Overexpression of Frequenin, a Modulator of Phosphatidylinositol 4-Kinase, Inhibits Biosynthetic Delivery of an Apical Protein in Polarized Madin-Darby Canine Kidney Cells*

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Polyphosphoinositides regulate numerous steps in membrane transport. The levels of individual phosphatidylinositols are controlled by specific lipid kinases, whose activities and localization are in turn regulated by a variety of effectors. Here we have examined the effect of overexpression of frequenin, a modulator of phosphatidylinositol 4-kinase activity, on biosynthetic and postendocytic traffic in polarized Madin-Darby canine kidney cells. Endogenous frequenin was identified in these cells by polymerase chain reaction, Western blotting, and indirect immunofluorescence. Adenoviral-mediated overexpression of frequenin had no effect on early Golgi transport of membrane proteins, as assessed by acquisition of resistance to endoglycosidase H. However, delivery of newly synthesized influenza hemagglutinin from the *trans*-Golgi network to the apical cell surface was severely inhibited in cells overexpressing frequenin, whereas basolateral delivery of the polymeric immunoglobulin receptor was unaffected. Overexpression of frequenin did not affect postendocytic trafficking steps including apical and basolateral recycling and basal-to-apical transcytosis. We conclude that frequenin, and by inference, phosphatidylinositol 4-kinase, plays an important and selective role in apical delivery in polarized cells.

We have been interested in the mechanisms that regulate cargo sorting and vesicle formation along the biosynthetic and postendocytic pathways. In recent years, rapidly accumulating evidence has implicated polyphosphoinositides as key players in membrane trafficking (reviewed in Ref. 1). In addition to their function as second-messenger precursors, these lipids are now thought to participate physically in the formation and release of vesicles from various compartments along the secretory pathway. The regulation of polyphosphoinositide formation by specific lipid kinases is therefore the subject of intense study.

Frequenin is a myristoylated ~22-kDa calmodulin-related calcium-binding protein that modulates regulated secretion in neuronal and neuroendocrine cells (2–4). Recently, frequenin

was demonstrated to regulate the activity of the yeast phosphatidylinositol 4-OH kinase (PI4K)[1] Pik1 (5), which is homologous to the mammalian PI4K β isoform (PI4Kβ (6)). Although *FRQ1* is an essential gene in yeast, overexpression of *PIK1* suppressed a frq1Δ mutation; however, overexpression of frequenin did not rescue a *pik1Δ* mutant, suggesting that PIK1 acts downstream of FRQ1 (5). *PIK1* activity was subsequently found to regulate secretion in yeast at the level of the *trans*-Golgi (7, 8). In addition, a kinase-defective *pik1* mutant showed a block in endocytic traffic (7).

Although initial reports suggested that frequenin expression was localized exclusively to neuronal tissues (3, 4), the recent discovery of a yeast homolog of frequenin led us to examine its expression in a non-neuronal cell line. Interestingly, we found that cultured Madin-Darby canine kidney (MDCK) cells expressed significant levels of frequenin mRNA and protein. Therefore, we tested whether mammalian frequenin interacts with PI4Kβ, and investigated the effect of frequenin overexpression on biosynthetic and postendocytic protein traffic in this polarized epithelial cell line.

MATERIALS AND METHODS

Cell Lines—Low passage MDCK T23 cells (9) were maintained in minimal essential medium (Cellgro, Fisher Scientific, Pittsburgh, PA) supplemented with 10% fetal bovine serum (Atlanta Biologicals, Norcross, GA), streptomycin (100 µg/ml), and penicillin (100 units/ml). MDCK T23 cells stably express the polymeric immunoglobulin receptor (pIgR) as well as the tetracycline transactivator, which is required for expression of influenza hemagglutinin (HA) from adenovirus. For all experiments, cells were seeded at high density (~2 × 10⁵ cells/well) on 12-mm Transwells (0.4 µm pore, Costar, Cambridge, MA) for 2–3 days prior to infection with recombinant adenovirus at the indicated multiplicity of infection (m.o.i.) as described in Ref. 9. Experiments were performed the following day.

Recombinant Adenoviruses and Adenoviral Infection—A cDNA fragment encoding frequenin from rat brain was subcloned into the pAdlox vector, and a recombinant adenovirus generated as described in Ref. 10. Generation of a control adenovirus encoding a nonsense sequence (influenza M2 inserted in the reverse orientation) and of adenovirus encoding influenza HA has been previously described (9).

