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Distinct Golgi Populations of Phosphatidylinositol 4-Phosphate Regulated by Phosphatidylinositol 4-Kinases*

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Kelly M. Weixel‡§, Anna Blumental-Perry¶‖, Simon C. Watkins¶, Meir Aridor¶, and Ora A. Weisz‡¶**

From the ‡Renal-Electrolyte Division, Department of Medicine and the ¶Department of Cell Biology and Physiology, University of Pittsburgh, Pittsburgh, Pennsylvania 15261

Phosphatidylinositol 4-phosphate (PI4P) regulates biosynthetic membrane traffic at multiple steps and differentially affects the surface delivery of apically and basolaterally destined proteins in polarized cells. Two phosphatidylinositol 4-kinases (PI4Ks) have been localized to the Golgi complex in mammalian cells, type III PI4Kβ (PI4KIIIβ) and type II PI4Kα (PI4KIIα). Here we report that PI4KIIIβ and PI4KIIα localize to discrete subcompartments of the Golgi complex in Madin-Darby canine kidney (MDCK) cells. PI4KIIIβ was enriched in early Golgi compartments, whereas PI4KIIα colocalized with markers of the *trans*-Golgi network (TGN). To understand the temporal and spatial control of PI4P generation across the Golgi complex, we quantitated the steady state distribution of a fluorescent PI4P-binding domain relative to *cis*/*medial* Golgi and TGN markers in transiently transfected MDCK cells. The density of the signal from this PI4P reporter was roughly 2-fold greater in the early Golgi compartments compared with that of the TGN. Furthermore, this ratio could be modulated *in vivo* by overexpression of catalytically inactive PI4KIIIβ and PI4KIIα or *in vitro* by the PI4KIIIβ inhibitor wortmannin. Our data suggest that both PI4KIIIβ and PI4KIIα contribute to the compartmental regulation of PI4P synthesis within the Golgi complex. We discuss our results with respect to the kinetic effects of modulating PI4K activity on polarized biosynthetic traffic in MDCK cells.

The synthesis and metabolism of phosphatidylinositols (PIs)[1] provide a mechanism to regulate myriad cellular processes. In addition to their function as intracellular second messengers, significant roles for PIs in the regulating interactions between the lipid bilayer and protein machinery involved in vesicle budding and traffic as well as in cytoskeletal organization have recently emerged (1, 2). Cellular signaling and intracellular trafficking events are activated independently, which requires spatial and temporal control of PI metabolism. This is mediated in part by the subcellular distribution of phosphatidylinositol kinases, which phosphorylate PIs in specific compartments of the cell, including the plasma membrane, endosomes, secretory granules, and the Golgi complex (3–6).

Recent studies from our laboratory and others have documented an emerging role for phosphatidylinositol 4-kinases (PI4Ks) in regulating traffic along the biosynthetic pathway. These enzymes selectively catalyze the phosphorylation of PI at the D-4 position of the inositol ring to produce phosphatidylinositol 4-phosphate (PI4P). Although PI4P serves as a major precursor of phosphatidylinositol 4,5-bisphosphate, this lipid also has been shown to directly regulate the delivery of proteins from the Golgi complex to the plasma membrane in yeast (7, 8) An important role for PI4P in the regulation of biosynthetic traffic appears to be preserved in mammalian cells as well. Our lab has demonstrated that changes in PI4P metabolism have complex effects on transport through and export from the Golgi complex in polarized MDCK cells (9). This is supported by work from other laboratories that demonstrates a role for PI4P in the recruitment of the adaptor protein complex AP-1 and EpsinR to the TGN (10–12).

To date, two PI4 kinases in mammalian cells have been reported to contribute to the functional pool of PI4P in the Golgi complex, PI4KIIIβ and PI4KIIα (12, 13). PI4KIIIβ is a peripheral protein that is recruited to the Golgi complex via ARF1, where it has been implicated in regulating the transport of newly synthesized proteins from the Golgi to the plasma membrane (9, 14). PI4KIIα appears to have a broader distribution in the cell and has been shown to be associated with several membrane bound organelles, including endosomes, a subcompartment of the endoplasmic reticulum, synaptic vesicles, and the Golgi complex (12, 15–17). Although these two proteins function in the same organelle to generate PI4P, the suborganellar distribution of these kinases and the locations of the pools of PI4P they generate are unknown. Our previous studies in polarized MDCK cells have shown that altered PI4P metabolism has differential effects on intra-Golgi transport *versus* cell surface delivery, as well as on apical and basolateral traffic. Overexpression of a catalytically inactive mutant of PI4KIIIβ inhibited intra-Golgi transport, but paradoxically stimulated TGN to cell surface delivery of the apical marker influenza hemagglutinin (HA) in these cells. By contrast, overexpression of wild-type PI4KIIIβ had no effect on early Golgi transport but inhibited TGN-to-apical membrane delivery (9).

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** To whom correspondence should be addressed: Renal-Electrolyte Division, University of Pittsburgh, 3550 Terrace St., Pittsburgh, PA 15261. Tel.: 412-383-8891; Fax: 412-383-8956; E-mail: weisz@pitt.edu.

[1] The abbreviations used are: PI, phosphatidylinositol; BFA, brefeldin A; HA, hemagglutinin; MDCK, Madin-Darby canine kidney; NRK, normal rat kidney; PH, pleckstrin homology; PI4K, phosphatidylinositol 4-kinase; PI4P, phosphatidylinositol 4-phosphate; TGN, *trans*-Golgi network; WMN, wortmannin; GST, glutathione *S*-transferase; YFP, yellow fluorescent protein; PBS, phosphate-buffered saline; GFP, green fluorescent protein.

These data demonstrate that PI4P can be regulated at the suborganellar level and has multiple and sometimes opposing roles in transport through the Golgi complex. Although changes in PI4K expression have been shown to affect total cellular phosphatidylinositol phosphate levels, it has been difficult to assess the temporal changes in PI4P distribution in the Golgi under conditions when PI4P metabolism is manipulated (9, 12). Here we have expressed the pleckstrin homology (PH) domain of Fapp1, which binds to the Golgi complex in a PI4P-dependent manner, as a reporter to identify and quantify the steady state distribution of PI4P across the Golgi complex of MDCK cells. Moreover, we have examined the effects of modulating PI4K activity on this distribution. These studies allow us for the first time to correlate changes in PI4P levels and localization with kinetic effects on biosynthetic membrane traffic.

