Phosphatidylinositol (PI) 3'-kinases are a family of lipid kinases implicated in the regulation of cell growth by oncogene products and tyrosine kinase growth factor receptors. The catalytic subunit of the p85/p110 PI 3'-kinase is homologous to VPS-34, a phosphatidylinositol-specific lipid kinase involved in the sorting of newly synthesized hydrolases to the yeast vacuole. This suggests that PI 3'-kinases may play analogous roles in mammalian cells. We have measured a number of secretory and endocytic trafficking events in Chinese hamster ovary cells in the presence of wortmannin, a potent inhibitor of PI 3'-kinase. Wortmannin caused a 40–50% down-regulation of surface transferrin receptors, with a dose dependence identical to that required for maximal inhibition of the p85/p110 PI 3'-kinase in intact cells. The redistribution of transferrin receptors reflected a 60% increase in the internalization rate and a 35% decrease in the recycling rate. Experiments with fluorescent transferrin showed that entry of transferrin receptors into the recycling compartment and efflux of receptors out of the compartment were slowed by wortmannin. Wortmannin altered the morphology of the recycling compartment, which was more vesiculated than in untreated cells. Using Semliki Forest virus as a probe, we also found that delivery of the endocytosed virus to its lysosomal site of degradation was slowed by wortmannin, whereas endosomal acidification was unaffected. In contrast to these effects on endocytosis and recycling, wortmannin did not affect intracellular processing of newly synthesized viral spike proteins. Wortmannin did induce missorting of the lysosomal enzyme cathepsin D to the secretory pathway, but only at a dose 20-fold greater than that required to inhibit p85/p110 PI 3'-kinase activity or to redistribute transferrin receptors. Our data demonstrate the presence of wortmannin-sensitive enzymes at three distinct steps of the endocytic cycle in Chinese hamster ovary cells: internalization, transit from early endosomes to the recycling and degradative compartments, and transit from the recycling compartment back to the cell surface. The wortmannin-sensitive enzymes critical for endocytosis and recycling are distinct from those involved in sorting newly synthesized lysosomal enzymes.

Phosphatidylinositol (PI) 3'-kinases are a family of lipid kinases that contain homologous catalytic domains, but distinct regulatory elements. The first known PI 3'-kinase was a heterodimeric lipid kinase composed of an 85-kDa regulatory subunit and a 110-kDa catalytic subunit (1–5). This enzyme was discovered in transformed cells, but is also stimulated by mitogens including platelet-derived growth factor and insulin (6). The p85/p110 PI 3'-kinase is activated when the two SH2 domains in p85 bind to phosphorylated YXXM motifs in tyrosine kinase receptors or their substrates (7, 8); activation by interactions with p21ras, Rho, and the SH3 domains of Src family kinases has also been described (9–11). Activation of the p85/p110 PI 3'-kinase increases the intracellular concentrations of PI(3,4)P2 and PI(3,4,5)P3 (12). Although these lipid products activate calcium-independent protein kinase C isoforms in vitro, their function in intact cells is not known (13, 14). p110 also contains a serine/threonine protein kinase activity with an apparently limited range of substrates (15). In addition to multiple isoforms of both the 85- and 110-kDa subunits, the recent description of several novel PI 3'-kinase isoforms with varying substrate specificities and regulatory mechanisms has added to the complexity of 3-phosphoinositide signaling (16–18).