Antibodies and Western Blotting—Generation and affinity purification of two rabbit polyclonal anti-frequenin antibodies (44162 and 44163) and chicken anti-frequenin antibody 21 generated against purified mammalian frequenin are described in detail elsewhere (11),[2] Polyclonal anti-PI4Kβ antibody was purchased from Upstate Biotech-

[1] The abbreviations used are: PI4K, phosphatidylinositol 4-kinase; AV, adenovirus; endo H, endoglycosidase H; HA, hemagglutinin; MDCK, Madin-Darby canine kidney; pIgR, polymeric immunoglobulin receptor; m.o.i., multiplicity of infection; PI3K, phosphatidylinositol 3-kinase; PI4Kβ, phosphatidylinositol 4-kinase β isoform; PIP₂, phosphatidylinositol 4,5-bisphosphate; TGN, *trans*-Golgi network; PAGE, polyacrylamide gel electrophoresis.

[2] A. Jeromin, M. R. Soranzo, F. Vita, C. Borchetta, J. Roder, and G. Zabucchi, submitted for publication.

nology (Lake Placid, NY). Detection of PI4Kβ by Western blotting was performed according to the manufacturer's protocol. The same procedure was used to detect frequenin in solubilized cell lysates, membrane, and cytosolic fractions.

Membrane Fractionation—MDCK cells grown to confluence on 6-cm dishes were mock-infected or infected with AV-frequenin. The following day, cells were rinsed with phosphate-buffered saline and scraped using a rubber policeman into 0.5 ml of buffer containing 30 mM Tris, pH 8.0, 100 mM NaCl, 5 mM MgCl$_2$, 0.5 mM CaCl$_2$, and 0.1 mM phenylmethyl-sulfonyl fluoride. The cells were then passed through a 20-gauge needle 10 times, and centrifuged for 1 h at 100,000 × *g*. The pellet was resuspended in the original volume of buffer, and the supernatant and resuspended pellet were trichloroacetic acid precipitated and solubilized with Laemmli sample buffer. Samples were run on 12% SDS-PAGE gels and Western blotted using rabbit polyclonal antibody 44163. Blots were exposed to film (BioMax MR, Eastman Kodak Co., Rochester, NY), and bands were quantitated after scanning using Quantity One software (Bio-Rad).

Indirect Immunofluorescence—Indirect immunofluorescence staining of filter-grown MDCK T23 cells expressing frequenin was performed using the pH shift protocol as described previously (9). Frequenin was detected using rabbit polyclonal anti-frequenin antibody 44163. Cy3-conjugated goat anti-rabbit secondary antibody (1:1000 dilution) was from Jackson ImmunoResearch Laboratories, Inc. (Avondale, PA). Imaging was performed on a Nikon Eclipse TE300 inverted microscope (Fryer Co. Inc., Huntley, IL) using a CFI plan apochromat × 100 oil-immersion objective (numerical aperture 1.4) with a DAPI/FITC/TRITC (4′,6-diamidino-2-phenylindole/fluorescein isothiocyanate/tetramethylrhodamine isothiocyanate) triple band filter set (single band exciters; Chroma Technology Corp., Brattleboro, VT). Vertical series of images, each 0.5 μm apart, were captured with a Hamamatsu C4742-95 digital CCD camera (Hamamatsu, Hamamatsu City, Japan) using Openlab software (Improvision, Coventry, United Kingdom) with the following settings: exposure time 400–600 ms, offset 20–40%, gain 69%, camera binning ×1, and 8-bit grayscale. Images were then cropped to a 600 × 600 pixel region of interest and processed using the Openlab multi-neighbor deconvolution module (4 neighbors) to remove out-of-focus information. Projections of two consecutive sections were saved in tag-information file format and the contrast levels of the images were adjusted using Photoshop software (Adobe, Mountain View, CA) on a Power PC G-3 Macintosh computer (Apple, Cupertino, CA).

Biosynthetic and Postendocytic Transport Assays—The rate of transport through the cis/medial Golgi was quantitated by monitoring acquisition of HA to endoglycosidase H (endo H) resistance as described in Ref. 12. Briefly, cells were starved for 30 min, radiolabeled with [^{35}S]methionine for 10 min, then chased for the indicated times. Cells were then solubilized and HA immunoprecipitated using a monoclonal antibody. After collection with fixed *Staphylococcus aureus* (Calbiochem, La Jolla, CA), antibody-antigen complexes were eluted, divided in half, and mock-treated or treated with endo H (New England Biolabs, Inc., Beverly, MA). Samples were electrophoresed on 10% SDS-PAGE gels and quantitated using a PhosphorImager with Quantity One software (Personal Molecular Imager FX; Bio-Rad). To measure delivery of HA from the *trans*-Golgi network (TGN) to the cell surface, cells were starved and radiolabeled as described above, then chased for 2 h at 19 °C to accumulate newly synthesized HA in the TGN. Cell surface delivery was measured using the trypsinization assay described in Ref. 12. Basolateral delivery of pIgR was quantitated as described in Ref. 13. Quantitation of transcytosis and apical recycling of ^{125}I-IgA, and of basolateral recycling of ^{125}I-transferrin was performed exactly as described in Ref. 9.