MATERIALS AND METHODS

Construction of Plasmids and Adenoviruses—Recombinant adenoviruses encoding PI4KIIIβ and PI4KIIIβ_{D656A} have been described previously (9). Plasmids encoding GFP-PI4KIIIβ, HA-PI4KIIα, and GFP-Fapp1(PH) were kindly provided by Tamas Balla (National Institutes of Health). A cDNA construct encoding the amino terminus of human β1–4-galactosyltransferase linked to yellow fluorescent protein (GalT-YFP) was a gift from Dr. Jennifer Lippincott-Schwartz (National Institutes of Health). To construct GST-Fapp1(PH), a SalI and *EcoR*I fragment containing the first 99 amino acids of Fapp1 was subcloned into the pGex 4t-1 vector. Recombinant expression of GST-Fapp1(PH) was carried out as described (18). Trp^{15} and Arg^{18} within the Fapp1 sequence of GFP-Fapp1(PH) were mutagenized to Asp and Lys, respectively, using the QuikChange® site-directed mutagenesis kit from Stratagene to generate the GFP-Fapp1(PH) N-K mutant.

Cell Culture and Transfections—Transient transfections of MDCK type II cells and NRK cells were performed using Lipofectamine 2000™ (Invitrogen) as per the manufacturer's protocol. The cells were seeded onto 12-mm coverslips and transfected 24 h later with 2 μg of plasmid DNA. Cells transfected with GFP-Fapp1(PH) or GFP-PI4KIIIβ were incubated for 12–16 h to allow for protein accumulation. The cells expressing HA-PI4KIIα were fixed and processed for indirect immunolocalization 8 h post-transfection (12). For some GFP-Fapp1(PH) quantitation studies, the cells were infected with recombinant adenoviruses encoding PI4KIIIβ and PI4KIIIβ_{D656A}, then transfected 2 h later with plasmid encoding GFP-Fapp1(PH), and examined the following day. Similar results were obtained using cells cotransfected with plasmids encoding PI4K and GFP-Fapp1(PH).

Fapp1(PH) Binding to Semi-permeabilized Cells—Cells transfected with a construct encoding GalT-YFP were grown on coverslips overnight. The indicated coverslips were incubated with 5 μM WMN for 1 h prior to permeabilization as described in Ref. 19. Following permeabilization on ice, the cells were incubated with rat liver cytosol supplemented with an ATP-regenerating system and 0.2 μM GST-Fapp1(PH) for 30 min at 32 °C. Cells pretreated with WMN were incubated with cytosol supplemented with 5 μM WMN to inactivate any cytosol-associated PI4KIIIβ. The cells were then fixed with 4% paraformaldehyde and processed for immunofluorescence.

Immunofluorescence and Confocal Microscopy—Transfected cells were grown on coverslips, washed with PBS, fixed for 20 min with 4% paraformaldehyde, washed, and permeabilized with 0.1% TX-100 in PBS containing 1% bovine serum albumin and 0.2% glycine. Cells were incubated for 30 min in blocking buffer (PBS containing 1% bovine serum albumin) followed by a 30-min incubation in PBS containing 5% normal goat serum. The coverslips were incubated for 1 h in primary antibodies diluted in blocking buffer, washed, incubated with secondary antibodies diluted in blocking buffer, washed, and mounted in Aqua-polymount (Polysciences, Inc., Warrington, PA). Rabbit anti-giantin (diluted 1:400) and mouse anti-GPP130 (diluted 1:500) were gifts from Adam Linstedt (Carnegie Mellon, Pittsburgh, PA), Rabbit anti-furin (diluted 1:400) was from Affinity Bioreagents (Golden, CO), mouse anti-γ-adaptin (diluted 1:300) was from BD Transduction Laboratories (San Diego, CA), and mouse anti-HA antibodies (diluted 1:1000) were from Covance (Princeton, NJ). Secondary antibodies (diluted 1:500; goat anti-rabbit 647 and goat anti-mouse 568 or TxRed) were from Molecular Probes (Eugene, OR). Imaging was performed on a TCS-SL confocal microscope equipped with argon and green and red helium-neon lasers (Leica, Dearfield, IL). Images were acquired by sequential scanning using a ×100 (1.4 numerical aperture) plan apochromat oil objective and the appropriate filter combination.

Quantitative Immunofluorescence Analysis—High expression levels of GFP-Fapp1(PH) disrupted cellular morphology; therefore only cells in which *cis*/*medial* and TGN compartment markers were distinctly separated were chosen for quantitative analysis. In experiments with cells coexpressing GFP-Fapp1(PH) and PI4KIIIβ_{D656A}, quantitation was performed on cells that retained detectable levels of GFP-Fapp1(PH) binding to the Golgi complex. Confocal images of GFP-Fapp1(PH)-expressing cells were saved as 12-bit images and imported into MetaMorph® software (Universal Imaging). Each channel was individually thresholded, and binary masks of the *cis*/*medial* and TGN staining profiles were generated. A minimal region of overlap between the two masks that could not be identified conclusively as either compartment was removed from each mask. Binary masks were used to extract the intensity, area, and mean voxel intensity of GFP-Fapp1(PH) staining in each compartment.

RESULTS

Both PI4KIIIβ and PI4KIIα have been localized to the Golgi complex of mammalian cells, but their sub-Golgi distribution is not known. To determine the intra-Golgi localization of these enzymes, MDCK cells were transiently transfected with plasmids encoding GFP-PI4KIIIβ (Fig. 1, *A–I*) or HA-tagged PI4KIIα (Fig. 1, *J–O*). The cells were fixed 6–8 h after transfection and processed for triple label immunofluorescence using antibodies against *cis*/*medial* Golgi and TGN marker proteins (Fig. 1, *A–I*). Confocal microscopy demonstrated that GFP-PI4KIIIβ localized primarily with the *cis*/*medial* Golgi protein giantin (Fig. 1*C*) but displayed little overlap with the TGN markers furin (Fig. 1*F*) or γ-adaptin (Fig. 1*I*). Similar to previous studies, we found that PI4KIIα localized not only to Golgi structures but also to punctate cytosolic structures consistent with endosomes (12, 16) In contrast to PI4KIIIβ, the Golgi-localized pool of PI4KIIα staining did not colocalize with giantin (Fig. 1*L*) but overlapped extensively with γ-adaptin (Fig. 1*O*). This is consistent with the reported function of this enzyme in generating a pool of PI4P that recruits AP-1 coat machinery (12). Coexpression of GFP-PI4KIIIβ and HA-tagged PI4KIIα confirmed the distinct localizations of PI4KIIIβ and PI4KIIα (Fig. 1*R*). These data suggest that PI4KIIIβ and PI4KIIα function independently to generate PI4P-rich domains in the *cis*/*medial* Golgi and TGN of MDCK cells.

