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Multiple Roles for Phosphatidylinositol 4-Kinase in Biosynthetic Transport in Polarized Madin-Darby Canine Kidney Cells*

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Phosphatidylinositols (PI) play important roles in regulating numerous cellular processes including cytoskeletal organization and membrane trafficking. The control of PI metabolism by phosphatidylinositol kinases has been the subject of extensive investigation; however, little is known about how phosphatidylinositol kinases regulate traffic in polarized epithelial cells. Because phosphatidylinositol 4-kinase (PI4K)-mediated phosphatidylinositol 4-phosphate (PI(4)P) production has been suggested to regulate biosynthetic traffic in yeast and mammalian cells, we have examined the role of PI4Kβ in protein delivery in polarized MDCK cells, at different levels of the biosynthetic pathway. Expression of wild type PI4Kβ had no effect on the rate of transport of influenza hemagglutinin (HA) through the Golgi complex, but inhibited the rate of *trans*-Golgi network (TGN)-to-cell surface delivery of this protein. By contrast, expression of dominant-negative, kinase-dead PI4Kβ (PI4Kβ_{D656A}) inhibited intra-Golgi transport but stimulated TGN-to-cell surface delivery of HA. Moreover, expression of PI4Kβ_{D656A} significantly increased the solubility in cold Triton X-100 of HA staged in the TGN, suggesting that altered association of HA with lipid rafts may be responsible for the enhanced transport rate. Both wild type and kinase-dead PI4Kβ inhibited basolateral delivery of vesicular stomatitis virus G protein, suggesting an effector function for PI4Kβ in the regulation of basolateral traffic. Thus, by contrast with the observed requirement for PI4Kβ activity and PI(4)P for efficient transport in yeast, our data suggest that changes in PI(4)P levels can stimulate and inhibit Golgi to cell surface delivery in mammalian cells.

Phosphatidylinositols (PIs)[1] play important roles in myriad cellular processes, including signaling, membrane trafficking, cell proliferation, and cytoskeletal organization (see Refs. 1 and 2 for reviews.) These lipids are synthesized by specific kinases localized to distinct regions of cells (3). In turn, numerous effector molecules alter the activity of these kinases to enhance the production of particular phosphoinositide species and thus control the regulation of these various cellular functions.

The function of PI metabolism in biosynthetic traffic has been the subject of recent investigation. Several studies suggest that synthesis of phosphatidylinositol 4-phosphate (PI(4)P) mediated by PIK1, a phosphatidylinositol 4-kinase (PI4K), plays an important role in regulating biosynthetic traffic in yeast (4–8). Additionally, an elegant study in mammalian cells demonstrated that ADP-ribosylation factor recruits the β isoform of PI4K, a homolog of PIK1, to the Golgi complex, where it stimulates synthesis of PI(4)P and ultimately increases phosphatidylinositol 4,5-diphosphate (PIP_2) levels (9). However, the effect of remodeling phosphatidylinositol composition on membrane traffic through the Golgi complex was not tested. Moreover, the role of PI kinases in regulating biosynthetic traffic in polarized cells, where proteins are segregated in the *trans*-Golgi network (TGN) and differentially targeted to the apical or basolateral plasma membrane, has not been examined.

We previously demonstrated that frequenin, a positive modulator of PI4Kβ, selectively inhibited the delivery of newly synthesized influenza hemagglutinin (HA) from the TGN to the apical cell surface in polarized MDCK cells (10). This finding was somewhat surprising, given previous studies demonstrating that *inhibition* of PI(4)P synthesis blocked secretory traffic in yeast (7, 8, 11). To investigate this further, we generated recombinant adenoviruses encoding wild type and kinase-dead PI4Kβ and tested their effects on cellular PI composition and on traffic through the secretory pathway in polarized cells. Our data reveal a complex role for PI4Ks at multiple steps along the biosynthetic pathway.

MATERIALS AND METHODS

Recombinant Adenoviruses—cDNA encoding HA-tagged wild type PI4Kβ was obtained from Dr. Rachel Meyers (Millenium Pharmaceuticals) and subcloned into the pAdtet vector (12). Protein expression from these viruses requires the presence of the tetracycline transactivator (stably expressed in the MDCK T23 cells used in this study; Ref. 13). The kinase-dead mutant PI4Kβ_{D656A} (9) was generated using the QuikChange mutagenesis kit from Stratagene. This mutant has been demonstrated previously to be defective in catalysis of PI(4)P (14). Recombinant adenoviruses (AVs) encoding vesicular stomatitis virus (VSV) G protein, PI4Kβ, and PI4Kβ_{D656A} were generated using the method described in Ref. 12. Polarized MDCK T23 cells, which stably express pIgR as well as the tetracycline transactivator were infected with recombinant AVs encoding PI4Ks (m.o.i. 250 unless otherwise indicated) and HA (m.o.i. 25) as described in Ref. 15 and used the following day. Protein expression in infected cells was confirmed by Western blotting using anti-PI4K and anti-HA epitope tag antibodies

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[1] The abbreviations used are: PI, phosphatidylinositol; AV, adenovirus; DMEM, Dulbecco's modified Eagle's medium; endo H, endoglycosidase H; HA, influenza hemagglutinin; MDCK, Madin-Darby canine kidney; m.o.i., multiplicity of infection; PA, phosphatidic acid; PBS, phosphate-buffered saline; pIgR, polymeric immunoglobulin receptor; PI4K, phosphatidylinositol 4-kinase; PIP, phosphatidylinositol phosphate; PI(4)P, phosphatidylinositol 4-phosphate; PIP_2, phosphatidylinositol 4,5-diphosphate; PM, plasma membrane; TGN, *trans*-Golgi network; TLC, thin layer chromatography; VSV, vesicular stomatitis virus.

(from Upstate Biotechnology and Roche, respectively), and performed as described in Ref. 10.

