Wortmannin, an Inhibitor of Phosphoinositide 3-Kinase, Inhibits Transcytosis in Polarized Epithelial Cells*

(Received for publication, July 17, 1995, and in revised form, September 15, 1995)

Steen H. Hansen‡, Anna Olsson, and James E. Casanova

From the Department of Pediatrics, Massachusetts General Hospital East, Charlestown, Massachusetts 02129

Wortmannin, an inhibitor of phosphoinositide 3-kinase, inhibits both basolateral to apical and apical to basolateral transcytosis of ricin in Fisher rat thyroid (FRT) cells by 50% at 100 nM in a continuous transcytosis assay. In MDCK cells, a similar effect of wortmannin on basolateral to apical transcytosis of ricin was found, whereas apical to basolateral transcytosis was inhibited to a lesser degree. Transcytosis of dimeric IgA in MDCK cells expressing the polymeric immunoglobulin receptor was also reduced to 50% of controls, suggesting that wortmannin inhibits membrane translocation rather than sorting of specific proteins in the transcytotic pathway. This effect of wortmannin is selective, however, in that endocytosis at the basolateral domain and recycling at both the basolateral and apical membrane domains are unaffected, and apical endocytosis and apical secretion are only moderately reduced. We have shown previously that cAMP stimulates a late stage in basolateral to apical transcytosis in MDCK cells through activation of protein kinase A (Hansen, S. H., and Casanova, J. E. (1994) J. Cell Biol. 126, 677–687). Elevation of cellular cAMP still induced a 100% increase in transcytosis in wortmannin-treated cells, but transcytosis was no longer increased when compared to cells which received no drugs. In contrast, in experiments using a 17°C block to accumulate ricin internalized from the basolateral surface in the apical compartment of MDCK cells, wortmannin had little effect on the stimulation of transcytosis by activators of protein kinase A observed under these conditions. The data thus suggest the existence of a wortmannin-sensitive step in the transcytotic pathway, positioned after endocytosis but prior to translocation into the protein kinase A-sensitive apical compartment, implying a role for phosphoinositide 3-kinase in an intermediate step in transcytosis in polarized epithelial cells.

Membrane homeostasis in eukaryotic cells is dependent on an interplay of membrane budding, fusion, and transport events, which must be regulated individually and collectively to maintain cell function. The generation of polarity in epithelial cells adds further complexity in that additional vesicle-mediated transport processes are required to allow selective traffic to and from the apical and basolateral membrane domains and between these domains (1, 2). The latter process, transcytosis, i.e. vesicle-mediated transepithelial transport, has been shown to be a brefeldin A-sensitive process (3, 4) which in the case of basolateral to apical transcytosis is inhibited by calmodulin antagonists (5, 6) and requires intact microtubules (7–9). However, little is known about how constitutive transcytosis is actually regulated.

Mammalian PI3K (p85/110), a heterodimer consisting of an 85-kDa and a 110-kDa subunit, has been implicated in a wide array of processes involving signaling through transmembrane receptors such as mitogenesis, cell transformation, cellular differentiation, apoptosis, membrane ruffling, and histamine secretion (for reviews, see Refs. 10–12). The 85-kDa regulatory subunit contains one SH3 domain and two SH2 domains which mediate interactions of PI3K with tyrosine-phosphorylated proteins (10–12). The 110-kDa catalytic subunit phosphorylates PtdIns, PtdIns-4-P, and PtdIns-4,5-P2 in the 3-position (13) and has also been shown to possess protein kinase activity in that it phosphorylates the 85-kDa subunit and the insulin receptor substrate 1 on serine (14–16). Phosphorylation of the 85-kDa subunit inhibits the lipid kinase activity of the 110-kDa subunit of PI3K, a possible mechanism for regulating PI3K activity (14, 15). Both the phosphoinositide and the protein kinase activities of PI3K are inhibited by nanomolar concentrations of the microbial product wortmannin (16–18), which has proven to be a useful tool in defining the role of PI3K in biological processes (10, 11).

The PI3K has also been implicated in vesicular trafficking. Mutagenesis studies have demonstrated that, upon ligand binding, the autophosphorylated PDGF receptor tail recruits PI3K to the membrane through binding of the SH2 domains in the 85-kDa subunit of PI3K to phosphotyrosine residues 740 and 751 of the PDGF receptor (19). It has been shown that these two tyrosine residues are required for efficient lysosomal transport of activated PDGF receptors (20) and that this process is inhibited by 25–50 nM wortmannin (21). A similar mechanism may be operating in the postendocytic sorting of the receptor for colony-stimulating factor (22, 23). Furthermore, insulin signaling through PI3K has been shown to recruit glucose transporters to the cell surface (24), which is also a vesicle-mediated process (25, 26).