To assess whether the PH domain of Fapp1 is an appropriate reporter of Golgi PI4P in MDCK cells, a GFP-tagged PH domain of Fapp1 (GFP-Fapp1(PH)) was transiently expressed in MDCK cells. Confocal microscopy images show GFP-Fapp1(PH) localized to the perinuclear region of the cell in structures that are also stained by antibodies specific for Golgi proteins (Fig. 2). To confirm that GFP-Fapp1(PH) localization to the Golgi complex requires binding of PI4P, a mutant protein was constructed in which selective recognition of PI4P is predicted to be disrupted. In this protein (GFP-Fapp1(PH) N-K), tryptophan and arginine residues in the conserved lipid recognition site within the PH domain have been mutagenized to asparagine and lysine, respectively. As predicted, these mutations largely inhibited Fapp1(PH) binding to PI4P as assessed by lipid strip binding and liposome pelleting assays.[2] However, GFP-Fapp1(PH) N-K retained weak binding specificity for other PI species, confirming that the mutant protein is not grossly misfolded. When expressed in MDCK cells, GFP-Fapp1(PH) N-K localized primarily to the cytoplasm, and no Golgi staining was observed (Fig. 2). In some cells, a small amount of staining was also observed at the cell surface and on non-Golgi membranes, possibly as a result of GFP-Fapp1(PH) N-K binding to distinct PI species. Thus, GFP-Fapp1(PH)

[2] A. Blumental-Perry, C. Haney, E. A. Harrison, K. M. Weixel, O. A. Weisz, S. C. Watkins, and M. Aridor, manuscript in preparation.

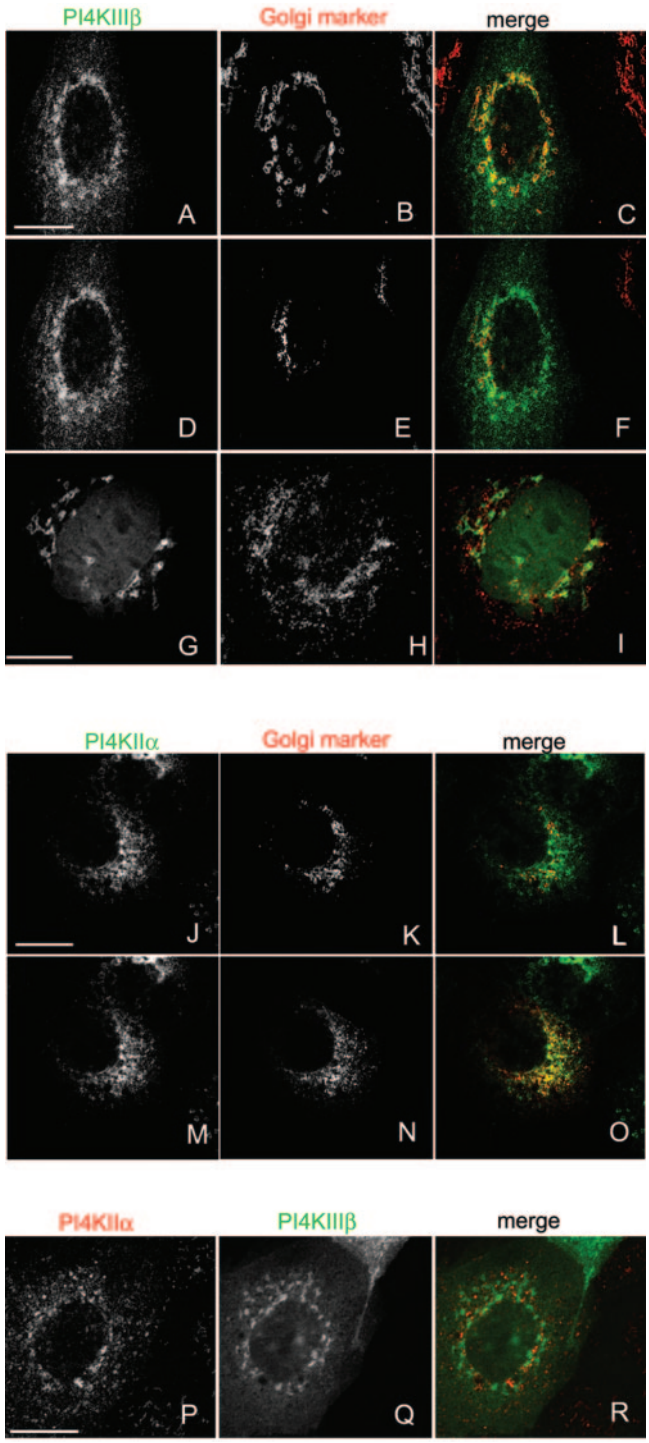

FIG. 1. **Golgi-associated phosphatidylinositol 4-kinases localize to distinct subcompartments.** MDCK cells transiently expressing GFP-PI4KIIIβ (*A–I*) or HA-tagged PI4KIIα (*J–O*) were immunostained with antibodies against the *cis/medial* Golgi marker giantin (*B* and *K*) or TGN markers furin (*E*) and γ-adaptin (*H* and *N*) and visualized by confocal microscopy. GFP-PI4KIIIβ colocalizes extensively with giantin (*C*), whereas little overlap is detected between GFP-PI4KIIIβ with either furin (*F*) or γ-adaptin (*I*). In contrast, HA-PI4KIIα colocalizes with γ-adaptin (*O*) and shows virtually no overlap with giantin (*L*). In *P* and *Q*, MDCK cells that transiently coexpress HA-tagged PI4KIIα and GFP-PI4KIIIβ were examined by confocal microscopy. The merged image *R* demonstrates the segregation of PI4KIIα (*P*) and PI4KIIIβ (*Q*) within distinct subcompartments of the Golgi complex. *Scale bars*, 4 μm.

localizes to the Golgi complex of MDCK cells in a PI4P-specific manner.

To examine the sub-Golgi distribution of PI4P, MDCK cells transiently expressing GFP-Fapp1(PH) were fixed and processed for indirect immunofluorescence using antibodies specific for markers of the *cis/medial* Golgi and TGN (GPP130 and furin, respectively; Fig. 3). The compartments in which GPP130 and furin reside could be readily resolved by confocal microscopy (Fig. 3*A*, *bottom left panel*). Comparison of the staining pattern of GFP-Fapp1(PH) with these markers revealed that GFP-Fapp1(PH) primarily localized with the *cis/medial* Golgi marker protein, GPP130, (Fig. 3*A*, *bottom middle panel*) and showed little overlap with furin in the TGN (Fig. 3*A*, *bottom right panel*). We used images from 20 cells to quantitate the relative *density* of Golgi-associated GFP-Fapp1(PH) in areas of colocalization with GPP130 *versus* furin. This quantitation method allows us to detect the average binding of GFP-Fapp1(PH) per unit pixel as opposed to the total intensity of staining in each compartment; importantly, the resulting values are independent of the relative area of each compartment that is captured in the optical section. The intensity of GFP-Fapp1(PH) staining was nearly twice as high in the *cis/medial* Golgi as in the TGN (see Fig. 5).