<center>RESULTS</center>

MDCK Cells Express Endogenous Frequenin—RNA isolated from MDCK cells using two methods was amplified by polymerase chain reaction using degenerate oligonucleotides directed against the sequence of rat frequenin. A single band of approximately 200 base pairs was observed, identical to that seen in control amplifications using authentic rat frequenin cDNA (Fig. 1). This is in agreement with the finding that frequenin cloned from mouse kidney is identical in sequence to the protein originally cloned from brain.[3] Western blotting of MDCK
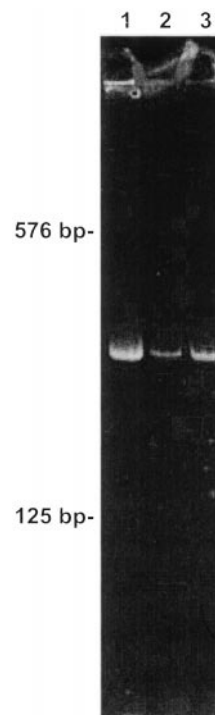
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[3] David Mount, personal communication.

FIG. 1. **MDCK cells express endogenous frequenin.** Random-primed (*lane 2*) and poly(A)$^+$ mRNA (*lane 3*) isolated from MDCK cells were subjected to polymerase chain reaction using degenerate primers directed against rat frequenin. A plasmid encoding authentic rat frequenin cDNA was used as a control (*lane 1*).

cells using a polyclonal antibody raised in either rabbit or chicken against purified rat/mouse frequenin revealed a single band at approximately 18 kDa, similar to the reported molecular weight of rat frequenin (Fig. 2). In MDCK cells infected with a recombinant adenovirus encoding rat frequenin (AV-frequenin), the intensity of this band increased by an average of 3.3-fold. Similar results (a range of between ~2 and ~4-fold increase in virally infected cells) were obtained by scanning and quantitating other blots from several experiments. Because frequenin associates with membranes via a myristoyl anchor, we tested whether overexpression of frequenin altered its membrane distribution. Crude membrane fractionation revealed that approximately 60% of endogenous frequenin was membrane associated, and overexpression of frequenin had no effect on the relative proportion of membrane-associated frequenin (Fig. 3). Digital deconvolution of uninfected MDCK cells processed for indirect immunofluorescence using rabbit polyclonal anti-frequenin antibody revealed punctate staining that was concentrated throughout the cytoplasm (Fig. 4, *panels A-H*), consistent with frequenin localization on membranous organelles. There was no effect on the overall staining pattern in cells overexpressing frequenin (Fig. 4, *panels I-P*), consistent with our fractionation data.

Mammalian Frequenin Interacts with PI4K—In yeast, the frequenin homolog Frq1 has been reported to interact with the *PIK1* gene product, PI4K. A mammalian homolog of Pik1 was recently isolated that encodes a wortmannin-sensitive β isoform of PI4K (6). We therefore performed coimmunoprecipitation experiments to determine whether PI4Kβ and frequenin associate in MDCK cells. Mock-infected or AV-frequenin-infected MDCK cells were solubilized and immunoprecipitated with anti-frequenin or anti-PI4Kβ antibodies. The samples were then electrophoresed and Western blotted with the converse antibody (Fig. 5). A ~97-kDa band, consistent with the molecular mass of PI4Kβ was observed when immunoprecipitates of frequenin from AV-infected but not mock-infected cells

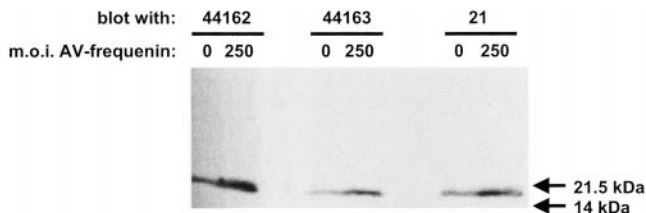| blot with:	44162		44163		21	
m.o.i. AV-frequenin:	0	250	0	250	0	250

← 21.5 kDa
← 14 kDa

FIG. 2. **Expression of frequenin in MDCK cells by adenoviral infection.** Filter-grown MDCK cells were mock-infected or infected with AV-frequenin at a m.o.i. of 250. The following day, cells were solubilized in Laemmli sample buffer. Electrophoresed samples were subjected to Western blotting using two different rabbit polyclonal anti-frequenin antibodies (44162 and 44163) and a chicken polyclonal antibody (21) as described under "Materials and Methods."
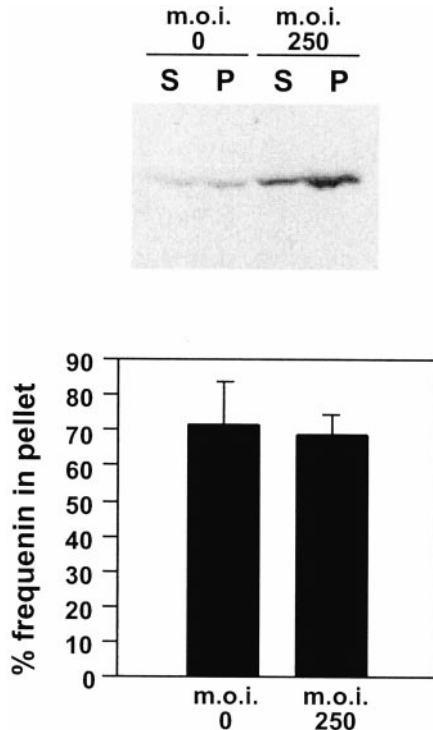