The most compelling evidence for the significance of phosphoinositide derivatives in membrane traffic has come from...
studies by Emr and co-workers (27) on vacuolar protein sorting in the yeast, Saccharomyces cerevisiae. These studies have shown that the Vps34 gene encodes a phosphatidylinositol-specific 3-kinase (PtdIns 3-kinase) with a catalytic domain homologous to that of the 110-kDa subunit of mammalian PI3K (27). Through interaction with Vps15p, a serine-threonine kinase, Vps34p mediates sorting of soluble hydrolytic enzymes to the vacuole (28). Extensive analysis of Vps15p and Vps34p mutants have shown that the Vps34 gene encodes a phosphatidylinositol-3-phosphate (PtdIns 3-phosphate) (29). In addition to its substrate specificity, this PtdIns 3-kinase differs from PI3K further in that it is less sensitive to wortmannin, requiring low micromolar concentrations for inhibition (30).

Furthermore, yeast deficient in Vps34 fail to complement the vacuole (28). Extensive analysis of Vps15 and Vps34 mutants has shown that PI3K activity (31). Very recently, the sorting and transport of lysosomal enzymes in mammalian cells has also been shown to require PI3K activity (32, 33).

The importance and diversity of PI3K activity in cell biology, including its significance in the previously described aspects of vesicular trafficking, led us to investigate a possible role for the PI3K in transcytosis in polarized epithelial cells, using wortmannin as a tool. As demonstrated in this work, we found that treatment of MDCK cells with 100 nM wortmannin resulted in a 50% reduction in basolateral to apical as well as apical to basolateral transcytosis of ricin, a toxic lectin which binds to terminal galactose residues on glycoproteins and glycolipids and thus serves as an effective marker of bulk membrane transport (4, 34).

Wortmannin had no effect on endocytosis from the basolateral membrane domain of FRT cells, while some reduction in apical endocytosis was detected. In MDCK cells expressing the polymeric immunoglobulin receptor (p.IgR), basolateral to apical transcytosis of both ricin and dimeric IgA (dIgA) was reduced to 50% of controls in the presence of 100 nM wortmannin, showing that bulk membrane and receptor-mediated transcytosis are affected similarly. It has previously been shown that transcytosis in MDCK cells is stimulated by activators of protein kinase A (PKA) (35, 36). To further determine exactly how PI3K activity affects transcytotic transport in transcytotic pathway (36). Using a 17°C block to accumulate ricin internalized from the basolateral domain in the apical compartment of MDCK cells, we provide evidence that wortmannin exerts its inhibitory effect on postendocytic transport earlier in the transcytotic pathway than the late stage where PKA stimulates the process (31). Very recently, the sorting and transport of lysosomal enzymes in mammalian cells has also been shown to require PI3K activity (32, 33).

Materials and Methods

Reagents—Wortmannin, obtained from Sigma, was dissolved in dimethyl sulfoxide at a concentration of 1 mM and distributed in 50- or 200-l aliquots which were stored at −20°C. An aliquot of this stock was thawed and diluted immediately prior to each experiment to minimize degradation of wortmannin, and the remainder was discarded. The concentration of dimethyl sulfoxide did not exceed 0.04% in any experiment in this work. All other reagents used in this work were obtained, prepared, and used as described previously (36).

Cell Culture—Fisher rat thyroid (FRT) cells were kindly provided by Dr. Michael P. Lisanti (Whitehead Institute of Biomedical Research) and grown in F12 Coon's modified medium, obtained from Biofluids, Inc. (Rockville, MD), supplemented with 5% fetal bovine serum (FBS) and antibiotics. MDCKII cells expressing the p.IgR were grown in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum and antibiotics. Both cell lines were maintained in a humidified atmosphere of 5% CO2, 10% O2, and 95% N2 and split 1:20 every 6 days. MDCK cells were trypsinized, the cells were resuspended in 12 ml of medium per plate, and 0.5/0.5 ml of cell suspension was seeded per 12/24-mm Transwell polycarbonate filter with a pore size of 0.4 μm (Costar Corp., Cambridge, MA).

Transcytosis Experiments with 125I-Ricin—A 12-well plate with 12-mm filters was processed in each experiment measuring transcytosis of ricin in FRT or MDCK cells, and these experiments were performed exactly as described previously for MDCK cells (36). Briefly, in a 37°C continuous basolateral to apical transcytosis experiment, the cells were rinsed with Hanks' balanced salt solution (HBSS) and incubated with vehicle only or with drugs (always in both chambers simultaneously) as detailed in the figure legends. Next, 125I-ricin (250 ng/ml, 1 × 104 cpm/ml), iodinated with chloramine T, was added from the basolateral side, and the filters were incubated for 15 or 60 min. Transport was arrested by transferring the plate to slush ice, the apical side was rinsed with HBSS, and the apical media containing 125I-ricin was sampled. The filters were then incubated for 5 min at 37°C in the presence of 0.1 M lactose in both chambers to release membrane-associated 125I-ricin, the basal and apical media were sampled, the filters were excised, and media and filters were counted. Total cell-associated counts were calculated as counts released from the basolateral side with 0.1 M lactose plus counts remaining in the cells. Intracellular ricin was expressed as the ratio of counts remaining in the cells to total cell-associated counts. Transcytosed ricin was expressed as the ratio of counts released into the apical medium plus counts released from apical membrane with 0.1 M lactose to total cell-associated counts. Apical to basolateral transcytosis experiments were performed identically except for the reversed polarity. More than 95% of transcytosed counts were transported across the basolateral membrane domain of ricin, a toxic lectin which binds to terminal galactose residues on glycoproteins and glycolipids and thus serves as an effective marker of bulk membrane transport (4, 34).

