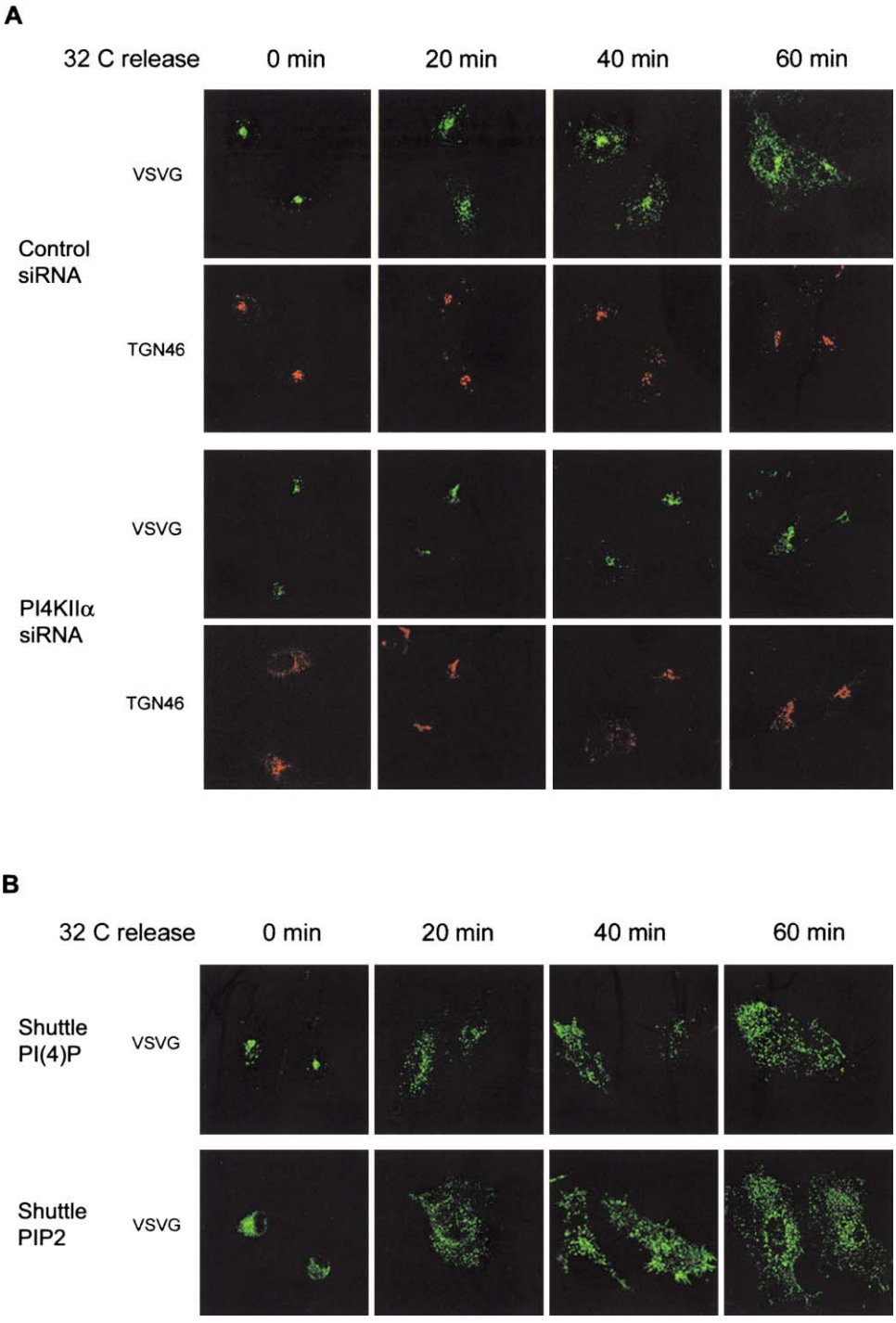

Figure 7. Rescue of VSVG Block by Shuttle PI(4)P and PIP2

Cells infected with ts045 VSVG virus were held at 19°C and warmed to 32°C at time 0.

(A) Control and PI4KIIα RNAi cells were fixed at time intervals, and triple labeled with anti-VSVG, anti-TGN46, and anti-PI4KIIα (not shown).

(B) The shuttle PI(4)P and shuttle PIP2 rescue experiments on PI4KIIα RNAi cells were performed by including PI(4)P or PIP2 during the 2 hr 19°C cold block. Only the VSVG images of cells with confirmed PI4KIIα knockdown were shown.

larly enriched in PIP2. Our discovery that AP-1 recruitment to the TGN is dependent on PI(4)P and that the Golgi accounts for the largest PI(4)P pool in cells strongly supports the possibility that PI(4)P is the predominant Golgi membrane marker (Munro, 2002). We therefore propose that PI(4)P is the third arm of the membrane lipid recognition system.