The *cis/medial* Golgi of MDCK cells is not disrupted by incubation with brefeldin A (BFA); however, the TGN becomes tubulated and dispersed under these conditions (20, 21). Therefore, to confirm the localization of GFP-Fapp1(PH) in MDCK cells, we examined the effect of BFA (10 min, 5 μg/ml) on the localization of this probe. The staining pattern of the TGN marker furin was dramatically altered in BFA-treated cells compared with untreated controls (Fig. 3*B*, *middle panels*). In contrast, the overall staining pattern of GFP-Fapp1(PH), although slightly more dispersed than in control cells, was relatively unaffected by this drug (Fig. 3*B*, *left-hand panels*). No change in these patterns was observed even upon significantly longer incubations with BFA.[3] Together, these data suggest that a sizable pool of PI4P exists in the *cis/medial* Golgi of MDCK cells at steady state.

A recent report has localized both full-length Fapp proteins and Fapp PH domains to primarily the TGN in NRK and COS7 cells (22). Indeed, we observed a similar distribution when we expressed GFP-Fapp1(PH) in NRK cells (Fig. 3*C*, compare colocalization with giantin *versus* furin). Furthermore, brief treatment with BFA caused the majority of GFP-Fapp1(PH) signal to dissociate from the Golgi complex in the majority of NRK cells, as previously reported (22).[3]

The compartmentalization of PI4KIIIβ to the *cis/medial* Golgi and the observation that GFP-Fapp1(PH) colocalized primarily with a *cis/medial* Golgi marker in MDCK cells suggested that PI4KIIIβ may be responsible for the synthesis and maintenance of this pool of PI4P. To test whether PI4KIIIβ activity is required to maintain the PI4P pool detected by GFP-Fapp1(PH), MDCK cells were cotransfected with plasmids encoding GFP-Fapp1(PH) and a dominant-negative, catalytically inactive mutant of PI4KIIIβ (PI4KIIIβ_{D656A}). Following fixation, the distribution of GFP-Fapp1(PH) was examined by confocal microscopy. In control cells, GFP-Fapp1(PH) was almost exclusively associated with the Golgi complex, with cytoplasmic staining observed only in cells expressing very high levels of the protein (Fig. 4*A*, *left panel*). The distribution of GFP-Fapp1(PH) was much more variable in cells expressing PI4KIIIβ_{D656A}. Golgi-associated GFP-Fapp1(PH) was detected in the majority of cells; however, it was significantly dimmer. Furthermore, the relative intensity of cytoplasmic GFP-Fapp1(PH) staining compared with Golgi staining was considerably higher than in control cells (Fig. 4*A*, *right panel*). In some PI4KIIIβ_{D656A}-expressing cells, GFP-Fapp1(PH) signal

[3] K. M. Weixel and O. A. Weisz, unpublished observation.

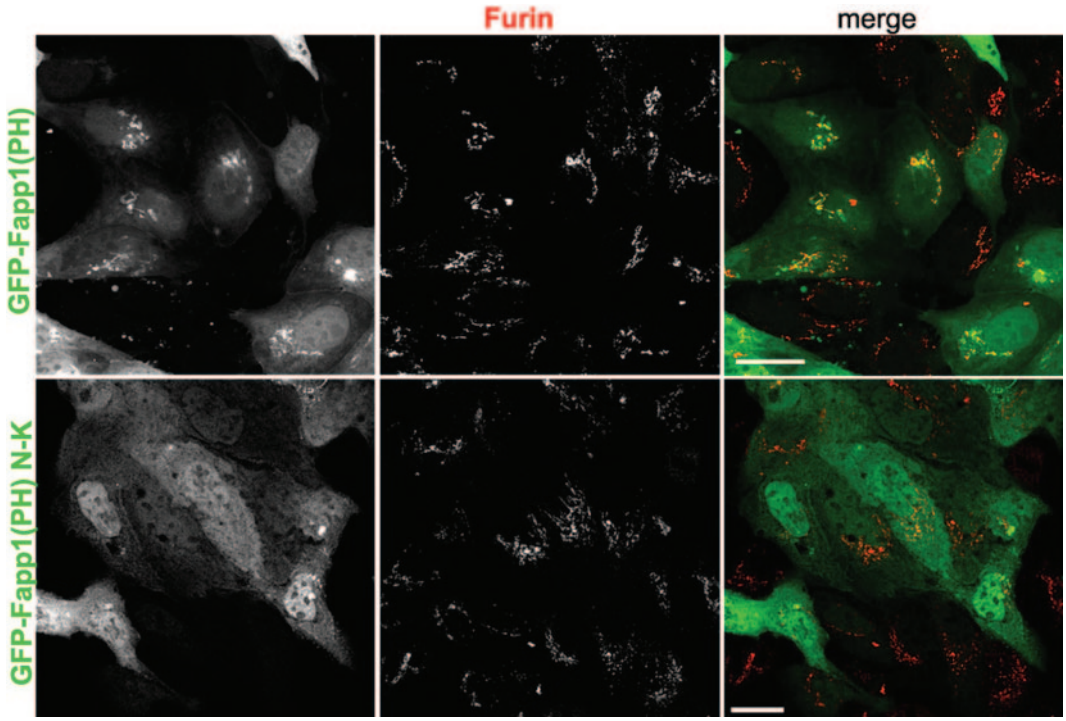

FIG. 2. **The pleckstrin homology domain of Fapp1 is targeted to the Golgi complex of MDCK cells in a PI4P-dependent manner.** MDCK cells transiently expressing GFP-Fapp1(PH) (*upper panels*) or GFP-Fapp1(PH) N-K, which has dramatically reduced binding affinity for PI4P (*lower panels*),[2] were fixed and immunostained with antibodies against the Golgi marker furin. GFP-Fapp1(PH) localization is similar to that of furin, whereas GFP-Fapp1-(PH) (N-K) is not targeted to the Golgi complex. *Scale bars*, 20 μm.

was completely dissociated from the Golgi; these cells were omitted from the quantitative immunofluorescence studies described below.