Indirect Immunofluorescence—To localize PI4K proteins, AV-infected MDCK cells grown on coverslips were fixed in 3% paraformaldehyde, rinsed with PBS containing 10 mM glycine (PBS-G), then permeabilized with 0.5% Triton X-100 in PBS-G for 3 min at ambient temperature. After washing, nonspecific binding sites were blocked by incubation for 5 min in PBS-G containing 0.25% w/v ovalbumin. Cells were then incubated for 1 h with primary antibodies (monoclonal anti-HA, 1:100 dilution and polyclonal anti-giantin, provided by Dr. Adam Linstedt; 1:100 dilution followed by extensive washing in blocking buffer and incubation for 1 h with secondary antibodies (Texas Red conjugated goat anti-mouse and fluorescein isothiocyanate-conjugated goat anti-rabbit (both from Jackson Laboratories, 1:500 dilution each). After washing, samples were post-fixed briefly with 3% paraformaldehyde, mounted in 1 M *n*-propyl gallate in glycerol, and viewed using a Nikon Eclipse TE300 inverted microscope using a 60× oil immersion objective. Images were captured with a Hamamatsu C4742–95 digital CCD camera using Openlab software (Improvision) and adjusted using Photoshop software (Adobe).

Analysis of Phosphatidylinositol Lipids—PI lipid analysis was performed essentially as described in Ref. 16. Briefly, AV-infected MDCK cells grown on 6 cm dishes were starved in phosphate-free DMEM for 30 min, radiolabeled for 4 h with 40 μCi/ml [^{32}P]orthophosphate (ICN) in phosphate-free DMEM, then rinsed and scraped into 0.5 ml of ice-cold PBS. 1.9 ml of chloroform:methanol:concentrated HCl (100:200:1) was added and the mixture incubated for 10 min on ice. Chloroform and 0.1 N HCl (3 ml each) were added to induce phase separation, and the organic phase was collected and washed twice with an equal volume of MeOH and 0.1 N HCl (1:1). Aliquots were counted using a scintillation counter, and equal counts/min were spotted onto oxalate-treated Silica gel 60 TLC plates (EM Science) and developed in 1-propanol plus 2 M acetic acid (65:35). Authentic lipid standards (Avanti Polar Lipids) were included in all runs and visualized using iodine vapor. Radiolabeled products were visualized and quantitated using a phosphorimager.

Protein Trafficking Assays—The kinetics of endoglycosidase H (endo H) resistance and sialylation of HA were determined as described in Ref. 17. Cell surface delivery of HA was quantitated using the trypsinization assay described in Ref. 18. The antibody used to immunoprecipitate HA does not recognize the HA epitope tag on PI4K. The rate of basolateral delivery of VSV G was quantitated by cell surface biotinylation using the method described in Ref. 19. VSV G was immunoprecipitated using antibody 8G5 (hybridoma provided by Dr. Douglas Lyles, Wake Forest University; Ref. 20).

Recovery of Triton-insoluble HA—Detergent solubility of HA was assessed using a modification of the method described in (21). Virally infected filter-grown MDCK cells were starved for 30 min, radiolabeled for 15 min with ^{35}S labeling mix (ICN), and chased at 19 °C for the indicated periods. The cells were rapidly chilled by placing the dishes on an aluminum sheet in a pan of ice and washing with a large volume of ice-cold PBS, and then solubilized for 20 min in ice cold TNET (25 mM Tris-HCl, pH 7.5, 150 mM NaCl, 5 mM EDTA, 1% (v/v) Triton X-100). Lysates were centrifuged for 10 min at 4 °C at maximum speed in an Eppendorf 5415C microcentrifuge, and the supernatants (cold Triton-soluble fraction) transferred to fresh tubes. The transwell filters were cut out of the inserts, combined with the detergent-insoluble pellets, and warm TNET (0.5 ml) was added. After 30 min at 37 °C, the samples (cold Triton-insoluble fraction) were centrifuged briefly to pellet nuclei. A 5-fold concentrated stock of detergent solution (1× is 50 mM Tris-HCl, 2% Nonidet P-40, 0.4% deoxycholate, 62.5 mM EDTA, pH 8.0) and SDS (final concentration 0.1%) were added, and samples were immunoprecipitated using monoclonal anti-HA antibody Fc125 and analyzed after SDS-PAGE using a Personal FX phosphorimager (Bio-Rad).

RESULTS

Expression and Localization of PI4Kβ and PI4Kβ$_{D656A}$ in MDCK Cells—To confirm that PI4K was efficiently expressed from our recombinant AVs, we blotted lysates from infected MDCK cells using commercially available antibodies against PI4Kβ and the HA epitope tag. As shown in Fig. 1, endogenous PI4Kβ was easily detected using the anti-PI4Kβ antibody; however, infection with as low as m.o.i. 25 resulted in dramatic overexpression of wild type PI4Kβ or PI4Kβ$_{D656A}$ relative to control levels. Blotting with anti-HA tag antibody confirmed the expression of PI4Kβ in these cells.

Endogenous PI4Kβ has been localized previously to the Golgi complex and cytoplasm of cells (9, 22). We therefore localized virally expressed PI4Kβ in MDCK cells using anti-HA antibody (Fig. 2). As predicted, we detected a significant amount of cytoplasmic PI4Kβ, as well as some Golgi staining. Interestingly, in this cell type, we did not observe any detectable effect of either wild type PI4Kβ or PI4Kβ$_{D656A}$ on overall Golgi morphology, in contrast to a previous report in which dominant-negative PI4Kβ dramatically disrupted Golgi structure when expressed in COS cells (9).

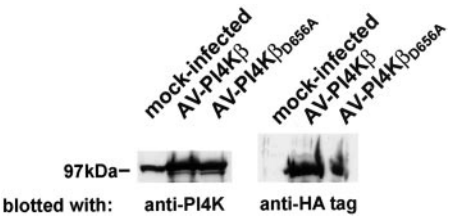

FIG. 1. **Expression of PI4Kβ and PI4Kβ$_{D656A}$ in MDCK cells.** MDCK cells were mock-infected or infected with adenoviruses encoding HA epitope-tagged wild type (PI4Kβ) or mutant kinase (PI4Kβ$_{D656A}$) at m.o.i. 25. The following day, cells were solubilized and lysates analyzed by Western blotting using commercially available anti-PI4K and anti-HA antibodies.