Experimental Procedures

Cell Culture, Plasmid Transfections, and Adenovirus Infections

Cells were cultured in DMEM with 10% (v/v) fetal bovine serum (FBS), 10 mM HEPES, and 1 mM sodium pyruvate at 37°C in a humidified 5% CO_2 incubator. Cells were transiently transfected with lipofectAMINE (Invitrogen), infected with recombinant adenovirus

(Yamamoto et al., 2001), or transfected with siRNA using oligofectAMINE (Invitrogen).

Antibodies, Constructs, and Reagents

The rabbit polyclonal anti-PI4KIIα (Wei et al., 2002), anti-PI4KIIβ (Wei et al., 2002), monoclonal anti-VSVG, and anti-HA antibodies (Lin et al., 1997, 1998) were described previously. Antibodies from commercial sources are as follows: rabbit anti-PI4KIIIβ (Upstate Biotechnology), goat anti-PIP5KIα (Santa Cruz Biotechnology), sheep anti-TGN46 (Serotec), monoclonal anti-PI(4)P and anti-PIP_2 (Assay Designs, Inc.), monoclonal anti-γ-adaptin (clone 100/3; Sigma), monoclonal anti-β-COP (clone M3A5; Sigma), anti-MPR300 (Affinity Bioreagents, Inc), and anti-actin (Sigma). Secondary antibodies were obtained from Jackson ImmunoResearch Labs, Inc., Amersham Life Sciences, or Santa Cruz Biotechnology.

The rat myc-PI4KIIα construct is as described in Barylko et al. (2001). GFP-OSBP-PH (Levine and Munro, 2002) was a gift of S. Munro, and pECFP-Golgi (CFP-β-GT) was from Clontech Labs, Inc. Recombinant adenovirus vectors expressing GFP, PI4KIIα, and a kinase-dead PI4KIIα (PI4KIIα K151A) were constructed using the AdEasy Adenoviral Vector System (Stratagene).

Most other nontissue culture reagents were from Sigma, except as noted in the text.

PI4KIIα RNA Interference

The siRNA sequence targeting human PI4KIIα (GenBank accession number NM_18425) spans nucleotides 888–908 and is specific for hPI4KIIα based on BLAST search (NCBI database). A siRNA sequence corresponding to nucleotides 695–715 of the firefly luciferase (U31240) was used as a negative control. siRNAs were synthesized by the Center for Biomedical Inventions (U. of Texas Southwestern Medical Center at Dallas), and annealed according to the protocol recommended by Dharmacon Research, Inc.

HeLa cells were plated in 6-well plates at 20%–30% confluence for 24 hr and transfected with 10 μl of 20 μM siRNA and 3 μl of oligofectAMINE in 1 ml of Opti-MEM. After 5 hr, cells were washed and cultured in DMEM containing FBS. They were either left alone, transfected with a cDNA (such as GFP-OSBP-PH), or infected with virus and used 72 hr after the initial siRNA treatment.

Immunofluorescence Microscopy

siRNA-treated cells were trypsinized after transfection, and reseeded on glass coverslips. In most cases, cells were fixed in 3.7% formaldehyde, permeabilized with 0.1% Triton X-100 on ice, and labeled with antibodies in blocking buffer (1% BSA, 3% donkey serum in PBS). In some cases, cells were fixed as above and "cracked open" by freeze-thawing without using detergents (Tran et al., 1999).

Cells were examined by a Zeiss 510 Laser Scanning Confocal Microscope using a 63 × 1.3 NA PlanApo objective. The Golgi is defined as the perinuclear region that is stained by anti-TGN46. This region of interest was selected, and the intensity of the other markers in this region is considered as Golgi-associated protein fluorescence. The time of image acquisition, the image gain, and enhancement were optimally adjusted at the outset and kept constant for all samples. In most cases, images were collected near the middle of the *z* axis. Captured images were analyzed using Metamorph Image software. Pixel intensity was used to quantitate fluorescence in the region of interest. Fluorescence intensity of Golgi AP-1 was expressed in arbitrary units after subtracting background cytosolic AP-1; VSVG and PI(4)P fluorescence in the Golgi were expressed as percentage of total fluorescence in the cell.

Phospholipid Analyses

Cells were labeled for 4 hr with 40 μCi/ml ^{32}P-PO_4 (NEN) in phosphate-free DMEM. Lipids were extracted with $CHCl_3$:methanol:HCl (volume ratio 5:10:4), resolved by thin layer chromatography (TLC) (Yamamoto et al., 2001), and detected using a Phosphorimager.

In Vitro AP-1 Membrane Binding Assay

HeLa cells were homogenized and centrifuged at 100,000 × g (Wei et al., 2002). The microsome pellet was resuspended and incubated for 10 min with ice-cold high salt alkaline solution (1 M NaCl, 0.1 M sodium carbonate [pH 10.0]) to remove membrane-bound AP-1. The stripped membranes were collected by centrifugation at 20,000 × g for 15 min, washed once, and resuspended in binding assay buffer (25 mM HEPES-KOH [pH 7.0], 250 mM sucrose, 125 mM potassium acetate, 5 mM magnesium acetate supplemented with 1 mM dithiothreitol [DTT]) at 1 mg protein/ml.