As an alternative test for the role of PI4KIIIβ activity in the production of the early Golgi pool of PI4P in MDCK cells, GFP-Fapp1(PH)-expressing cells were treated with 10 μM WMN for 1 h at 37 °C prior to fixation. WMN is traditionally used as an inhibitor of PI3Ks (23) and at higher concentrations functions as a potent inhibitor of type III but not type II PI4Ks (13, 24, 25). Under these treatment conditions, MDCK cells developed large vacuoles that began to fill the cell interior. As a result, the Golgi complex became slightly dispersed (Fig. 4*C*); however, the distribution of furin remained essentially coincident with that of giantin, and biosynthetic transport was maintained for several hours.[3] Consistent with our observations in MDCK cells expressing PI4KIIIβ_{D656A}, WMN treatment caused a significant portion of the GFP-Fapp1(PH) signal to dissociate from the Golgi complex (Fig. 4*C*). In some cells, no residual Golgi staining was observed (Fig. 4*C*, *arrows*). These data suggest that PI4KIIIβ is largely responsible for maintaining the steady state pool of PI4P in the Golgi complex at steady state in MDCK cells.

To confirm the requirement for PI4KIIIβ activity in this process, we determined the effect of WMN treatment on *de novo* synthesis of Golgi PI4P using an *in vitro* reconstitution assay. MDCK cells transiently expressing the TGN marker GalT-YFP were incubated with 5 μM WMN for 1 h. Following WMN treatment, the cells were permeabilized with digitonin, then washed, and incubated with rat liver cytosol containing an ATP-regenerating system, GST-Fapp1(PH) and 5 μM WMN (to inhibit cytosolic PI4KIIIβ). The cells were then incubated at 32 °C for 30 min followed by fixation and immunostaining with antibodies against GST and GPP130. Under control conditions where no WMN was added, GST-Fapp1(PH) localized almost exclusively with GPP130, consistent with our observations in intact cells (Fig. 4*B*, *upper left panel*; GST-Fapp1(PH) *green*, GPP130 *red*). In contrast, little overlap between GST-Fapp1(PH) staining and GalT-YFP was observed (Fig. 4*B*, *lower left panel*; GST-Fapp1(PH), *green*, and GalT-YFP, *red*). The distribution of GST-Fapp1(PH) was significantly altered in WMN-treated cells. Under these conditions, GST-Fapp1(PH) staining was adjacent to but no longer coincident with GPP130 and instead localized almost completely with GalT-YFP (Fig. 4*B*, *right panels*). In control cells only 35% of the total GST-Fapp1(PH) colocalized with GalT-YFP-positive compartments, consistent with the fraction of GFP-Fapp1(PH) staining we observed in the late Golgi of intact cells (Fig. 5). In contrast, 62% of the GST-Fapp1(PH) staining colocalized with GalT-YFP in cells reconstituted with WMN-treated cytosol.

The experiments described above demonstrate that modulation of PI4KIIIβ activity *in vivo* and *in vitro* alters the recruitment of GFP-Fapp1(PH) to the Golgi complex as well as its suborganellar distribution. To quantitate the effects of altered PI4K expression on the steady state distribution of PI4P, MDCK cells expressing GFP-Fapp1(PH) alone (control) or co-expressing either wild-type PI4KIIIβ, the catalytically inactive mutant PI4KIIIβ_{D656A}, or PI4KIIα were immunolabeled using antibodies against GPP130 and furin. The cells were examined by confocal microscopy and processed for quantitative immunofluorescence as described above and under "Materials and Methods" (Fig. 5). In control cells, GFP-Fapp1(PH) staining was nearly twice as intense per unit area in the *cis/medial* Golgi compared with the TGN. We previously demonstrated that overexpression of PI4KIIIβ results in a severalfold increase in total cellular PIP levels (9); however, the *distribution* of GFP-Fapp1(PH) intensity across the Golgi complex was unchanged by overexpression of PI4KIIIβ, (*cis/medial versus* TGN ratios). These data suggest that overexpression of the kinase does not overwhelm the compartmentalization for this kinase or, alternatively, that substrate is limiting. In contrast, expression of PI4KIIIβ_{D656} nearly collapsed the skewed GFP-Fapp1(PH) distribution across the Golgi complex and resulted