To confirm PI4Kβ activity in our system, we examined the effect of PI4K expression on cellular PI lipid composition (Fig. 3). Cells infected with control AV or AVs encoding PI4Ks or frequenin were labeled with [^{32}P]orthophosphate and lipids extracted as described under "Materials and Methods." Samples were analyzed by TLC and compared against authentic lipid standards. Only four lipids were recovered by this procedure: phosphatidic acid (PA), PI, PIP, and PIP_2. AV infection did not significantly affect the levels or ratios of radiolabel incorporation into these lipids compared with mock-infected cells (data not shown). Roughly equivalent incorporation of ^{32}P into PA, PI, and PIP was observed in control cells, with little incorporation into PIP_2. Expression of PI4Kβ$_{D656A}$ did not appreciably alter the ratios of radiolabeled lipids recovered. However, expression of wild type PI4Kβ or frequenin significantly increased the amount of radiolabeled PIP recovered in MDCK cells at the expense of PA and PI. Coexpression of equivalent amounts of PI4Kβ and PI4Kβ$_{D656A}$ resulted in a radiolabeled lipid profile equivalent to that observed with PI4Kβ alone.

Effects of PI4Kβ and PI4Kβ$_{D656A}$ on Intra-Golgi Traffic—We previously showed that overexpression of frequenin had no effect on the kinetics of endo H resistance of HA, but selectively inhibited TGN-to-cell surface delivery of this marker protein. Therefore, we expected that overexpression of wild type PI4Kβ would mimic the effect of frequenin on protein traffic. We first examined the effects of PI4Kβ and PI4Kβ$_{D656A}$ on traffic through the Golgi complex by monitoring the kinetics of acquisition of endo H resistance and sialylation. MDCK cells were coinfected with AV-HA and either control or PI4Kβ-encoding viruses, and transport assays performed as described under "Materials and Methods." Importantly, the level of HA expression was unaffected by coexpression with PI4Kβs (Fig. 4*A*). As predicted, PI4Kβ expression had no effect on the rate of HA transport through the Golgi complex (Fig. 4, *A–C*). However, expression of PI4Kβ$_{D656A}$ slightly but very reproducibly slowed the rate of HA acquisition of endo H resistance and sialylation. The effect of PI4Kβ$_{D656A}$ on sialylation kinetics was always greater than the effect on endo H resistance (Fig. 4, compare *C* and *B*), suggesting that this mutant acts at multiple steps in intra-Golgi transport. Coexpression of wild type PI4Kβ with PI4Kβ$_{D656A}$ fully restored normal kinetics of sialylation, demonstrating that the effect of PI4Kβ$_{D656A}$ was not caused by viral toxicity (Fig. 4*D*). Complete recovery was observed even when wild type PI4Kβ was expressed at half the level of PI4Kβ$_{D656A}$.

PI4Kβ$_{D656A}$ and PI4Kβ$_{D656A}$ Have Opposite Effects on Post-Golgi Transport of HA—We next examined the effect of PI4Ks on TGN-to-plasma membrane (PM) delivery of HA. Because PI4Kβ$_{D656A}$ inhibited transport through the early Golgi, we staged HA in the TGN using a 19 °C temperature block. Initial control experiments showed that after a 2-h chase at 19 °C, significantly less endo H-resistant, sialylated HA had accumulated in PI4Kβ$_{D656A}$-expressing cells compared with control cells, but that this discrepancy was nearly equalized by 4 h of chase (data not shown). However, the amount of HA reaching the plasma membrane after 4 h of chase was significantly lower in PI4Kβ$_{D656A}$-expressing cells compared with control cells. Therefore, to simplify the kinetic analysis of TGN-to-PM delivery, we subtracted the amount of HA present at 0 min of chase under each condition from subsequent time points. Expression of wild type PI4Kβ inhibited HA TGN-to-PM delivery (Fig. 5*A*), whereas PI4Kβ$_{D656A}$ stimulated this step in transport (Fig. 5*B*). Although the rate of HA delivery in control cells varied significantly between experiments, making statistical analysis difficult, inhibition by PI4Kβ and stimulation by PI4Kβ$_{D656A}$ were very reproducible and were observed in >25 experiments. However, neither PI4Kβ$_{D656A}$ nor wild type PI4Kβ affected the ultimate polarity of HA delivery measured after long chase times (Fig. 6), suggesting that these proteins exert kinetic but not sorting effects on apical membrane traffic. Coexpression of wild type PI4Kβ blocked PI4Kβ$_{D656A}$ stimulation of HA delivery (data not shown).

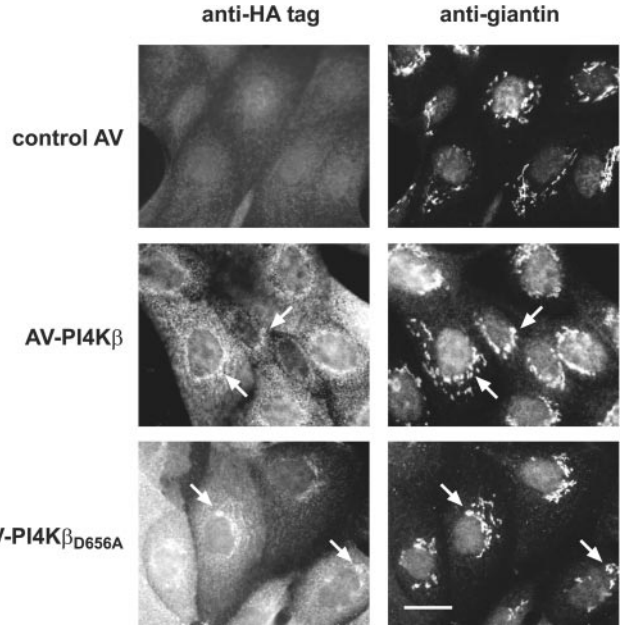

FIG. 2. **Localization of PI4Kβ in MDCK cells.** MDCK cells expressing HA epitope-tagged PI4Kβ or PI4Kβ$_{D656A}$ were fixed and processed for double label indirect immunofluorescence using antibodies against the HA epitope tag and the Golgi marker giantin. *Arrows* indicate areas where colocalization is apparent. *Scale bar*, 10 μm.