25 μl of the stripped membranes was incubated with 10 μl of 1 mg/ml of antibodies at 25°C for 15 min. 170 μl of HeLa cytosol (Traub et al., 1993) was added. and the final incubation mixture contained 100 μM GTPγS, 1 mM ATP, 8 mM creatine phosphate, 80 μg/ml creatine kinase, and 1 mM DTT. After incubation at 37°C for 15 min, samples were diluted with 400 μl binding assay buffer without sucrose, centrifuged at 20,000 × g for 15 min at 4°C, washed once, and analyzed by SDS-PAGE and Western blotting.

Protein-Lipid Overlay Assay

Bovine AP-1 was purified from bovine brain clathrin-coated vesicles by gel filtration followed by hydroxyapaptite chromatography as described previously (Rapoport et al., 1998). AP-1 binding to phospholipids was performed at room temperature using PIP-Strip and PIP-Array (Echelon Biosciences, Inc) following the manufacturer's protocol. Bound AP-1 was detected with anti-γ-adaptin and HRP-conjugated anti-mouse IgG.

Rescue of AP-1 Binding in Intact and Semi-Intact Cells by Exogenous Phospholipids

Intracellular delivery of PPIs into intact cells

We used the Echelon ShuttlePIP kit (Echelon Biosciences, Inc.) (Ozaki et al., 2000). Cells were washed with serum free DMEM twice, followed by incubation at 37°C for 30 min in serum-free DMEM with ShuttlePIP components. 10–20 μM diC16 PI(4)P and diC16 PIP_2 were delivered intracellularly (shuttled) via Echelon's polyamine carriers 3 and 2, respectively.

Addition to semi-intact cells

AP-1 recruitment in semi-intact cells was performed as described by Zhu et al. (1998). siRNA-treated HeLa cells were permeabilized with 20 μg/ml digitonin on ice for 10 min in 25 mM HEPES-KOH (pH 7.2), 125 mM potassium acetate, 5 mM magnesium acetate, 1 mM DTT, and 1 mg/ml D-glucose. They were further incubated at 37°C for 5 min in permeabilization buffer without digitonin to strip off endogenous AP-1.

The permeabilized cells were incubated with 20 μM sonicated PI(4)P or PIP2 in 25 mM HEPES-KOH (pH 7.2) and 1 mg/ml D-glucose for 15 min at room temperature. Four volumes of HeLa cytosol were added together with 100 μM GTPγS and an ATP-regeneration system. After 15 min incubation at 4°C, the coverslips were washed twice with PBS and cells were fixed with methanol for immunofluorescence analysis.

Transport Assays

Biosynthetic HA transport assays

HeLa cells in 35 mm dishes were transfected with either control or PI4KIIα siRNA. After 68 hr, cells were infected with influenza (HA) virus at 37°C for 5 hr (at >10 pfu/cell), washed, and incubated for 30 min in serum-free DMEM lacking methionine and cysteine. They were labeled with 200 μCi/ml Trans-^{35}S-label (ICN) at 37°C for 5–15 min (Lin et al., 1998).

To monitor acquisition of endoH resistance, cells labeled for 5 min were lysed in high salt RIPA buffer (50 mM Tris-HCl [pH 8.0], 150 mM NaCl, 1% NP40, 0.1% SDS, 0.5% sodium deoxycholate, 2 mM EDTA, 2 mM EGTA and a protease inhibitor cocktail [Roche]) at timed intervals and HA was immunoprecipitated with anti-HA/protein G-Sepharose. Immunoprecipitated proteins were released from the Sepharose beads with 1% SDS, 50 mM Tris-HCl (pH 6.8) and treated with endo H (25 U, New England Biolabs) or mock-treated at 37°C for 4 hr. Samples were analyzed by SDS-PAGE followed by exposure to Phosphorimager.

To monitor accessibility of HA to externally added trypsin, cells labeled for 15 min were kept at 19°C cold block for 1 hr and switched to 37°C in medium containing 10 μg/ml TPCK treated trypsin or no trypsin. At timed intervals, cells were solubilized in high-salt RIPA buffer containing 100 μg/ml soybean trypsin inhibitor. HA was immu-

noprecipitated with anti-HA and analyzed by SDS-PAGE and Phosphorimager analysis.

Immunofluorescence VSVG export assay

68 hr after exposure to siRNA, cells were infected with ts045VSVG virus (>10 pfu/cell) in serum-free DMEM (300 μl/well) for 30 min at 32°C, washed extensively, and placed at 40°C for 3.5 hr (Hirschberg et al., 1998). At the last 0.5 hr of incubation, 100 μg/ml cyclohexamide was added to block further protein synthesis. Cells were placed in low-carbonate DMEM supplemented with FCS at 19°C for 2 hr to block export from the TGN. Cells were switched to 32°C, fixed at timed intervals, and triple labeled with anti-VSVG, anti-PI4KIIα, and anti-TGN46. When indicated, 2 μM PI(4)P or PIP2 shuttle was included in the 19°C step for 2 hr.

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