MDCK cells expressing the rabbit polymeric immunoglobulin receptor (39), cultured on 12-mm Transwells were pretreated with 100 nM wortmannin for 15 min before or after addition of agonist. Filters were then washed 4 times rapidly with HBSA, placed in fresh HBSA with or without wortmannin, and chased for the times indicated in Fig. 2. At each time point, apical and basolateral media were harvested and replaced with fresh media. After the final time point, filters were cut from their holders and placed in Microfuge tubes. All samples were precipitated on ice for 15 min by addition of an equal volume of 20% trichloroacetic acid. Trichloroacetic acid-soluble counts did not exceed 4% of the total in any experiment. Apical recycling experiments were performed essentially as described (7) except that cells were treated with or without wortmannin as described above.

Determination of Cellular cAMP—Cellular cAMP levels were determined essentially as described by Shimizu et al. (40). Briefly, cells cultured on 12-mm Transwell filters were removed from their holders and placed in 1.0 ml of stop solution containing 5% trichloroacetic acid, 1 mM ATP, and 1 mM cAMP. After 10 min on ice, the samples were filtered and washed 4 times with 1 ml of 0.1 M HEPES, pH 7.0, containing 1 mM EGTA and 1 mM Mg2+. The filters were cut from their holders and placed in Microfuge tubes. All samples were precipitated on ice for 15 min by addition of an equal volume of 20% trichloroacetic acid. Trichloroacetic acid-soluble counts did not exceed 4% of the total in any experiment. Apical recycling experiments were performed essentially as described (7) except that cells were treated with or without wortmannin as described above.
7.4, and pulse-labeled with 1 mCi/ml EXPRE35S35S (Dupont NEN) for 15 min at 37 °C. The cells were then chased for 2 h at 20 °C to accumulate labeled proteins in the TGN (41) and during the last 30 min at 37 °C. The medium containing soluble transcytosed 125I-ricin was harvested directly, while membrane-associated 125I-ricin was recovered by incubation with 0.1 M lactose for 5 min at 37 °C. Finally, the filters containing the cells with intracellular 125I-ricin were excised, and all samples were counted. In a basolateral to apical transcytosis experiment, total cell-associated counts were calculated as counts removed with 0.1 M lactose from the basolateral side of the filter plus counts remaining in the cells. Intracellular ricin was expressed as the ratio of counts in the cells to total cell-associated counts. Transcytosed ricin was expressed as the ratio of soluble transcytosed counts plus transcytosed counts recovered with 0.1 M lactose to total cell-associated counts. Data were expressed similarly in apical to basolateral transcytosis experiments except for the reversed polarity. A, basolateral to apical transcytosis of ricin in 60 min at 37 °C. B, apical to basolateral transcytosis of ricin in 60 min at 37 °C. C–F, intracellular accumulation of ricin administered for 60 min at 37 °C from the basolateral side (C), 60 min at 37 °C from the apical side (D), 15 min at 37 °C from the basolateral side (E), and 15 min at 37 °C from the apical side (F). A and C and B and D, respectively, are derived from the same experiment. Negligible amounts of 125I-ricin were transcytosed in 15 min in either direction. The data shown are mean ± S.D. (n = 3) from experiments, each representative of at least three independent experiments.

RESULTS

Wortmannin Inhibits Bidirectional Transcytosis of Ricin in FRT Cells—To investigate a possible role for PI3K in membrane traffic in polarized epithelial cells, we tested the effect of wortmannin on transcytosis of ricin in FRT cells in a 1-h continuous transcytosis assay. As shown in Fig. 1, wortmannin inhibited both basolateral to apical and apical to basolateral transcytosis of ricin in FRT cells by 20–25% at 10 nM and by 50% at concentrations ≥50 nM. The dose-response of wortmannin on transcytosis with significant effects at nanomolar concentrations is highly suggestive of a role for PI3K in transcytosis and similar to other processes in which PI3K has been implicated (18, 21, 42–46). The effect of wortmannin on basolateral to apical transcytosis of ricin reached a plateau at 50–100 nM, whereas apical to basolateral transcytosis continued to decrease at higher wortmannin concentrations (Fig. 1, A and B; data not shown). Wortmannin did not affect internalization of ricin from the basolateral domain, whereas endocytosis from the apical surface was diminished in the presence of wortmannin (Fig. 1, C–F). However, as seen by comparing B and D of Fig. 1, the reduction in apical endocytosis following treatment with wortmannin is too small to account for the effect on apical to basolateral transcytosis in FRT cells. In all subsequent experiments, we used 100 nM, a concentration at which wortmannin almost completely inhibits PI3K activity without any known effect on other lipid or protein kinases. It
Wortmannin Inhibits Transcytosis in Polarized Epithelial Cells