Effects of PI4Kβ or PI4Kβ$_{D656A}$ on Transport to the Basolateral Cell Surface—We demonstrated previously that overexpression of frequenin has no effect on basolateral delivery of pIgR in polarized MDCK cells (10). Similarly, expression of wild type PI4Kβ or PI4Kβ$_{D656A}$ had no effect on the rate of pIgR delivery (data not shown). However, because it is difficult to accumulate newly synthesized pIgR in the TGN, it was not possible to test for differential effects of PI4Ks on early *versus* late biosynthetic transport of this protein. Therefore, we examined the effect of PI4K expression on the basolateral surface transport of another well characterized protein, VSV G, which is easily staged in the TGN. Preliminary experiments demonstrated that a 2-h chase at 19 °C resulted in intracellular accumulation of fully mature VSV G with little leakage to the PM, independent of PI4K expression. Surprisingly, expression of either wild type PI4Kβ or PI4Kβ$_{D656A}$ inhibited the rate of transport of newly synthesized VSV G to the basolateral surface, with PI4Kβ routinely having a more potent effect than PI4Kβ$_{D656A}$ (Fig. 7).

PI4Kβ$_{D656A}$ Expression Alters HA Insolubility in Cold Triton X-100—Because PI(4)P and PIP_2 are components of lipid rafts, and because raft association has been suggested to play a role in HA delivery to the apical membrane (23, 24), we examined the effects of wild type PI4K-β and PI4Kβ$_{D656A}$ expression on HA insolubility in cold Triton X-100. HA is thought to associate with detergent-insoluble lipid microdomains in the Golgi complex after addition of complex sugars (25, 26). Cells infected with AV-HA and either control virus or viruses encoding PIKs were starved, radiolabeled for 15 min, and chased at 19 °C. The cells were then rapidly chilled and solubilized in ice-cold Triton X-100-containing solution. Detergent-soluble and -insoluble fractions were isolated, immunoprecipitated with anti-HA an-

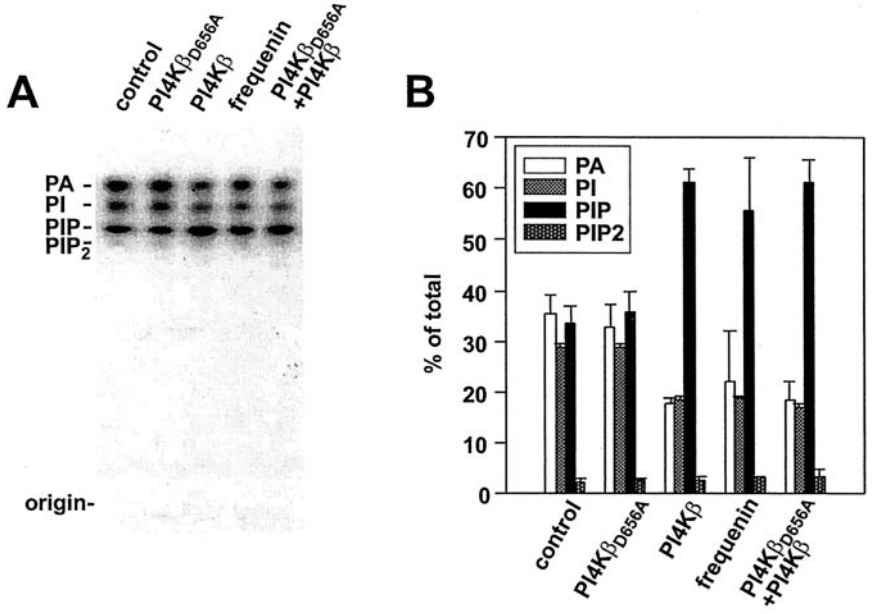

FIG. 3. **Effects of PI kinases on total cellular phosphatidylinositol ratios.** MDCK cells were infected with control AV or AVs encoding PI4Kβ$_{D656A}$, PI4Kβ, frequenin, or PI4Kβ$_{D656A}$ plus PI4Kβ (m.o.i. 250 each). The following day, cells were starved in phosphate-free DMEM, labeled for 4 h with 40 μCi/ml of [^{32}P]orthophosphate, then rinsed and phosphatidylinositol lipids extracted as described under "Materials and Methods." Equivalent counts/min from each sample were analyzed by TLC. A representative TLC plate is shown in *panel A*, and the origin and migration position of PA, PI, PI(4)P, and PIP_2 lipid standards are noted. Quantitation of the lipid composition determined in two independent experiments (average ± range) is plotted in *panel B*.