The p110 catalytic subunit is 55% homologous to the Saccharomyces cerevisiae protein VPS-34 (2, 19). Disruption of the VPS-34 gene causes misrouting of vacuolar hydrolases to the secretory pathway and also abolishes PI 3'-kinase activity in yeast (20). Although VPS-34 is a PI 3'-kinase, its substrate specificity is limited to phosphatidylinositol, unlike the p85/p110 mammalian enzyme that also utilizes PI(4)P and PI(4,5)P2 (21). Moreover, mammalian p110 is lethal when expressed in yeast (22). However, a mammalian homologue to VPS-34 has been cloned, which, like VPS-34, is a lipid kinase activity specific for phosphatidylinositol (16). Several experiments suggest that PI 3'-kinases play a role in vesicular trafficking in mammalian cells. Mutagenesis of the two PI 3'-kinase-binding sites in the platelet-derived growth factor receptor disrupts the post-endocytic sorting of this receptor (23). However, these same sites bind to SH2-containing mole-
ules other than PI 3'-kinase (24), which complicates the interpretation of this finding. PI kinase inhibitors such as wortmannin and LY294002 inhibit histamine secretion in RBL-2H3 cells, exocytosis in neutrophils stimulated with chemotactic agents, and translocation of the GLUT4 glucose transporter in insulin-stimulated adipocytes and skeletal muscle (25–31). These data have been interpreted as demonstrating a role for the p85/p110 PI 3'-kinase in these processes. However, a number of PI kinase isoforms are sensitive to wortmannin, including both PI 3-kinases and PI 4-kinases (16, 24, 32). Despite consistent with these changes, the morphology of the recycling compartment is altered by wortmannin, and movement of transferrin into and out of the recycling compartment is slowed. We also find that wortmannin slows the delivery of endocytosed Semliki Forest virus to its lysosomal site of degradation. However, we were unable to detect any changes in the transit of newly synthesized viral glycoproteins through the Golgi apparatus and to the plasma membrane, and we detected alterations in the sorting of newly synthesized lysosomal enzymes only at micromolar concentrations of wortmannin. Our data demonstrate the presence of wortmannin-sensitive enzymes that regulate receptor-mediated endocytosis and recycling. These enzymes are pharmacologically distinct from those involved in the sorting of newly synthesized lysosomal proteases.

**EXPERIMENTAL PROCEDURES**

**CHO Cell Lines—**TRVb-1 cells are derived from CHO-WTB cells and express 10^5 human transferrin receptors/cell (33). CHO/IR cells are derived from CHO-K1 cells and express 10^6 wild-type human insulin receptors/cell (exon 11-minus isofrom (34, 35)).

Inhibition of PI 3'-Kinase by Wortmannin—Wortmannin (Sigma) was stored in dimethyl sulfoxide at –70 °C and diluted into protein-free medium just prior to use. Inhibition of PI 3'-kinase in intact cells was measured by incubating CHO/R cells with the indicated concentrations of wortmannin or dimethyl sulfoxide (0.1%, v/v) for 30 min at 37 °C. After an additional incubation in the absence or presence of 100 nm wortmannin, the cells were lysed, and PI 3'-kinase activity in anti-p85 immunoprecipitates was determined as described previously (7).

Transferrin Uptake and Recycling—TRVb-1 cells cultured in 6-well dishes were incubated in the absence or presence of wortmannin (100 nm unless otherwise indicated, in a final concentration of 0.1% dimethyl sulfoxide) for 45 min at 37 °C; control experiments showed that 0.1% dimethyl sulfoxide does not affect trafficking parameters in TRVb-1 cells. Transferrin endocytosis was measured, and endocytic rate constants were calculated using the ln/Sur method as described previously (33, 36). In experiments examining both internalization and surface binding at 37 °C, parallel sets of untreated or wortmannin-treated cells were incubated with 125I-labeled transferrin for 0–10 min and then washed with neutral buffer, to determine surface-bound radioactivity, or with acidic buffer, to determine intracellular radioactivity. Uptake of 35S-labeled transferrin was measured by incubating untreated or wortmannin-treated cells (100 nm; 45 min) in the presence of 10 μg/ml [35S]methionine-labeled transferrin for 2 h at 37 °C, followed by an additional 45 min with 125I-labeled transferrin in the absence or presence of 100 nm wortmannin. The cells were washed with 50 ml MES (pH 5.0) and 150 ml NaCl to remove surface-bound transferrin and incubated in 600 μg/ml unlabeled transferrin and 100 μg/ml desferoxamine in the continued absence or presence of wortmannin. At various times, the medium and one wash were collected, and the cells were solubilized and determined to determine recycled and cell-associated radioactivity.

**Inulin Endocytosis—**Cells were pretreated with 100 nm wortmannin or carrier (0.1% (v/v) dimethyl sulfoxide) for 30 min at 37 °C. Insulin internalization was measured, and internalization rate constants were calculated as described previously (37).