Fig. 2. Transcytosis and recycling of dIgA. MDCK cells expressing the plgR were pretreated for 15 min with 100 nM wortmannin (open symbols) or with vehicle alone (closed symbols), then allowed to internalize $^{[125]}$-dIgA from either the basolateral (A) or apical (B) surface for 10 min. After washing, cells were placed in fresh medium with or without wortmannin and chased for the times indicated. A, basolateral internalization: closed circles, transcytosis (control); open circles, transcytosis (+ wortmannin); closed squares, basolateral recycling (control); open squares, basolateral recycling (+ wortmannin). B, apical internalization: closed circles, transcytosis (control); open circles, transcytosis (+ wortmannin); closed squares, apical recycling (control); open squares, apical recycling (+ wortmannin). Data are the mean ± S.D. of triplicate determinations and are representative of four experiments.

Wortmannin Inhibits Transcytosis of Ricin and dIgA in MDCK Cells—100 nM wortmannin also reduced basolateral to apical transcytosis in MDCK cells to nearly 50% of control, whereas transport in the opposite direction was inhibited by only 30% (data not shown). To further characterize the putative role for PI3K in transcytosis in polarized epithelial cells, we determined the effect of wortmannin on receptor-mediated transport of dIgA in MDCK cells expressing the plgR. As for ricin, basolateral to apical transcytosis of dIgA was reduced to 50% of the control level in the presence of 100 nM wortmannin (Fig. 2A). A small stimulation in apical to basolateral transcytosis of dIgA was observed following treatment with wortmannin, but the transcytosed amounts under these conditions are so low that it is difficult to derive any conclusion based on this result (Fig. 2B). Neither endocytosis (data not shown) nor recycling from either surface (Fig. 2, A and B) were significantly affected by wortmannin. This is consistent with reports that PI3K (21) and Vps34p/End12 (31) play no significant roles in internalization of PDGF receptors in mammalian cells or α-factor in yeast, respectively. Thus, the main effect of wortmannin on vesicular transport of dIgA is a selective inhibition of basolateral to apical transcytosis. Since bulk-membrane (ricin) and receptor-mediated (dIgA) transport are similarly affected by wortmannin, the data implicate a role for PI3K in postendo-

cytic membrane translocation, rather than sorting of specific proteins, in the transcytotic pathway.

Activators of PKA Stimulate Transcytosis Bidirectionally in FRT Cells—We have previously found that activators of PKA stimulate both receptor-mediated and bulk membrane transcytosis in MDCK cells, and that this stimulation is more pronounced for basolateral to apical transcytosis than transport in the opposite direction (36). The same polarity was found for the inhibition of transcytosis by wortmannin in MDCK cells in the present study. To examine whether activators of PKA affected transcytosis in FRT cells with the same (lack of) polarity as wortmannin, we performed many of the experiments previously carried out with MDCK cells. In FRT cells, forskolin (FSK) and 8-Br-cAMP in the presence of the phosphodiesterase inhibitor Ro 20–1724, strongly stimulate both basolateral to apical and apical to basolateral transcytosis of ricin in a 1-h continuous transcytosis assay (Fig. 3). We also found that cholera toxin, which constitutively activates Gs through ADP-ribosylation, stimulates transcytosis of ricin bidirectionally in FRT cells (data not shown). In further agreement with the data obtained with MDCK cells, the stimulation in transport in both directions by FSK was significantly reduced in the presence of H-89, a selective inhibitor of PKA, and completely eliminated when FSK was substituted with dideoxyforskolin, an analog which does not activate adenylyl cyclase (Fig. 3). The stimulation in transcytosis correlated with a 10-fold elevation of cellular levels of cAMP (data not shown). Thus, in two polarized epithelial cell lines with distinct differences in protein sorting, wortmannin inhibits the same vesicular transport processes that are stimulated by activators of PKA.

Wortmannin Inhibits an Intermediate Step in Transcytosis—The data presented above implicate a role for both PI3K and PKA in bidirectional transcytosis in FRT cells as well as basolateral to apical transport in MDCK cells. This similarity made it interesting to study the relationship between the effects of wortmannin and activators of PKA on transcytosis. Furthermore, since PKA exerts its stimulatory role at a late stage in the basolateral to apical transcytotic pathway in MDCK cells, after internalized ligand has been translocated into the apical compartment, it was possible to use activators of PKA to position the inhibitory effect of wortmannin in the transcytotic pathway.

Wortmannin did not affect the generation of cAMP by Ro 20–1724 and FSK (data not shown). In the presence of 100 nM wortmannin, the combination of forskolin and Ro 20–1724 still increased transcytosis by 100% above the level of wortmannin-treated cells in a continuous 1-h transcytosis assay in both FRT and MDCK cells (Fig. 4, A–C). However, transcytosis was no longer increased when compared to cells which received no drugs (Fig. 4, A–C). In contrast, in pulse-chase experiments using a 17 °C block to accumulate ricin internalized from the basolateral surface in the apical compartment of MDCK cells, the presence of wortmannin had little effect on the stimulation in transcytosis by forskolin and Ro 20–1724 observed under these conditions (Fig. 4D). These data suggest that the effects of wortmannin and agents which increase cellular levels of cAMP are separable and implicate a role for PI3K in an intermediate step in transcytosis after internalization but before translocation to the PKA-sensitive compartment.