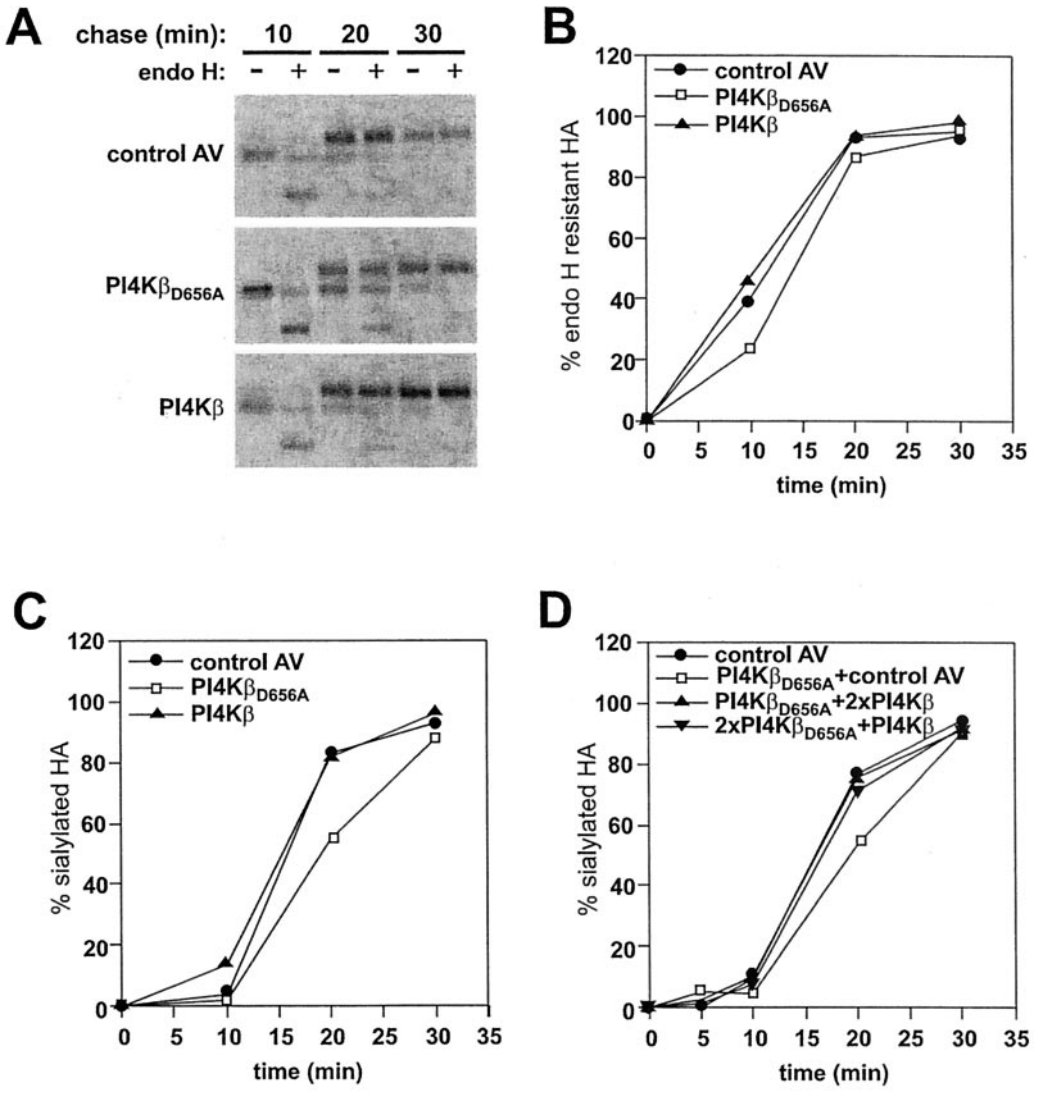

FIG. 4. **PI4Kβ$_{D656A}$ inhibits intra-Golgi transport kinetics.** MDCK cells were co-infected with AV-HA and the indicated viruses. The following day, cells were starved, radiolabeled for 5 min, then chased for the indicated periods at 37 °C. HA was immunoprecipitated from the cell lysates. The gel is shown (*panel A*), and the kinetics of endo H resistance (*panel B*) and sialylation (*panel C*) were determined. Similar results were obtained in three experiments. *Panel D* shows the quantitation of a similar experiment in which the ability of PI4Kβ to block PI4Kβ$_{D656A}$ inhibition of intra-Golgi transport was tested. Cells were co-infected with AV-HA (m.o.i. 25) plus the indicated viruses or mixture of viruses (m.o.i. 750 total in each case), and the kinetics of HA sialylation determined. PI4Kβ expressed at half the concentration of PI4Kβ$_{D656A}$ (*upside-down triangles*) fully restored the kinetics of HA sialylation.

tibody (which does not recognize the HA epitope on PI4Kβ), and the fraction of sialylated HA that was detergent-insoluble was calculated (Fig. 8). Surprisingly, whereas expression of wild type PI4Kβ had no effect on the fraction of HA that was detergent-insoluble, HA recovered from cells expressing PI4Kβ$_{D656A}$ was considerably more soluble in cold Triton X-100 than HA recovered from control cells. Moreover, co-expression of wild type PI4Kβ blocked the effect of PI4Kβ$_{D656A}$ and restored HA insolubility to control levels. Similar results were obtained regardless of whether the cells were chased for 2 or 4 h at 19 °C. However, when the cells were warmed to 37 °C for 1 h to allow HA transport to the PM, overall HA insolubility increased slightly and there was no longer a difference between control and PI4Kβ$_{D656A}$-expressing cells (54 ± 5.0% insoluble in control cells *versus* 51 ± 4.4% insoluble in PI4Kβ$_{D656A}$ cells, $n = 6$). Thus, the increased detergent solubility in cells expressing PI4Kβ$_{D656A}$ may be a transient characteristic of TGN-staged HA.

DISCUSSION

PIKs have been implicated in the regulation of numerous transport steps along the biosynthetic and endocytic pathways (reviewed in Refs. 1, 2, and 27). These proteins can act directly to alter PI composition in cells, but can also exert their effects by modulating the activity of other proteins that regulate membrane traffic. The development of dominant-negative, kinase-dead mutants that compete with endogenous PIKs for effector binding has aided in the analysis of this complex field. Here we report that expression of a kinase-dead mutant of PI4Kβ inhibits intra-Golgi traffic but selectively stimulates delivery of an apical marker from the TGN to the cell surface of polarized MDCK cells. Moreover, expression of mutant PI4Kβ transiently alters the solubility properties of influenza HA in cold Triton X-100. Together, our data demonstrate that PI4Kβ acts as both a positive and negative regulator of biosynthetic transport.