FIG. 3. **Overexpression of frequenin does not alter its membrane association.** MDCK cells (mock-infected or infected with AV-frequenin) were homogenized and cytosolic and crude membrane fractions recovered as described under "Materials and Methods." After SDS-PAGE, samples were analyzed by Western blotting, and the proportion of total cellular frequenin in each fraction was quantitated. Cells infected with AV-frequenin expressed 3.4-fold more frequenin than mock-infected cells. The graph represents quantitation (mean ± S.D.) of an experiment performed in quadruplate, and a representative blot is shown. Similar results were obtained in three independent experiments.

were blotted with anti-PI4Kβ antibody. The same band was observed in MDCK cell lysates that had been immunoprecipitated using anti-PI4Kβ antibody, confirming the identity of this protein (not shown). Interestingly, in some experiments, an additional band of ~110 kDa was also observed in cell lysates and in anti-frequenin immunoprecipitates blotted with anti-PI4Kβ antibody (Fig. 5, *upper band*); however, this band was not specific to cells overexpressing frequenin. The identity of this protein is unknown. By indirect immunofluorescence, PI4K localized to numerous intracellular punctae, reminiscent of the localization of frequenin; in addition, some nuclear localization was also observed (not shown). Overexpression of frequenin had no effect on the gross distribution of PI4K.

Overexpression of Frequenin Does Not Affect Postendocytic Traffic in Polarized MDCK Cells—We tested whether overexpression of frequenin affects endocytic trafficking pathways in

polarized MDCK cells. The MDCK T23 cells stably express pIgR, which is a useful marker to follow the basolateral-to-apical transcytotic and apical recycling pathways. Previously we demonstrated that adenoviral infection with a control virus (expressing a nonsense construct) had no effect on the rate of postendocytic traffic in cells (9). Thus, in these experiments, we compared the rates of basolateral-to-apical transcytosis and apical recycling of ^{125}I-IgA in cells infected with frequenin and cells infected with the control virus (Fig. 6, *A* and *B*). Overexpression of frequenin had no effect on the rate of IgA transcytosis or recycling, even when 5-fold higher levels of virus were used (not shown). In addition, frequenin had no effect on the rate of basolateral recycling in polarized MDCK cells as measured using ^{125}I-transferrin as a marker (Fig. 6C). Thus, overexpression of frequenin does not appear to alter the delivery of pre-endocytosed proteins to the apical or basolateral plasma membrane of polarized cells.

Overexpression of Frequenin Selectively Perturbs Delivery of Proteins from the TGN to the Apical Membrane—We next examined whether overexpression of frequenin affects biosynthetic delivery in MDCK cells. To test whether frequenin overexpression affects the rate of protein traffic through the early secretory pathway, we monitored acquisition of endo H resistance of newly synthesized influenza HA (Fig. 7). This assay measures the rate of delivery of proteins from their synthesis to arrival at the cis/medial Golgi. The endo H kinetics of HA were identical in cells infected with a control AV compared with cells overexpressing frequenin, suggesting that frequenin does not normally modulate transport through the early secretory pathway. However, when transport of radiolabeled HA from the TGN to the cell surface was monitored, we observed a dramatic delay in apical delivery of this protein in cells overexpressing frequenin (Fig. 8). However, overexpression of frequenin did not cause missorting of HA, as the cell surface distribution of HA measured after long chase times (6 h) was unaffected (85.2 ± 6.9% apical in control cells *versus* 93 ± 10.4% apical in frequenin-expressing cells, mean ± S.D., n = 3). Treatment with concentrations of wortmannin (10 μM) that inhibit PI4K had a similar effect on HA delivery from the TGN to the cell surface (not shown).

The effect of frequenin on the initial rate of HA TGN-to-apical delivery but not on its ultimate sorting could reflect a delay in apical delivery alone or a generalized defect in TGN-to-cell surface delivery. Because only a small fraction of HA is delivered to the basolateral surface, we could not determine whether frequenin affected the kinetics of delivery of this pool; therefore, we tested whether frequenin affects the delivery of pIgR, which is rapidly and efficiently delivered to the basolateral cell surface after synthesis. Interestingly, overexpression of frequenin had no effect on the kinetics of pIgR delivery to the basolateral surface of polarized MDCK cells (Fig. 9). In addition, frequenin overexpression delayed apical but not basolateral secretion of another protein, a glycosylated form of the human growth hormone (not shown) that we and others have demonstrated is secreted predominantly apically (13, 14).