Effect of Wortmannin on Apical Secretion of gp 80 in MDCK Cells—To test whether wortmannin affects the secretory pathway, we examined the effect of wortmannin on apical secretion of the endogenous sulfated glycoprotein gp 80 in MDCK cells (47, 48). The 80-kDa preprotein is cleaved intracellularly into a group of 35–45-kDa peptides which are easily detected by SDS-polyacrylamide gel electrophoresis and fluorography of
apical media sampled from metabolically labeled cells (47, 48). In one set of experiments, the cells were pulse-labeled for 15 min with a mixture of [35S]methionine and [35S]cysteine and finally chased for 60–120 min at 37 °C. The presence of 100 nM wortmannin throughout these experiments had no effect on the amount of gp 80 secreted into the apical or the basolateral medium (data not shown). In another set of experiments, following pulse-labeling, the cells were incubated for 2 h at 19.5 °C to accumulate labeled proteins in the TGN (41). During the last 30 min at 19.5 °C, the cells were treated with or without 100 nM wortmannin and, in some experiments, were incubated further in the presence or absence of Ro 20–1724 and FSK for the last 15 min. Next, the cells were warmed to 37 °C for 15, 30, or 60 min with or without drugs. Under these conditions, treatment with 100 nM wortmannin reduced apical delivery of gp 80 by 25% after 15 min at 37 °C, whereas no reduction was observed at later time points (Fig. 5A). Furthermore, wortmannin did not reduce the previously demonstrated strong stimulation in gp 80 secretion by Ro 20–1724 and FSK after 15 min (Fig. 5B) or at later time points (data not shown). Treatment with wortmannin thus causes a slight but reproducible reduction in the rate of constitutive delivery of gp 80 from the TGN to the apical surface. Similar results were found for apical secretion of soluble endogenous proteins from FRT cells (data not shown).

DISCUSSION

In an attempt to identify mechanisms regulating vesicular transport in polarized epithelial cells, we have investigated a possible role for PI3K in transcytosis, using the fungal metabolite wortmannin as a tool. At nanomolar concentrations, wortmannin is an irreversible, noncompetitive, and specific inhibitor of this kinase (17, 18). Indeed, we found that as little as 10 nM wortmannin gave a significant (25%) reduction in bidirectional transcytosis of ricin in FRT cells, while, at a concentration of 100 nM, transcytosis was reduced to 50% of control values. In MDCK cells, both receptor-mediated (dIgA) and bulk-membrane (ricin) transcytosis was inhibited by 50% in the basolateral to apical direction, whereas apical to basolateral transport of ricin was inhibited by 25%. Recycling of dIgA was unimpaired at either the basolateral or apical surface. These data suggest a role for PI3K in transcytosis, a postendocytic vesicular trafficking pathway that is unique to epithelial cells.

As mentioned in the introduction, a role for PI3K in postendocytic sorting of activated PDGF receptors has been described. The observation that mutant receptors lacking PI3K binding sites are efficiently internalized but not degraded has led to the assumption that PI3K is involved in the sorting of the internalized receptors into the lysosomal pathway (20). This hypothesis has been supported by the demonstration that 25–50 nM wortmannin also inhibits degradation of receptors in the presence of PDGF (21). Our results, however, using ricin and dIgA as markers of bulk membrane and receptor-mediated transcytosis, respectively, suggest that wortmannin at similar concentrations significantly inhibits the constitutive flow of membrane into the transcytotic pathway rather than sorting of individual proteins.

Another constitutive membrane transport process that is known to require PI3K activity is the sorting of soluble hydrolytic enzymes to the yeast vacuole. Vps34p, the Ptdlns 3-kinase which is essential to this process, has an IC50 of 4 μM and requires 10 μM wortmannin for complete inhibition, a 100-fold higher concentration than mammalian p110 (30). Vps34p appears to be required for a specific sorting event since trafficking of vacuolar membrane proteins remains unaffected following inactivation of Vps34p (29). Yet, the possibility remains that soluble and membrane vacuolar proteins are cycled in two separate vesicle populations, of which only the first requires Vps34p for a budding or other membrane translocation event. The organelle in the biosynthetic pathway where the action of Vps34p is required is probably the TGN, although Munn and
Riezman (31) have recently determined that Vps34p is also required for transport of internalized α-factor to the vacuole, suggesting that it may function at the exit from late endosomes as well. Very recently, Brown et al. (33) have presented evidence in favor of a similar role for a PI3Kinase in transport of newly synthesized lysosomal enzyme (cathepsin D) from the TGN to late endosomes/prelysosomes in mammalian cell lines NRK and CHO. As for Vps34p, high wortmannin concentrations (1–3 μM) were required for maximal inhibition of cathepsin D processing in late endosomes. Furthermore, this mammalian enzyme appears to function in a M6PR-dependent sorting event in the TGN, since M6PRs were detected at normal levels in the TGN but were depleted from the late endosomes in wortmannin-treated cells (33). These authors suggest that the most likely candidate is a PtdIns3-kinase characterized by Stephens et al. (49), which, like Vps34p, has a high IC50 for wortmannin. Davidson (32), however, has found that in K562, NRK, and Cos 1 cells, the sorting of procathepsin D is inhibited by low nanomolar concentrations of wortmannin. Davidson suggests that the transport of lysosomal enzymes in these cells is regulated by a mammalian homolog of Vps34p, which very recently has been cloned by Volinia et al. (50) and shown to interact with a mammalian homolog of Vps15p. This PtdIns3-kinase is indeed inhibited by concentrations of wortmannin in the low nanomolar range (50). Thus, it is possible that homologs of Vps34p function in transport of soluble hydrolytic enzymes to the lysosome in mammalian cells.