Regulation of Intra-Golgi Traffic by PI4Kβ—There is consid-

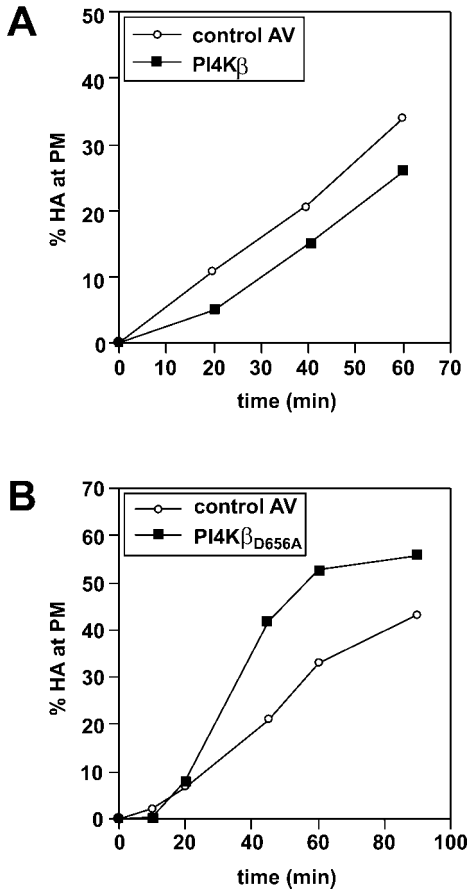

FIG. 5. **Effect of PI kinases on TGN-to-PM delivery of HA.** MDCK cells were infected with AV-HA and control or PI4Kβ-encoding viruses (wild type or mutant) as indicated below. Cells were starved, radiolabeled for 15 min, and chased for 4 h at 19 °C. The medium was replaced with pre-warmed medium, the cells were transferred to 37 °C for the indicated periods, and HA delivery to the plasma membrane was quantitated. *Panel A*, PI4Kβ inhibits HA apical delivery from the TGN. *Panel B*, PI4Kβ$_{D656A}$ stimulates HA apical delivery from the TGN. To directly compare the rates of HA TGN-to-PM delivery in this graph, the amount of HA that had reached at the PM prior to warm-up after the 19 °C chase was subtracted from each time point (9% for control, 2% for PI4Kβ$_{D656A}$-expressing cells).

erable evidence from previous studies that decreased PI(4)P levels in yeast cause defects in secretion. Yeast with a temperature-sensitive mutation in SEC14, which encodes an essential PI transfer protein required for formation of Golgi-derived transport vesicles (4, 5), have decreased levels of PI(4)P at the nonpermissive temperature and overexpression of Pik1 suppressed the temperature-sensitive defect in SEC14 cell transport (11). Moreover, cells carrying temperature-sensitive Pik1 mutations had decreased PI(4)P levels and delayed kinetics of invertase secretion and vacuolar delivery of carboxypeptidase Y (6–8, 11). Because Pik1 localizes to the *trans*-Golgi and because mutants accumulate Golgi membranes (see below), this enzyme appears to play an important role in Golgi-to-plasma membrane delivery in yeast (7). By contrast, the other PI4K in yeast, Stt4p, plays a distinct, non-overlapping function and appears to be important in regulating actin cytoskeleton organization, vacuolar morphology, and cell wall integrity (8).

We found that expression of wild type PI4Kβ in polarized MDCK cells had no effect on intra-Golgi traffic. These data are consistent with our previous studies, which showed that overexpression of the PI4Kβ activator frequenin had no effect on this step in biosynthetic transport (10). By contrast, expression of dominant-negative PI4Kβ reduced the rate at which newly synthesized HA traversed the Golgi complex. We observed a slight decrease in the rate of acquisition of endo H resistance, and greater effect on the rate of HA sialylation in cells expressing PI4Kβ$_{D656A}$ compared with control. This suggests that PI4Kβ regulates transport in multiple Golgi compartments, resulting in a cumulative slowing of transport as proteins traverse each cisterna of the Golgi complex. One possibility is that a threshold level of PI(4)P is required for efficient transport through the Golgi complex, but that increased amounts are not stimulatory.

PI4Kβ Regulates TGN to Apical Cell Surface Delivery—As predicted by our previous findings with frequenin, overexpression of wild type PI4Kβ inhibited TGN-to-PM delivery of HA, whereas kinase-dead PI4Kβ stimulated this step in transport. These data contrast with the observed requirement for PI4Kβ activity and PI(4)P for efficient transport in yeast (see above) and suggest that changes in PI(4)P levels can both positively and negatively regulate Golgi-to-cell surface delivery.

The stimulation of HA cell surface delivery by PI4Kβ$_{D656A}$ could be caused by an increase in the rate of HA exit from the TGN or to more efficient vesicle trafficking to/fusion with the apical plasma membrane. We believe that the effect we observe occurs at the level of the TGN, because both wild type and kinase-dead PI4Kβ localize to the Golgi complex. Moreover, mutant PI4Kβ does not stimulate apical release from vesicles carrying recycling or transcytosed proteins in polarized MDCK cells.[2] Although this does not preclude a specific role for PI4Kβ in fusion of Golgi-derived vesicles with the apical plasma membrane, there does not appear to be a general enhancement of vesicle fusion with the apical plasma membrane in these cells.

We demonstrated previously that overexpression of frequenin has no effect on the basolateral delivery of newly synthesized pIgR in polarized MDCK cells (10). Additional experiments demonstrated that neither wild type PI4Kβ nor PI4Kβ$_{D656A}$ affected the overall transport rate of pIgR to the basolateral cell surface. However, because this protein is not efficiently staged in the TGN at 19 °C,[3] these experiments cannot identify differential effects on pre- and post-TGN transport. Therefore we examined the effects of wild type PI4Kβ and PI4Kβ$_{D656A}$ on transport of another basolateral marker, VSV G. Surprisingly, both wild type and dominant-negative PI4Kβ significantly inhibited TGN-to-basolateral delivery of this protein. One possible explanation for this disparity is that the pIgR transport assay is not sensitive enough to detect inhibition by the PI4Ks. In this regard, because PI4Kβ$_{D656A}$ decreases the rate of intra-Golgi transport, we expected to observe at least a slight inhibition of transport by this protein, yet we saw no effect. However, another perturbant of biosynthetic traffic, bafilomycin A1, significantly and reproducibly inhibited pIgR transport in this assay. Alternatively, it is possible that basolateral transport of pIgR and VSV G are regulated by different mechanisms. Regardless, the inhibition of VSV G transport by both wild type and dominant-negative PI4Kβ that we observed suggests that basolateral trafficking of this protein is not directly regulated by PI4K enzymatic activity, but rather via effector molecules that interact with PI4Kβ. For example, protein kinase C has been shown to be important for efficient budding of TGN-derived vesicles, but its phosphorylating activity was not required (28).