DISCUSSION

We have demonstrated that frequenin is endogenously expressed in MDCK cells, and that overexpressed frequenin co-immunoprecipitates with the wortmannin-sensitive β isoform of PI4K. Overexpression of frequenin inhibited delivery of newly synthesized influenza HA from the *trans*-Golgi network to the apical surface of polarized MDCK cells; however, the proper sorting of this protein was ultimately unimpaired. By contrast, transport through the early secretory pathway, cell surface delivery of a basolaterally directed protein, basolateral-to-apical transcytosis, and recycling of preinternalized proteins

Frequenin Overexpression Inhibits Apical Delivery

m.o.i. 0   m.o.i. 250

FIG. 4. **Indirect immunofluorescence localization of frequenin in polarized MDCK cells.** MDCK cells grown on Transwell inserts were mock-infected (m.o.i. 0) or infected with AV-frequenin (m.o.i. 250). The following day, cells were fixed and processed for indirect immunofluorescence to localize frequenin (*panels A-D and I-L*) as described under "Materials and Methods." Nuclei (*panels E-H and M-P*) were detected using DAPI. Vertical series of images, each 0.5 μm apart, were captured using Openlab software and processed using digital deconvolution. Projections of two consecutive optical sections are shown from the apex of the cells (*panels A, E, I,* and *M*), 0.5 μm below the previous sections (*panels B, F, J,* and *N*), at the level of the nucleus (*panels C, G, K,* and *O*), and at the base of the cells (*panels D, H, L,* and *P*). *Bar*, 10 μm.
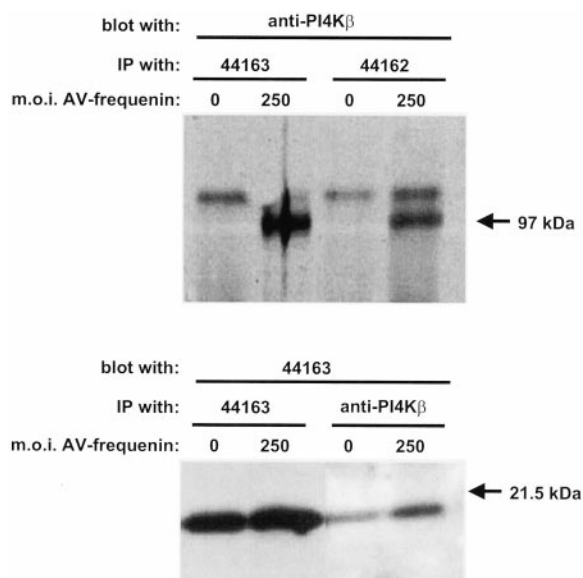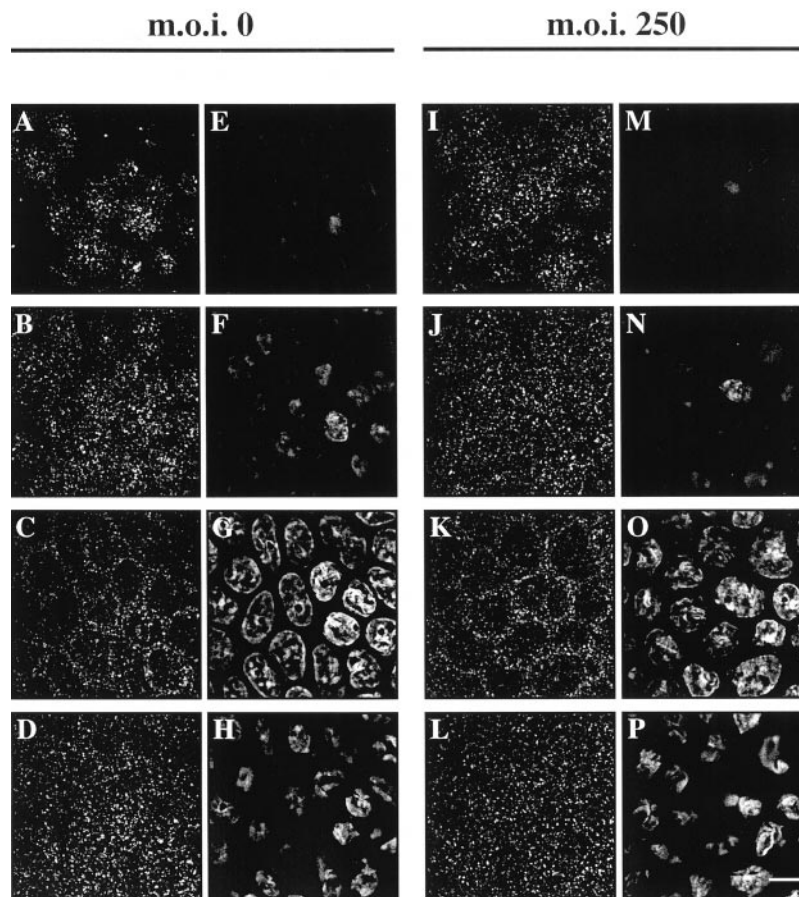