The effect of wortmannin on transcytosis was further characterized through experiments that included activators of PKA. These experiments were prompted by the finding that wortmannin appeared to inhibit vesicular transport processes that are stimulated by PKA activators. In 37°C continuous transcytosis experiments, the inhibition by wortmannin in percent was the same in cells treated with PKA activators as in controls. In contrast, in experiments with MDCK cells employing a 17°C temperature block where marker internalized from the basolateral domain is accumulated in the apical compartment (8), the presence of wortmannin had little effect on stimulation by PKA activators on transport of ricin to the apical membrane. These results show that wortmannin exerts its inhibitory effect on transcytosis prior to the late stage in the transcytotic pathway where activators of PKA stimulate. In addition, it was found that wortmannin has a small inhibitory effect on apical secretion of gp 80 which also is stimulated strongly by activa-

**FIG. 4.** Wortmannin inhibits an intermediate step in the transcytotic pathway. A–D, continuous transcytosis experiments. Monolayers of FRT cells (A and B) or MDCK cells (C) were treated with or without 100 nM wortmannin for 15 min at 37°C and then further preincubated with or without 20 μM Ro 20–1724 and 1 μM FSK for 15 min at 37°C before addition of 125I-ricin to the basal or apical side of the filters. The cells were then incubated for 60 min at 37°C, and counts representing transcytosed, intracellular, and bound 125I-ricin were recovered and the data were expressed as explained in Fig. 1. A, basolateral to apical transcytosis in FRT cells. B, apical to basolateral transcytosis in FRT cells. C, basolateral to apical transcytosis in MDCK cells. D, summary of the protocol for the experiments in A–C. E and F, transcytosis experiments utilizing a 17°C block. E, monolayers of FRT cells were incubated with 125I-ricin (400 ng/ml; 1 × 104 cpm/ng) in the basal chamber for 4 h at 17°C. The basal side was then rinsed six times, and the cells were incubated with or without 100 nM wortmannin for 15 min at 17°C and then further incubated in the presence or absence of 20 μM Ro 20–1724 and 1 μM FSK for another 15 min at 17°C. The cells were then chased for 15 min at 37°C with or without drugs in the presence of 0.1 M lactose in both chambers. Finally, bound, internalized, and transcytosed 125I-ricin were recovered and counted, and the data were expressed as in continuous transcytosis experiments (see Fig. 1). F, summary of the protocol for the experiments in E. Shown are the means ± S.D. from experiments, each representative experiment of at least three independent experiments.
tors of PKA. A simple hypothesis compatible with these data is that wortmannin inhibits vesicle budding from a postendocytic compartment whereas activators of PKA exert their effect by stimulating fusion of transcytosing and secretory vesicles with the apical membrane in MDCK cells (and possibly with both apical and basolateral membranes in FRT cells).

Very recently, an isoform of the 110-kDa subunit of PI3K, p110\textsubscript{g}, has been cloned which has no binding site for the 85-kDa regulatory subunit and is activated by G protein subunits (51). Although it is tempting to speculate that this isoform is involved in the regulation of transcytosis, there are several discrepancies which are difficult to reconcile with this possibility. First, as discussed above, the data obtained in this work implicate PKA (activated by G\textsubscript{s}a, adenylyl cyclase, and cAMP) and PI3K at different stages in the transcytotic pathway. Second, the p110\textsubscript{g} is less sensitive to wortmannin requiring higher concentrations for complete inhibition than those significantly inhibiting transcytosis. Third, in addition to \beta\gamma subunits of G proteins (51, 52), the activity of p110\textsubscript{g} appears to be stimulated strongly by the G\textsubscript{13} group of G\textsubscript{13} subunits (51), whereas pertussis toxin, an inhibitor of this group, has no effect on transcytosis (36).

Serine/threonine kinases clearly serve important roles in regulating transcytosis. In addition to PKA, it has been shown that transcytosis of the plgR is also stimulated by phorbol esters which activate protein kinase C (53). It remains unknown, however, if this effect of phorbol esters is specific for the plgR or, like PKA, affects bulk membrane transport as well.