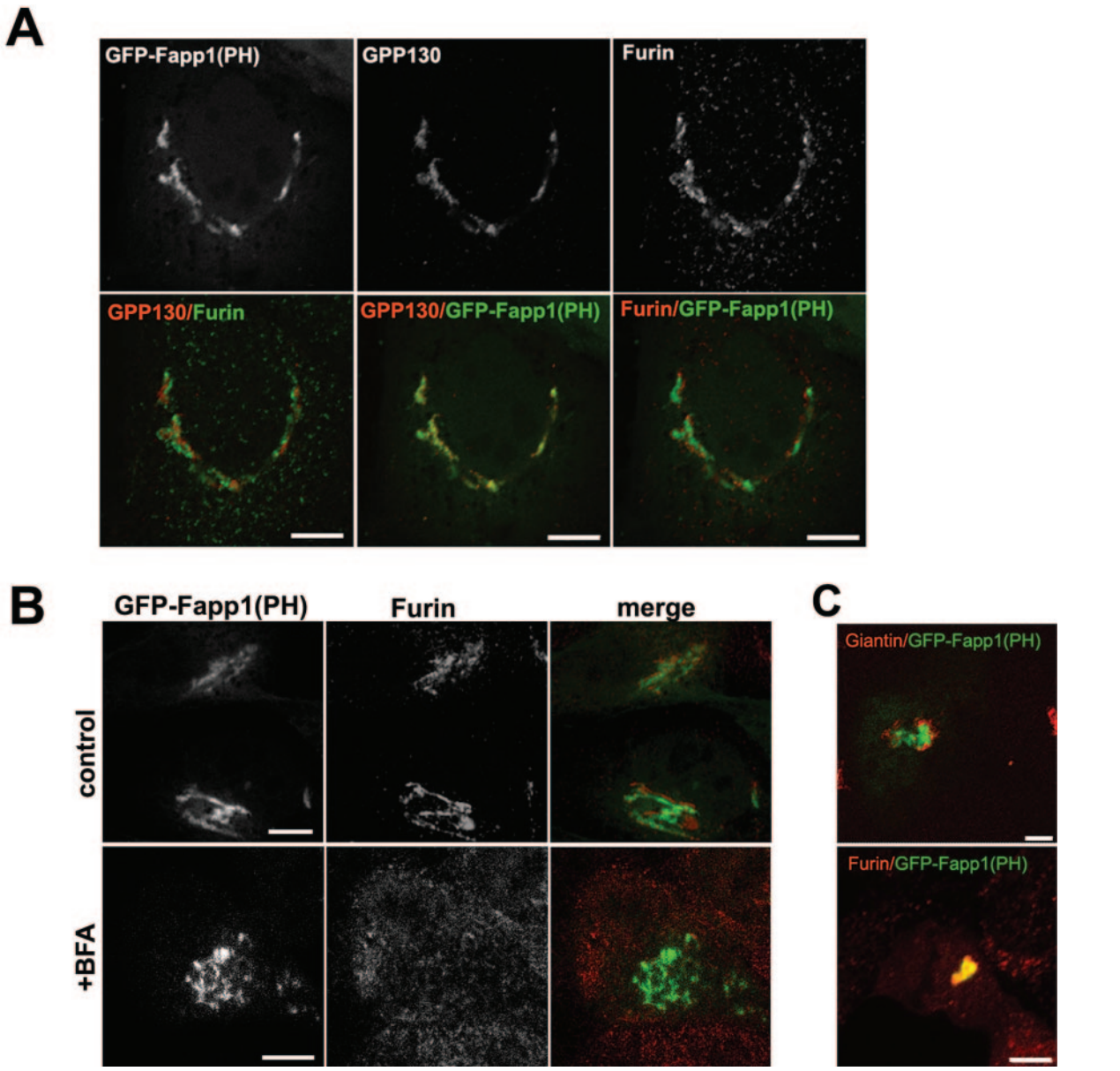

FIG. 3. **GFP-Fapp1(PH) localizes primarily to the early Golgi in MDCK cells.** *A*, MDCK cells expressing GFP-Fapp1(PH) were double-labeled with antibodies against the *cis/medial* Golgi marker GPP130 and the TGN marker furin and examined by confocal microscopy. The individual staining patterns are shown in the *upper panels*; the *lower panels* show the overlay of the GPP130 (*red*) and furin (*green*) channels (*left*), GPP130 (*red*) and GFP-Fapp1(PH) (*green*) channels (*center*), and furin (*red*) and GFP-Fapp1(PH) (*green*) channels (*right*). The data demonstrate that *cis/medial* Golgi and TGN subcompartments can be clearly distinguished by confocal microscopy and that GFP-Fapp1(PH) colocalizes predominantly with GPP130 rather than with furin. *Scale bars*, 5 μm. *B*, MDCK expressing GFP-Fapp1(PH) were incubated with 5 μg/ml BFA for 10 min prior to fixation and immunolabeling with anti-furin antibody. In contrast to the TGN, which is selectively dispersed by BFA in MDCK cells, GFP-Fapp1(PH) staining remained largely unaffected, consistent with its colocalization primarily with early Golgi markers. *Scale bars*, 5 μm. *C*, NRK cells expressing GFP-Fapp1(PH) were immunostained with giantin (*red*, *upper panel*) or furin (*red*, *lower panel*). In these cells, GFP-Fapp1(PH) localizes primarily to the TGN. *Scale bars*, 10 μm.

in similar densities of GFP-Fapp1(PH) intensity in the *cis/medial* Golgi and TGN. Overexpression of PI4KIIα had a similar effect on the distribution of PI4P, resulting in virtually equal mean voxel intensity in *cis/medial* Golgi and TGN compartments.

DISCUSSION

The goal of this study was to examine the distribution of PI4P across the Golgi complex of MDCK cells and to correlate changes in this distribution with the kinetic effects on polarized biosynthetic traffic that we observe when PI4P metabolism is perturbed. We found that two PI4Ks reported to maintain PI4P levels in the Golgi complex of mammalian cells occupy distinct subcompartments of this organelle, with PI4KIIIβ localizing primarily with *cis/medial* Golgi markers and PI4KIIα coincident with γ-adaptin in the TGN. Interestingly, the majority of PI4P localized with PI4KIIIβ to early Golgi compartments in MDCK cells at steady state. However, modulation of the activity of either of these kinases, either by overexpression or by expression of a dominant-negative mutant, was able to alter the distribution of PI4P across the Golgi complex. Together, these data reveal a previously unappreciated pool of PI4P in the early Golgi of these cells and suggest that compartmentalized pools of PI4P are generated by the action of distinct PI4Ks within the this organelle. These data allow us for the first time to correlate PI4K-mediated kinetic effects on intra- and post-Golgi traffic with alterations in the