FIG. 5. **Overexpressed frequenin in MDCK cells coprecipitates with PI4K.** MDCK-T23 cells were mock infected or infected with AV-frequenin at a m.o.i. of 250 (*upper panel*). The following day, cells were solubilized and immunoprecipitated using polyclonal anti-frequenin antibodies 44163 or 44162. Samples were electrophoresed and blotted with anti-PI4Kβ antibody. Overexpression of frequenin coprecipitates a protein of ~97 kDa that is recognized by anti-PI4Kβ (*lower panel*). Samples were immunoprecipitated using anti-frequenin or PI4Kβ antibodies, then blotted with anti-frequenin antibody. Anti-PI4Kβ antibody precipitates a band comigrating with authentic frequenin. A 2-min exposure is shown of the *two left lanes*; the *two right lanes* were visualized after overnight exposure.

to either membrane domain were unaffected by frequenin overexpression. Although we have not determined whether frequenin overexpression inhibits the budding of vesicles from the TGN or their fusion with the apical membrane, our data are consistent with recent observations demonstrating a role for PI4K in Golgi-to-cell surface delivery in yeast (7, 8).

The mechanism by which frequenin regulates PI4K activity is not clear, and is likely to be complex. Frequenin may mediate membrane targeting and localization of PI4K; alternatively, frequenin might regulate PI4K activity directly. Membrane targeting of frequenin requires a myristate anchor and is dependent on Ca^{2+} (5). However, both the physical interaction between Pik1 and frequenin and frequenin's stimulation of Pik1 activity *in vitro* were shown to be independent of Ca^{2+} concentration and frequenin myristoylation (5). Thus, it is possible that frequenin regulates PI4K localization and activity independently. Balla and colleagues[4] have demonstrated that overexpression of frequenin stimulates activity of heterologously expressed PI4K in COS cells, however, the mechanism is unknown. We did not detect any effect of frequenin overexpression on the localization of PI4K by indirect immunofluorescence. Numerous attempts to examine the membrane distribution of PI4K in mock-infected *versus* frequenin-overexpressing cells gave highly variable and irreproducible results, although the majority of PI4K was recovered in the soluble fraction, consistent with previous results by Wong *et al.* (15). Thus, we conclude that overexpression of frequenin does not have a dramatic effect on PI4K distribution, although subtle localized changes would be difficult to detect.

Polyphosphoinositide metabolism has been demonstrated to play a critical role in vesicular traffic through the Golgi com-

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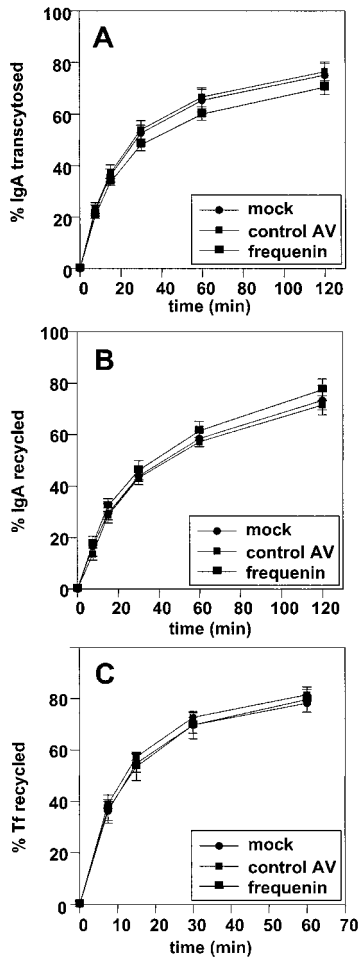[4] Tamas Balla, personal communication.