Furthermore, it has been demonstrated that sorting of the plgR into the transcytotic pathway is stimulated by phosphorylation of the receptor itself by an as yet unidentified kinase (38). The catalytic subunit of PI3K is also a protein kinase (see introduction), and it cannot be excluded that it is the protein and not the lipid kinase activity of PI3K which is involved in the regulation of constitutive transcytosis, as both activities are inhibited by wortmannin. However, there is to our knowledge no precedence for a role for the serine kinase activity of PI3K in membrane traffic.

How the PI3K functions in membrane traffic is not known in detail, but it has been suggested that the negative charge of the phosphoinositide products of the kinase may be important in membrane invagination and/or recruitment of adaptor proteins (12). The negative charge of phospholipid products have also been implicated in the possible function of other lipid kinases in membrane traffic (54), like phosphatidylinositol 4-kinase and phosphatidylinositol 4-phosphate 5-kinase, which may operate through a mechanism that involves ADP-ribosylation factor (55, 56). These suggestions fit our data well in that the effect of wortmannin does not appear to distinguish between receptor-mediated and bulk membrane transport, arguing against a role for PI3K in sorting but in favor of a possible function in formation of vesicles that mediate transcytosis. This could also explain the differential effect of wortmannin on the transcytotic pathways in MDCK cells, since the apical and basolateral membranes of these cells have been shown to exhibit distinct differences in lipid composition (57). It seems

**Fig. 5.** Effect of wortmannin on apical secretion of gp 80 in MDCK cells. Monolayers of MDCK cells were starved for 20 min at 37°C, pulse-labeled with EXPRE \textsuperscript{35}S (1 mCi/ml) for 15 min at 37°C and chased for 2 h at 19.5°C, to accumulate proteins in the TGN, in the presence or absence of 100 nM wortmannin for the last 30 min (A–F) and with or without 20 \textmu M Ro 20–1724 and 1 \textmu M FSK for the last 15 min at 19.5°C (D–F). Next, the cells were warmed to 37°C for 15 (A–F) or 30 (A–C) min in the presence or absence of drugs. The apical medium was sampled, and aliquots thereof were subjected to SDS-polyacrylamide gel electrophoresis and fluorography as detailed under “Materials and Methods.” gp80 is the major secretary product which under reducing conditions is easily identifiable as three polypeptides of 35–45 kDa. A–C, effect of wortmannin on the constitutive level of apical delivery of gp 80. D–F, effect of wortmannin on apical transport of gp 80 in the presence of PKA activators. The data shown are from experiments representative of at least three separate experiments of each kind. The data in B are mean ± S.D. (n = 3). C and F, summary of the protocols for the experiments in A and B and D and E, respectively.
likely that either the extent to which the charged phospholipids are generated and/or their effect on vesicle formation would differ depending on whether the membrane derives from the apical or basolateral domain of MDCK cells. FRT cells, in contrast to most other polarized epithelial cells studied (including MDCK), have been shown to sort most of their glycosylphosphatidylinositol-anchored proteins to the basolateral domain (58). This suggests that the lipid compositions of the apical and basolateral membrane domains in FRT cells differ substantially from those of MDCK cells and could provide an explanation for the lack of polarity in the effect of wortmannin on transcytosis in FRT cells. However, whether this is actually the case remains speculative until more is known about the role of lipids and their kinases in membrane traffic.

Acknowledgment—We thank Dr. Marianne Wessling-Resnick, Dept. of Nutrition, Harvard School of Public Health, for helpful discussions.