**Analysis of Transferrin Trafficking by Immunofluorescence—**In pulse-chase experiments, TRVb-1 cells grown on coverslips were incubated with 100 nm wortmannin or carrier for 30 min at 37 °C. Cells were incubated with CY3-transferrin (10 μg/ml) for 3 min, washed, and chased in the continued absence or presence of wortmannin. At various times, the cells were washed and fixed in 3.7% formaldehyde as described (38) and examined using a Bio-Rad MRC 600 laser scanning confocal microscope with Nikon ×60 NA 1.4 planapo lenses. Serial sections were collected at 0.6 μm steps over the depth of the cells and projected using the maximum pixel method and were printed using a Sony Movigraph color video printer. In the sorting experiments, cells were incubated in CY3-transferrin for 15 min, washed, and then chased with medium containing FITC-transferrin (10 μg/ml) in the absence or presence of 100 nm wortmannin. Cells were washed three times after the chase period, fixed in 3.7% formaldehyde, and examined using a Zeiss Axiowert microscope at ×63 or ×100. Images were photographed directly from the microscope using identical exposure times.

**Uptake and Degradation of 35S-Labeled Semliki Forest Virus—**35S]methionine-labeled Semliki Forest virus was prepared and purified as described (39). CHO/IR or TRVb-1 cells grown in 35-mm plates were pretreated in the absence or presence of 100 nm wortmannin for 30 min at 37 °C. The cells were then incubated with 100 nM wortmannin and 200 nM desferoxamine in the continued absence or presence of 100 nm wortmannin. Cells were incubated with [35S]methionine-labeled SFV (10 units/ml) for 30 min at 37 °C, washed three times after the incubation, and resuspended in medium containing 0.15 mg/ml desferoxamine. The cells were infected with vesicular stomatitis virus spike protein (VSV-G) was analyzed as described previously (42). Briefly, CHO cells cultured in 35-mm dishes were infected with vesicular stomatitis virus (20 plaque-forming units/cell). The cells were treated without or with 100 nm wortmannin for 30 min at 37 °C in methionine-free medium, labeled with [35S]methionine (50 μCi/well) for 5 min, and chased for various periods of time. Lysates were incubated for 1 h at 4 °C with a rabbit polyclonal antibody against the SFV spike protein or with monoclonal antibody E1a-1, which specifically recognizes the SFV E1 glycoprotein only after it has undergone an acid-dependent conformational change (40). Immunoprecipitated proteins were visualized using a PhosphorImager (Molecular Dynamics, Inc.).

**Measurement of Endosomal Acidification with Conformation-specific Antibodies—**The effect of wortmannin on endosomal acidification was determined by following the acid-induced conformational change in the Semliki Forest virus E1 spike protein as described previously (41). Briefly, TRVb-1 cells were incubated for 30 min at 37 °C in the presence of 100 nm wortmannin or carrier and incubated with [35S]labeled SFV at 4 °C as described above. The cells were washed rapidly to 37 °C to initiate endocytosis and lysed at various times. Lysates were incubated with [35S]labeled Semliki Forest virus in the continued absence or presence of wortmannin. After a rinse in cold medium to remove unbound virus, internalization and degradation of surface-bound SFV were determined as described (40).

**Transit of Newly Synthesized Vesicular Stomatitis Virus Glycoproteins—**Biosynthetic trafficking of the vesicular stomatitis virus spike protein (VSV-G) was analyzed as described previously (42). Briefly, CHO cells cultured in 35-mm dishes were infected with vesicular stomatitis virus (20 plaque-forming units/cell). The cells were treated without or with 100 nm wortmannin for 30 min at 37 °C in methionine-free medium, labeled with [35S]methionine (50 μCi/ml) for 5 min, and chased for various periods of time. Lysates were incubated for 1 h at 4 °C with a rabbit polyclonal antibody against the SFV spike protein or with monoclonal antibody E1a-1, which specifically recognizes the SFV E1 glycoprotein only after it has undergone an acid-dependent conformational change (40). Immunoprecipitated proteins were visualized using a PhosphorImager (Molecular Dynamics, Inc.).

**Processing of Newly Synthesized Semliki Forest Virus Spike Proteins—**Processing of the SFV E1 and E2 spike proteins in wortmannin-treated cells was analyzed as described previously (40). Briefly, CHO cells grown in 35-mm dishes were infected with SFV (100 plaque-forming units/ml) with or without 100 nm wortmannin for 30 min at 37 °C in methionine-free medium, labeled with [35S]methionine (50 μCi/ml) for 5 min, and chased for 1 h at 4 °C with [35S]methionine (50 μCi/well) for 30 min, and chased in medium containing excess methionine in the continued absence or presence of wortmannin. At various times, 35S-labeled spike proteins were immunoprecipitated from solubilized cells and analyzed as described (40). Viral budding was determined as described (40).