Expression of Dominant Negative PI4β Alters HA Detergent Solubility—Expression of PI4Kβ$_{D656A}$ but not wild type PI4Kβ significantly decreased the amount of HA that was insoluble in cold Triton X-100 after a 19 °C chase. However, no difference in HA solubility was detected when the cells were warmed to

[2] J. R. Bruns, M. T. Miedel, and O. A. Weisz, unpublished data.
[3] G. Apodaca, personal communication.

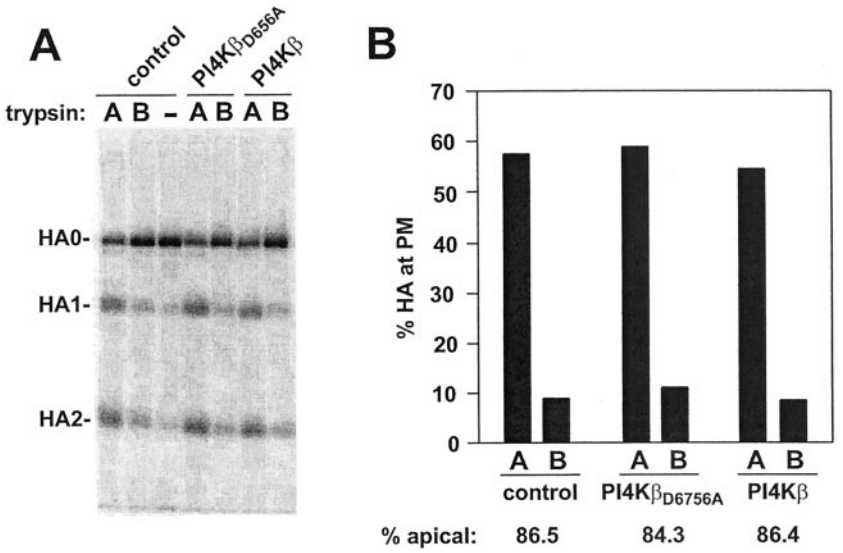

FIG. 6. **PI4Kβ expression does not affect the polarity of HA delivery.** MDCK cells coinfected with AV-HA and the indicated viruses were starved, radiolabeled for 15 min, then chased for 5 h at 37 °C. The filters were rapidly chilled and incubated with apically (*lanes* marked *A*) or basolaterally (*lanes* marked *B*) added trypsin for 30 min on ice. One control sample was left untreated (−) to determine the amount of HA cleavage by endogenous proteases. After multiple washes, residual trypsin was quenched with soybean trypsin inhibitor, the cells were solubilized, and HA (along with the trypsin cleavage products HA1 and HA2) were immunoprecipitated. The gel is shown in *panel A*. The amount of HA at each plasma membrane (after subtraction of endogenously cleaved HA, 4.5%) is plotted in *panel B*, and the calculated polarity of cell surface HA is noted *below* each set of samples. Similar results were obtained in two independent experiments.

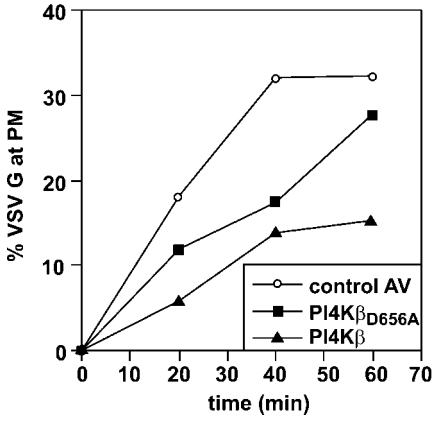

FIG. 7. **PI4Ks inhibits TGN-to-basolateral surface delivery of VSV G.** MDCK cells were coinfected with AV-VSV G and the indicated viruses. The following day, cells were starved, radiolabeled for 15 min, and chased for 2 h at 19 °C. The medium was then replaced with prewarmed medium, the cells were transferred to 37 °C for the indicated periods, and VSV G delivery to the plasma membrane was quantitated by biotinylation. The amount of VSV G that had reached at the PM prior to warm-up after the 19 °C chase (<6% in each case) was subtracted from each time point. Similar results were obtained in three experiments.

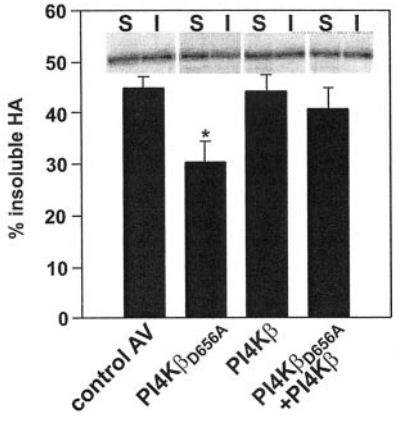

FIG. 8. **PI4Kβ$_{D656A}$ reduces detergent solubility of HA in the TGN.** MDCK cells coinfected with AV-HA and the indicated viruses were starved, radiolabeled for 15 min, then chased at 19 °C for 4 h. In these experiments, PI4Kβ was expressed at twice the m.o.i. of PI4Kβ$_{D656A}$. The cells were rapidly chilled by rinsing with ice-cold PBS, then solubilized in ice-cold Triton X-100-containing solution and soluble (*S*) and insoluble (*I*) HA separated and immunoprecipitated as described under "Materials and Methods." The fraction of mature HA that was detergent insoluble (mean ± S.E. of four experiments performed in quadruplicate) is plotted, and representative samples are shown in the *inset*. Expression of PI4Kβ$_{D656A}$ significantly reduced the fraction of HA that was insoluble in cold Triton X-100 ($p < 0.05$ *versus* all other conditions), and the effect of PI4Kβ$_{D656A}$ was reversed by coexpression of excess PI4Kβ.