REFERENCES

1. Rodriguez-Boulan, E., and Nelson, W. J. (1989) Science 245, 718–725
2. Mostov, K., Apodaca, G., Arceci, B., and Okamoto, C. (1992) J. Cell Biol. 116, 577–583
3. Hunziker, W., Whitney, J. A., and Mellen, H. (1991) Cell 67, 617–627
4. Prydz, K., Hansen, S. H., Sandvig, K., and van Deurs, B. (1992) J. Cell Biol. 119, 259–272
5. Apodaca, G., Enrich, C., and Mostov, K. E. (1994) J. Biol. Chem. 269, 19005–19013
6. Mostov, K., Apodaca, G., Arceci, B., and Okamoto, C. (1992) J. Cell Biol. 116, 577–583
7. Hunziker, W., Male, P., and Mellen, I. (1990) EMBO J. 9, 3515–3525
8. Matzer, K., Bucher, K., and Haupi, R. P. (1990) EMBO J. 9, 3163–3170
9. Diveau, N., and Irvine, R. F. (1995) Cell 80, 269–278
10. Kapferer, R., and Cantley, L. C. (1994) Bioessays 16, 565–576
11. Panayotou, G., and Waterfield, M. D. (1992) Trends Cell Biol. 2, 358–360
12. Whitman, M., Downes, C. P., Keeler, M., Keller, T., and Cantley, L. (1988) Nature 332, 644–646
13. Davidow, M., Ehinger, B., and Waterfield, M. (1994) J. Biol. Chem. 269, 2195–2204
14. Carlberg, K., Tapley, P., Haystead, C., and Rohrschneider, L. (1991) EMBO J. 10, 877–883
15. Shapiro, S. A., Downing, J. J., Rock, C. O., Hawkins, S. A., Rousset, M. F., and Sherr, C. J. (1992) EMBO J. 11, 2415–2421
16. Cheatham, B., Vlahos, C. J., Cheatham, L., Wang, L., Blenis, J., and Kahn, C. R. (1994) Mol. Cell. Biol. 14, 4902–4911
17. Proud, G. D.,ഷ, 432–437
18. Slot, J. W., Geuze, H. J., Gigengack, S., Lienhard, G. E., and James, D. E. (1991) J. Cell Biol. 113, 123–135
19. Stack, J. H., Takegawa, K., and Totty, N. F. (1994) J. Cell Biol. 127, 373–386
20. Davidson, H. W. (1995) J. Cell Biol. 130, 797–805
21. Brown, J. W., DeWals, D. B., Emr, S. D., Plutner, H., and Balch, E. W. (1995) J. Cell Biol. 120, 2195–2204
22. Cheatham, B., Vlahos, C. J., Cheatham, L., Wang, L., Blenis, J., and Kahn, C. R. (1994) Mol. Cell. Biol. 14, 4902–4911
23. Hudson, A. W., Ruiz, M. L., and Birnbaum, M. J. (1992) J. Cell Biol. 119, 259–272
24. Slot, J. W., Geuze, H. J., Gigengack, S., Lienhard, G. E., and James, D. E. (1991) J. Cell Biol. 113, 123–135
25. Schwartz, M., Takegawa, K., and Totty, N. F. (1994) J. Cell Biol. 127, 373–386
26. Stack, J. H., Takegawa, K., and Totty, N. F. (1994) J. Cell Biol. 127, 373–386
27. Stack, J. H., DeWals, D. B., Emr, S. D., Plutner, H., and Balch, E. W. (1995) J. Cell Biol. 127, 802–813
28. van Deurs, B., Hansen, S. H., Petersen, O. W., Løkken Melby, E., and Sandvig, K. (1990) Eur. J. Cell Biol. 51, 96–109
29. Eker, P., Holm, K. V., van Deurs, B., and Sandvig, K. (1994) J. Cell Biol. 126, 1807–1815
30. Hansen, S. H., and Casanova, J. E. (1994) J. Cell Biol. 126, 677–687
31. Brandtzaeg, P., and Baklien, K. (1997) Clin. Exp. Immunol. 10, 77–88
32. Casanova, J. E., Brettfield, P. P., Ross, S. A., and Mostov, K. E. (1990) Science 248, 742–745
33. Mostov, K. E., and Eltchler, D. L. (1986) Cell 46, 613–621
34. Shimizu, H., Daly, J. W., and Cereveling, C. R. (1969) J. Neurochem. 16, 1699–1619
35. Matlin, K. S., and Simons, K. (1983) Cell 32, 332–344
36. Huang, J., Kazlauskas, A., and Blenis, J. (1993) Science 260, 54, 690–693
37. Carrano, C. A., and Wymann, M. P. (1993) Bioessays 15, 296, 297–301
38. Yao, R., and Cooper, G. M. (1995) Science 267, 2003–2006
39. Urban, J., Parczyk, K., Leutz, A., Kayne, M., and Kondor-Koch, C. (1987) EMBO J. 6, 2735–2743
40. Kondor-Koch, C., Bravo, R., Fuller, S. D., Cutler, D., and Garoff, H. (1985) Cell 43, 297–306
41. Shima, Y., Dorris, C. F., Watts, R., Jackson, T., Volinia, S., Gout, I., Waterfield, M. D., and Hawkins, P. T. (1994) EMBO J. 13, 522–533
42. Carpenter, C. L., Auger, K. R., Duckworth, B. C., Hsu, W.-M., Schaffhausen, S., and Cantley, L. C. (1993) Mol. Cell. Biol. 13, 1657–1665
43. Liscovitch, M., and Cantley, L. C. (1995) Science 269, 718–725
44. Liscovitch, M., Chalifa, V., Pertile, P., Chen, C.-S., and Cantley, L. C. (1994) EMBO J. 13, 83–93
45. Munn, A. L., and Riezman, H. (1994) Cell 81, 659–662
46. Parker, P., and Cantley, L. C. (1995) Science 269, 373–386
47. Urban, J., Parczyk, K., Leutz, A., Kayne, M., and Kondor-Koch, C. (1987) EMBO J. 6, 2735–2743
Wortmannin, an Inhibitor of Phosphoinositide 3-Kinase, Inhibits Transcytosis in Polarized Epithelial Cells
Steen H. Hansen, Anna Olsson and James E. Casanova

J. Biol. Chem. 1995, 270:28425-28432.
doi: 10.1074/jbc.270.47.28425

Access the most updated version of this article at http://www.jbc.org/content/270/47/28425

Alerts:
• When this article is cited
• When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts

This article cites 57 references, 34 of which can be accessed free at http://www.jbc.org/content/270/47/28425.full.html#ref-list-1