FIG. 6. **Overexpression of frequenin has no effect on postendocytic traffic in polarized MDCK cells.** Filter grown MDCK-T23 cells were infected with AV-frequenin or control AV at a m.o.i. of 250. The following day, the rates of basolateral-to-apical transcytosis of ^{125}I-IgA (*panel A*), apical recycling of ^{125}I-IgA (*panel B*), and basolateral recycling of ^{125}I-transferrin (*panel C*) were quantitated as described under "Materials and Methods." The mean $\pm$ S.D. of triplicate samples is shown. Each experiment was repeated at least three times with similar results.
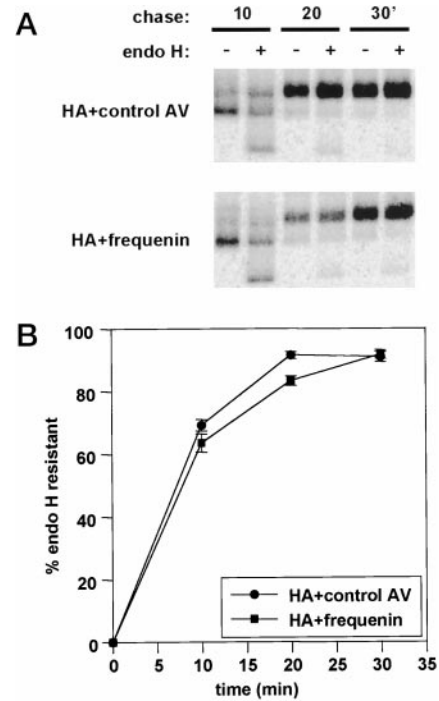

FIG. 7. **Frequenin overexpression has no effect on the rate of transport through the early secretory pathway.** MDCK cells were infected with AV-HA (m.o.i. 75) and either AV-frequenin or control AV (m.o.i. 250). The following day, cells were starved, radiolabeled for 10 min, then chased for the indicated periods. Samples were solubilized and the immunoprecipitated HA was mock treated or treated with endo H as described under "Materials and Methods." A typical gel is depicted in *panel A*, and quantitation of the rate of endo H kinetics of HA (average $\pm$ range) from this and another experiment performed in an identical manner is shown in *panel B*.
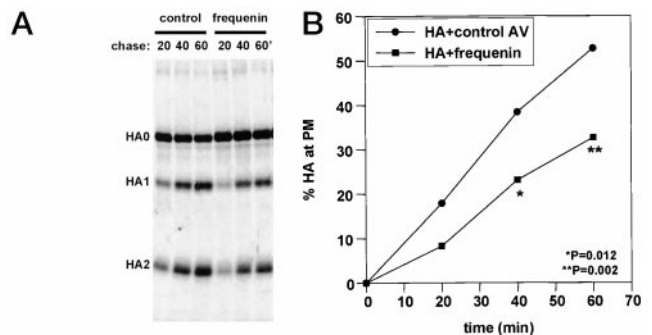

FIG. 8. **Frequenin overexpression inhibits HA TGN-to-apical cell surface delivery.** Polarized MDCK T23 cells were infected with AV-HA and either AV-frequenin or control AV. Cells were starved, radiolabeled, and chased for 2 h at 19 °C. The medium was replaced with prewarmed medium and delivery of HA to the apical plasma membrane quantitated using a surface trypsinization assay as described under "Materials and Methods." A representative gel is shown in *panel A*; *HA0* marks the position of uncleaved HA, and the migration of cleavage products HA1 and HA2 are noted. Quantitation of the rate of HA delivery to the cell surface is shown in *panel B*. Similar results were obtained in six experiments, however, the total amount and rate of cell surface and HA delivery varied somewhat between experiments. The raw data from six independent experiments was subjected to paired *t* test analysis; *asterisks* denote time points in which HA delivery in frequenin-expressing cells is statistically different from control.

plex, and particularly in vesicle release from the TGN (see Refs. 1, 16, and 17, for review). Recent insight into the mechanism by which PI4K might function in this context comes from the observation that ARF recruits PI4Kβ (as well as an unidentified PI5K) to the Golgi complex, and thus causes an increase in phosphatidylinositol 4,5-bisphosphate (PIP$_2$) levels (18). In addition, ARF also activates phospholipase D activity, which stimulates PI5K activity and further increases PIP$_2$ levels (19, 20). Negatively charged phospholipids such as PIP$_2$ have been suggested to play numerous roles in vesicle formation, including altering membrane fusogenicity and curvature (21, 22). In addition, increased PIP$_2$ levels may serve to recruit or modulate proteins that contain pleckstrin-homology domains, such as dynamin and spectrin. Dynamin is present on the TGN and has been found to participate in vesicle budding from the TGN (23). Phosphoinositide-mediated alterations in the Golgi-specific cytoskeleton have also been postulated to drive vesicle formation from the TGN (20).

A further clue to the role of polyphosphoinositide formation in post-Golgi biosynthetic traffic comes from a recent report on the effect of PI5K overexpression in 3T3 cells, which along with other nonpolarized cell lines, can differentially regulate delivery of heterologously expressed "apical" and "basolateral" proteins (24). Recently, Rozelle *et al.* (25) reported that overexpres-