37 °C to allow cell surface transport to proceed. There are several possible explanations for these observations. One is that HA staged at 19 °C in control cells has access to a later, possibly post-TGN, compartment than HA in cells expressing PI4Kβ$_{D656A}$; in this regard, cell surface HA was slightly more detergent-insoluble than HA staged at 19 °C. This would be consistent with our observation that more HA had reached the PM after 4 h of chase in control cells than in PI4Kβ$_{D656A}$-expressing cells; however, this is difficult to reconcile with the overall stimulation in transport rate of HA that we observed in PI4Kβ$_{D656A}$-expressing cells. Another more intriguing possibility is that expression of PI4Kβ$_{D656A}$ alters Golgi/TGN lipid composition in a manner that selectively affects HA association with lipid microdomains in this organelle. The selective effect on HA solubility we observed at 19 °C *versus* 37 °C would be consistent with the presence of distinct, non-miscible pools of PIP at different cellular locations (6, 8).

Although a lipid-mediated effect on HA solubility is unexpected because lipid rafts are thought to be held together by lipid-lipid interactions in the luminal rather than the cytoplasmic domain, this has never been rigorously tested. Interestingly, several reports have demonstrated enrichment of PI(4)P and PI(4,5)P_2 in detergent-insoluble lipid domains (29–32). This is of particular interest because lipid rafts have been suggested to function as platforms from which signal transduction cascades can be initiated (33, 34). Our data suggest that a linkage may exist between generation of PI species and the regulation of apical transport.

Role of PI(4)P in Biosynthetic Transport—Although we observed a clear increase in ^{32}P-labeled PIP recovered from metabolically labeled cells overexpressing wild type PI4Kβ, expression of PI4Kβ$_{D656A}$ did not appreciably alter radiolabeled PIP levels. This is likely a result of the constitutive turnover of large pools of PIP in other compartments that would be insensitive to PI4Kβ$_{D656A}$ expression (see above). Nevertheless, although it is impossible to assess how PI4Kβ$_{D656A}$ affects PI composition in the Golgi, let alone in individual Golgi cisternae, we suspect that altered PI(4)P levels directly contribute to both the stimulation and inhibition of traffic that we observed because: 1) PI4Kβ$_{D656A}$ has different effects on both intra-Golgi

transport and apical delivery kinetics of HA compared with wild type PI4Kβ; 2) PI4Kβ_{D656A} alters the detergent solubility properties of HA staged in the TGN, suggesting that it may affect Golgi complex lipid composition; and 3) coexpression of wild type PI4Kβ blocks the effects of PI4Kβ_{D656A} on intra-Golgi transport, cell surface delivery, and detergent solubility of HA. Thus, it is likely that changes in PI(4)P levels can directly affect transport rates along the biosynthetic pathway.

Several studies suggest that changes in PI(4)P levels may regulate Golgi complex morphology as well as function. Cells expressing temperature-sensitive mutants of SEC14 or PIK1 accumulate Berkeley bodies, which are thought to represent hypertrophy of the Golgi cisternae in response to the transport blockade (7, 8, 35). Similarly, Godi *et al.* (9) found that overexpression of dominant-negative but not wild type PI4Kβ in COS-7 cells resulted in dramatic alterations in the distribution of the Golgi marker giantin, resulting in a disorganized pattern with irregular filamentous and punctate structures. By contrast, we saw no observable changes in giantin staining at the light microscope level in MDCK cells overexpressing large amounts of either wild type or mutant PI4Kβ. The reason for this discrepancy is not clear, but it could be caused by differences in the cell type or expression level used in our study.

Although PI(4)P is the most abundant phosphatidylinositol in the Golgi complex, other downstream metabolites may also play a role in regulating biosynthetic transport. Several roles for PIP_2 in Golgi transport have been suggested, including recruitment and regulation of a Golgi membrane cytoskeleton (36, 37) and participation in a positive feedback loop involving ADP-ribosylation factor and phospholipase D that would drive protein transport (see Ref. 38 for review.) In addition, overexpression of the α isoform of phosphatidylinositol 5-kinase in cells resulted in actin polymerization selectively on membrane vesicles containing lipid rafts to form actin comets (39), although the consequences of this on membrane transport rates were not assessed. The phosphatidylinositol 5-kinase responsible for generation of PIP_2 in the Golgi has not yet been identified. We found that expression of wild type and mutant PI4Kβ had little effect on total cellular PIP_2 levels, but the subcellular distribution of this pool could not be ascertained. However, we saw no observable change in the overall distribution of an adenovirally expressed construct encoding the green fluorescent protein-tagged pleckstrin homology domain from phospholipase Cδ (which avidly binds PIP_2), when either wild type or mutant PI4Kβ were coexpressed in MDCK cells (data not shown).

In summary, our data demonstrate that PI4Kβ regulates transport at multiple compartments in the Golgi complex and can play both positive and negative regulatory roles in membrane traffic. The opposing effects of dominant-negative PI4Kβ_{D656A} on intra-Golgi transport *versus* Golgi-to-cell surface delivery of HA reinforces the complex role that phosphatidylinositol metabolism plays in the regulation of biosynthetic traffic. PIs can regulate traffic via their structural role as membrane components or via their ability to recruit proteins via specific domains (such as pleckstrin homology, Src homology 2, and FYVE domains). It is likely that both of these functions contribute to the overall role of PI4Kβ in biosynthetic transport. Recent technological developments, including the generation of lipid-specific probes (40) and the ability to perform detailed kinetic analyses in live cells (41, 42) should be useful tools for sorting out this complexity.

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