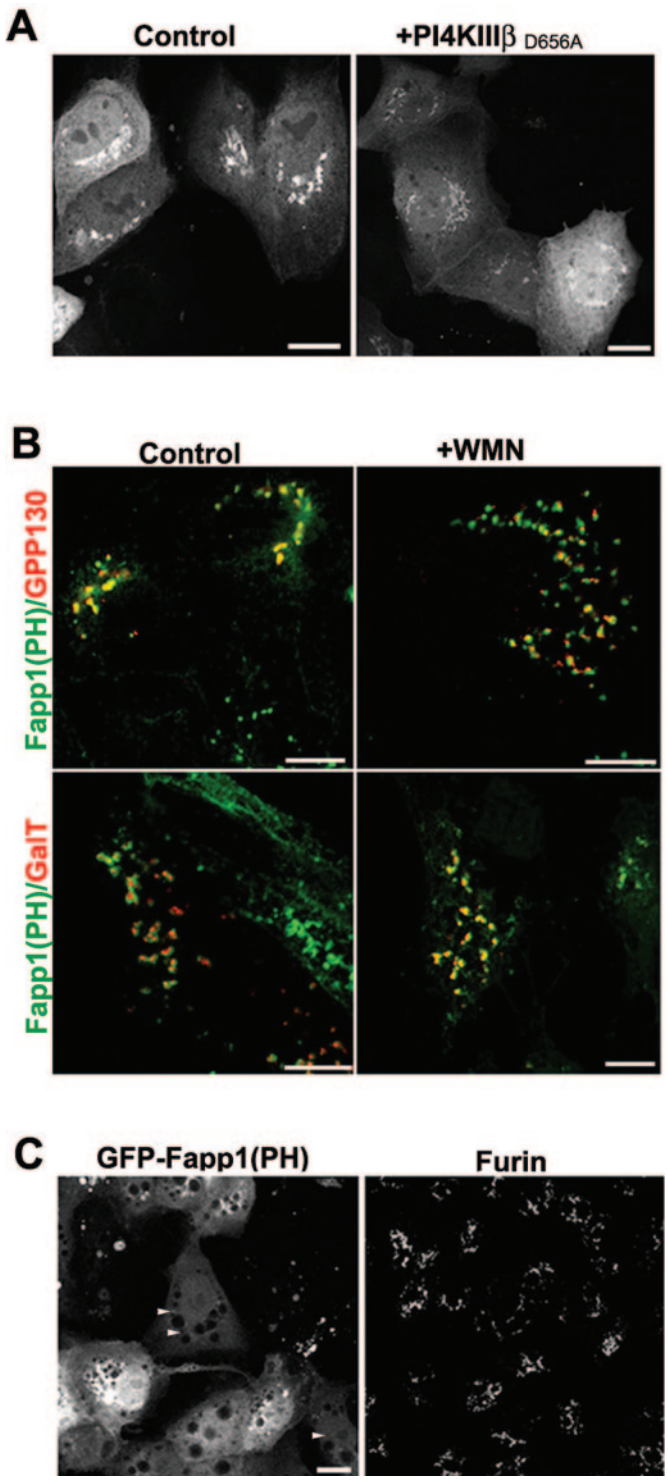

FIG. 4. **Inhibition of PI4KIIIβ activity reduces the association of GFP-Fapp1(PH) with the *cis/medial* Golgi.** *A*, MDCK cells transiently expressing GFP-Fapp1(PH) alone (control) or with the catalytically inactive mutant PI4KIIIβ_{D656A} were examined by confocal microscopy. Expression of PI4KIIIβ_{D656A} significantly reduced GFP-Fapp1(PH) targeting to the Golgi complex. *Scale bar*, 10 μm. *B*, WMN blocks resynthesis of the *cis/medial* Golgi pool of PI4P in semipermeabilized MDCK cells. MDCK cells transiently expressing the *trans*-Golgi marker GalT-YFP were treated with 5 μM WMN in culture for 1 h prior to permeabilization with digitonin. The cells were subsequently washed and reconstituted with an ATP-regenerating system and either control or WMN-treated cytosol containing 0.2 μM GST-Fapp1(PH). After incubation for 30 min at 32 °C, the cells were processed for immunofluorescence, and the distribution of GST-Fapp1(PH) (*green*), GPP130 (red, *upper panels*), and GalT-YFP (red, *lower panels*) was examined. GST-Fapp1(PH) was recruited to GPP130-positive compartments in cells reconstituted with control cytosol. By contrast, GST-Fapp1(PH) staining largely overlapped with GalT-YFP in cells reconstituted with WMN-treated cytosol. *Scale bars*, 10 μm. *C*, WMN largely abolishes GFP-Fapp1(PH) Golgi targeting in intact cells. MDCK cells transiently expressing GFP-Fapp1(PH) were incubated with 10 μM WMN for 1 h prior to fixation. GFP-Fapp1(PH) is redistributed in many cells, whereas furin staining remains intact. *Scale bar*, 10 μm.

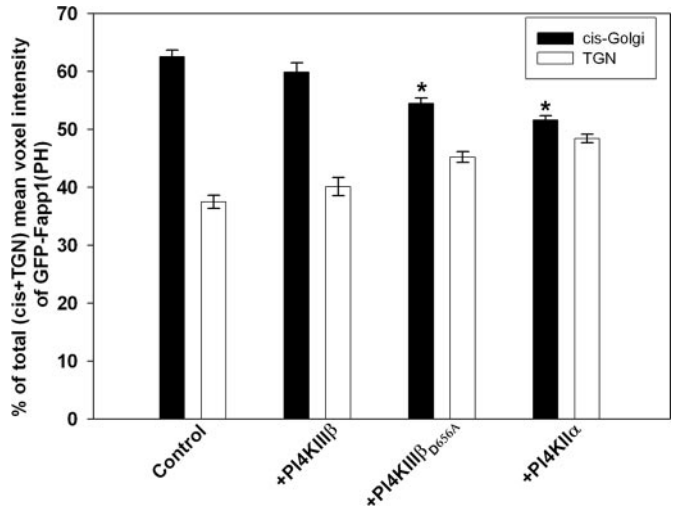

FIG. 5. **The intra-Golgi distribution of GFP-Fapp1(PH) in MDCK cells is modulated by PI4K activity.** MDCK cells transiently expressing GFP-Fapp1(PH) were infected prior to transfection with a control adenovirus or viruses encoding PI4KIIIβ or PI4KIIIβ_{D656A} or were cotransfected with PI4KIIα. The cells were processed for triple-labeled immunofluorescence using antibodies against GPP130 and furin as in Fig. 3. Confocal images of individual optical sections were imported into Metamorph™, and the mean voxel intensity of GFP-Fapp1(PH) in regions that colocalized with *cis/medial* Golgi *versus* TGN markers was quantitated as described under "Materials and Methods." The graph shows the percentage of GFP-Fapp1(PH) voxel intensity in *cis/medial versus* TGN compartments calculated for each condition. Whereas overexpression of wild-type PI4KIIIβ has no effect on the relative intra-Golgi distribution of GFP-Fapp1(PH), expression of either PI4KIIIβ_{D656A} or PI4KIIα significantly alters the distribution of this probe across the Golgi complex. *, $p < 0.001$ *versus* control by Student's *t* test; control, $n = 24$; PI4KIIIβ, $n = 24$; PI4KIIIβ_{D656A}, $n = 23$; PI4KIIα, $n = 7$.

levels and distribution of PI4P across the Golgi complex.

The ability of PI-selective PH domains to report changes in lipid composition has been previously documented (26, 27). In these studies, we have used the PH domain of Fapp1, which is highly specific for PI4P, to assess the roles of distinct PI4Ks in maintaining the steady state distribution of PI4P across the Golgi complex. Previous studies have documented PI4P selective targeting of the PH domains of Fapp1 and the oxysterol-binding protein to this organelle, and it is generally accepted that the Golgi complex is the major repository of this lipid in the cell at steady state (28–31). However, several issues potentially complicate the use of these PH domains as PI sensors. For example, although these probes clearly label the Golgi complex, they do not generally recognize smaller or more transient pools of PI4P in other compartments (32). It may be that other factors contribute to PH domain targeting (31), that these pools are masked by other proteins, or that a threshold level of PI4P is required for binding to be detected.

Another consistent theme that has emerged from previous studies using Fapp1 and oxysterol-binding protein is that PH domain targeting to this organelle is mediated by multiple interactions, which complicates the use of the PH domain as a direct probe for PI4P. The small G protein ARF1 has been implicated in recruiting both of these PH domains to the Golgi complex (22, 28, 29). Differential binding to ARF1 or other proteins may explain the differential localization of oxysterol-

binding protein homologs domains in yeast (30). Clearly, some relationship between Fapp1(PH) binding and ARF1 exists as BFA treatment abolished Fapp1 targeting to the Golgi complex in NRK cells (22). We did not observe BFA-dependent redistribution of GFP-Fapp1(PH) in MDCK cells; however, the ARF-GEFs that regulate ARF1 binding to the early Golgi of MDCK cells are insensitive to BFA treatment (21, 33). Furthermore, the asymmetric distribution of GFP-Fapp1(PH) is in contrast to the distribution of ARF1 in MDCK cells, which colocalized with all markers of the Golgi tested in the studies presented here (data not shown). Nevertheless, targeting of GFP-Fapp1(PH) to the Golgi complex is ultimately dependent upon its ability to bind PI4P, because a mutant construct with drastically reduced PI4P binding affinity was not targeted to this organelle. Furthermore, the localization of Fapp1(PH) was sensitive to changes in local PI4P metabolism because its distribution was shifted in response to changes in the expression of Golgi-localized PI4Ks. Moreover, Fapp1-PH binding was largely confined to the TGN when permeabilized cells were incubated with concentrations of wortmannin that inhibit PI4KIIIβ activity. Collectively, the data do not rule out other regulating features in Fapp1 binding to the Golgi complex but indicate that in monomer form, the PH domain of this protein is an effective reporter of relative *changes* in PI4P across this organelle.