sion of PI5K in 3T3 fibroblasts resulted in increased cellular PIP$_2$ levels accompanied by a dramatic elevation in the formation of actin comets around vesicles. Comet formation was found to be regulated by N-WASP, a member of the Wiskott-Aldrich syndrome protein family, which has previously been shown to induce actin comet formation around vesicles in a PIP$_2$ dependent manner (26). The majority of comets in PI5K-
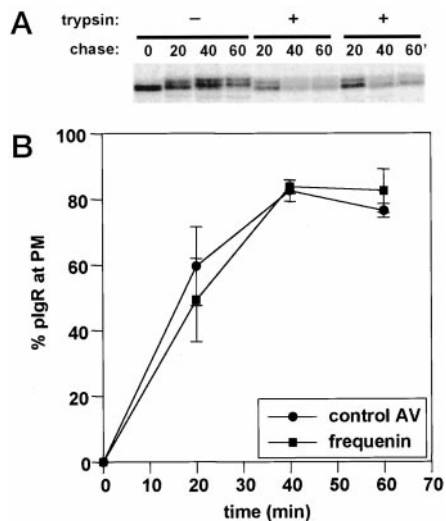
FIG. 9. **Frequenin overexpression has no effect on the rate of basolateral delivery of the polymeric immunoglobulin receptor.** Polarized MDCK T23 cells were infected with either AV-frequenin or control AV. Cells were starved, radiolabeled for 15 min, and chased for the indicated periods in the presence or absence of basolaterally added trypsin. Cell surface delivery of newly synthesized pIgR was determined as described under "Materials and Methods." A representative gel is shown in *panel A*, and the mean ± S.E. from five independent experiments is plotted in *panel B*.

overexpressing cells were associated with Golgi-derived vesicles, and intriguingly, were selectively associated with vesicles carrying the apical marker HA, whereas vesicles carrying a basolateral marker were rarely seen associated with comets (25). Actin polymerization is known to nucleate from cholesterol- and sphingolipid-rich membrane domains (lipid rafts) under some conditions (27), and these rafts are also enriched in PIP_2 (28). Segregation into lipid rafts has been suggested as a mechanism for the sorting of apically destined proteins (29); thus, one could envision a selective role for PIP_2 formation in generation or release of apically destined vesicles. However, the rate of HA delivery to the cell surface was not quantified in the study described above, and it is not clear whether actin comet formation ultimately stimulates or inhibits apical membrane delivery. In fact, we observed an inhibition of HA cell surface delivery in both MDCK and HeLa cells (data not shown) infected with AV-frequenin; this might suggest that elevated PIP_2 levels somehow inhibit the delivery of apical cargo. We did not observe any effects of frequenin overexpression on the steady state localization of actin, PI4K, or the TGN marker furin (data not shown).

We found no effect of frequenin overexpression on the rate of recycling of preinternalized apical and basolateral proteins, even at very high expression levels. In addition, we did not observe any obvious effect of frequenin on the intracellular accumulation of apical or basolateral markers, although we did not measure internalization rates. The selective effect of frequenin overexpression on biosynthetic delivery was surprising given that overexpression of PI4K has been demonstrated to modulate endocytosis in other systems. For example, Walch-Solimena and Novick (7) reported that *PIK1* mutants show a defect in endocytosis at the level of the endosome, although the affected step was not defined. Moreover, a recent report demonstrated a role for PI4Kβ in agonist-induced endocytosis of G protein-coupled receptors in neuroblastoma cells (30). Because several mammalian homologs of Pik1 have been identified, it is possible that another PI4K isoform regulates this step in MDCK cells. To our knowledge, the expression and localization

of PI4K isoforms in MDCK cells have not yet been investigated. Immunoblots of MDCK cell lysates using PI4Kβ-specific antiserum sometimes revealed a ~110-kDa band in addition to the reproducible 97-kDa protein that coprecipitated with overexpressed frequenin; interestingly, coprecipitation of this protein with frequenin occurred even in the absence of frequenin overexpression (Fig. 4). This protein might represent an additional form of PI4K that interacts strongly with endogenous frequenin; however, we do not know the identity of this protein at present.

Several studies have also shown that overexpression of frequenin results in stimulated release of regulated secretory vesicles (2–4). In addition, PI4K activity was recently demonstrated to be essential for stimulated secretion from isolated nerve terminals and permeabilized adrenal chromaffin cells (31, 32). Together these observations suggest that frequenin acts as a positive regulator of PI4K activity on synaptic vesicles or chromaffin granules. By contrast, we observed that overexpression of frequenin inhibits protein delivery from the TGN to the plasma membrane of polarized cells. Because MDCK cells do not have a significant regulated secretion pathway, we cannot determine whether this pathway is affected by frequenin overexpression in our current system.

In summary, our results suggest that frequenin-mediated modulation of PI4K activity disrupts the delivery of an apical protein to the plasma membrane of polarized MDCK cells. Thus, in addition to its function in regulating synaptic vesicle release in neuronal cells, frequenin may play a more ubiquitous role in membrane trafficking than has previously been appreciated. Studies are underway to pinpoint the exact step(s) affected by frequenin, as this is likely to reveal interesting mechanistic parallels and differences between constitutive membrane transport and neuronal release.

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