The observation that Fapp1(PH) localized primarily to the *cis/medial* Golgi in MDCK cells is in marked contrast to its predominantly TGN localization in other systems (Ref. 22 and Fig. 3). The localization of GFP-Fapp1(PH) to early Golgi compartments in MDCK cells is consistent with our observation that the majority of PI4KIIIβ also localizes to these subcompartments and with the inability of BFA to dislodge Fapp1(PH) binding from the Golgi complex. The existence of a sizable pool of Fapp1(PH) in the early Golgi is consistent with the previously reported requirement for PI4P in intra-Golgi transport in mammalian cells and yeast (8, 9). Moreover, lipid remodeling leading to transient elevation of PI4P levels is required to regulate the export of newly synthesized biosynthetic cargo from the endoplasmic reticulum (34).[2] The differences in steady state distribution of Fapp1(PH) could therefore reflect cell type differences in the distribution or activity of the Golgi-associated PI kinases or of PI-catabolizing phosphatases or, alternatively, in the availability of substrate within distinct Golgi subcompartments.

Our data suggest that PI4KIIIβ is responsible for the majority but not all of the PI4P synthesis in the Golgi complex at steady state. Expression of a dominant-negative mutant of this enzyme or treatment with WMN dramatically reduced Golgi targeting of GFP-Fapp(PH) in intact cells. Moreover, when *de novo* synthesis of PI4P was reconstituted in permeabilized cells, the majority of staining colocalized with *cis/medial* Golgi markers. In contrast, inhibition of PI4KIIIβ activity upon treatment of the cells and cytosol with WMN resulted in *de novo* synthesis of PI4P primarily in later Golgi compartments. A tentative conclusion from these results is that PI4KIIIβ and PI4KIIα compete for the same pool of substrate, and under normal conditions, the majority of this pool is available first to PI4KIIIβ. The primary role for PI4KIIIβ in Golgi PI4P synthesis is consistent with the observations of Godi *et al.* (22); however, another group has suggested that PI4KIIα is responsible for the majority of PI4P production in this organelle (12).

Quantitative analysis of the GFP-Fapp1(PH) distribution in PI4KIIIβ_{D656A}-expressing cells that had residual Golgi staining revealed a significant shift toward the TGN in the intra-Golgi distribution of this reporter, presumably because of loss of PI4P synthesis in early Golgi compartments. Similarly, overexpression of PI4KIIα increased the relative amount of GFP-Fapp1(PH) staining in the TGN, most likely because of increased synthesis of PI4P in this compartment. It is important to note, however, that although the quantitative immunofluorescence method we used allows us to detect changes in the intra-Golgi distribution of GFP-Fapp1(PH) under these different conditions, we cannot measure changes in the total amount of PI4P in each compartment or in the Golgi as a whole.

How does the PI4K-mediated modulation of Fapp1(PH) distribution we observed correlate with the effects of these enzymes on biosynthetic traffic? We previously demonstrated that overexpression of PI4KIIIβ had no effect on intra-Golgi transport, whereas expression of PI4KIIIβ_{D656A} slowed the acquisition of endoglycosidase H resistance of an itinerant glycoprotein. Our observation here that the majority of Fapp1(PH) binds to the *cis/medial* Golgi is consistent with the hypothesis that a threshold level of PI4P is required for efficient intra-Golgi transport; however, production of excess PI4P does not stimulate this step.

In contrast to its inhibitory effect on intra-Golgi transport, expression of PI4KIIIβ_{D656A} stimulated TGN-to-apical membrane transport of HA. Conversely, overexpression of wild-type PI4KIIIβ inhibited apical delivery of this protein (9). Here we found that GFP-Fapp1(PH) binding to the Golgi complex is markedly reduced upon expression of PI4KIIIβ_{D656A}, consistent with an overall decrease in Golgi PI4P levels. Thus, our data support a negative regulatory role for PI4P in apical membrane delivery. Although ARF1 has been suggested to recruit PI4KIIIβ to the Golgi complex in some cell types (14), we have recently determined that HA export from the TGN does not require ARF1 (35). We conclude that PI4KIIIβ regulates apical membrane traffic via an ARF1-independent mechanism.

Interestingly, recent reports have demonstrated a requirement for PI4P synthesis in other post-Golgi transport steps. Targeting of EpsinR and the adaptor protein complex AP-1 to the TGN was recently shown to require PI4P, suggesting a role for this lipid in the TGN export of newly synthesized proteins destined for delivery to endosomes and lysosomes (10–12). Knockdown of PI4KIIα by small interference RNA blocked the binding of AP-1 to the TGN (10, 12), consistent with our observation that this enzyme localizes to AP-1-positive domains in the TGN. Biosynthetic delivery of vesicular stomatitis virus was also inhibited in these cells, although this appeared to be due to the inability to synthesize phosphatidylinositol 4,5-bisphosphate rather than to a direct role for PI4P (10, 12). Thus, PI4P in the TGN appears to have multiple and opposing roles in regulating biosynthetic transport of cargo directed toward distinct compartments.

The regulated production and distribution of PI4P is clearly very complex and highly dynamic. The activity and localization of competing enzymes, changes in substrate levels, and the metabolism to different PIs or lipids all contribute to a steady state balance of PI4P across the Golgi complex. Our studies demonstrate that this distribution is cell type-dependent and that PI4K-mediated changes in PI4P distribution can be detected across the Golgi complex using a PI4P selective reporter. At steady state, there exists a sizable pool of PI4P in early Golgi compartments of MDCK but not NRK cells. Both PI4KIIIβ and PI4KIIα appear to contribute to the generation of this PI4P pool, and modulation of the relative activities of these enzymes alters the distribution of PI4P across the Golgi complex. The ability to quantitate such changes allows us, for the first time, to correlate alterations in PI4P production and distribution with specific effects on the kinetics and fidelity of membrane trafficking and sorting pathways. Taken together, our data suggest that alterations in the level or compartmentalization of

PI4P may serve as an inverse regulator for the efficiency of distinct biosynthetic pathways that emanate from the Golgi complex.

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