**Secretion of Cathepsin D—**Analysis of cathepsin D synthesis was performed as described by Richo and Conner (45). TRVb-1 or CHO/R cells, grown in 35-mm dishes, were incubated for 30 min in wortmannin as indicated in methionine-free medium, labeled with [35S]methionine for 30 min, and chased in medium containing excess methionine in the continued absence or presence of wortmannin. At various times, the medium was removed, SDS was added (0.4% final concentration), and the samples were boiled. The samples were solubilized in 0.5 ml of boiling...
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RESULTS

Inhibition of PI 3'-Kinase by Wortmannin—We determined the concentration of wortmannin required to inhibit the p85/p110 PI 3'-kinase in intact cells. A 30-min incubation with 100 nM wortmannin reduced both basal and insulin-stimulated p85/p110 activity by 70–80% (data not shown). Basal PI 3'-kinase activity showed half-maximal inhibition at 30 nM wortmannin and maximal inhibition at 100 nM, with no additional decrease in activity at 1 μM wortmannin (Fig. 1). Subsequent experiments used 100 nM wortmannin unless otherwise indicated.

Effect of Wortmannin on the Endocytic Pathway—Previous studies suggest a role for wortmannin-sensitive enzymes in the endocytic pathway (23). We examined the effect of wortmannin on transferrin receptor trafficking using TRVb-1 cells, which overexpress the human transferrin receptor. Treatment of TRVb-1 cells with wortmannin resulted in a depletion of cell-surface transferrin receptors (Fig. 2A). Half-maximal depletion was observed at 30 nM wortmannin, and a maximal 40–50% depletion was achieved at 100 nM wortmannin. No further decrease in cell-surface receptors was observed at concentrations as high as 1 μM wortmannin (data not shown). The effect of wortmannin on cell-surface transferrin receptors corresponded with the dose response for inhibition of the p85/p110 PI 3'-kinase.

We then measured the uptake of 125I-labeled transferrin during a 10-min incubation at 37 °C in TRVb-1 cells. Pretreatment with wortmannin for 30 min decreased the accumulation of surface-bound and intracellular transferrin (Fig. 2B). The change in cell-surface transferrin binding reflected a redistribution of cell-surface transferrin receptors into an intracellular compartment, as total cellular transferrin binding was unaffected by wortmannin (data not shown). To determine the mechanism for this redistribution, we measured the rates of internalization and recycling in wortmannin-treated cells. The internalization rate constant for 125I-labeled transferrin was calculated by the In/Sur method (36); a representative experiment is shown in Fig. 2C. In data pooled from nine experiments, the rate constant for transferrin receptor endocytosis was increased 55% by wortmannin (Table I). Similarly, the rate of 55Fe uptake was increased 60% by treatment of cells with wortmannin (Table I). Although the calculated rate constants differed using the two methods, in both cases, treatment of cells with 100 nM wortmannin caused a similar increase in the internalization rate (Table I). Although there was a decrease in the absolute amount of internalized 125I-labeled transferrin in wortmannin-treated cells (Fig. 2B), the internalization rate constants are calculated on a per receptor basis and are in fact increased by wortmannin.

In addition to the effects on internalization, the rate of transferrin recycling was reduced by treatment of cells with wortmannin. Cells were preloaded with 125I-labeled transferrin in the absence of wortmannin (to prevent any differences in the concentration of intracellular ligand) and then incubated in...
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Rate constants for transferrin endocytosis and recycling

| Endocytosis | Recycling, \(k_r\) | Change in surface binding |
|-------------|---------------------|--------------------------|
| \(k_e\) (InSur) | \(k_e\) (55Fe) | Predicted | Observed | % |
| Control | 0.27 ± 0.03 | 0.024 ± 0.007 | 0.084 ± 0.005 | -41.7 | -45 |
| Wortmannin | 0.42 ± 0.07 | 0.035 ± 0.008 | 0.056 ± 0.003 | (−33.3%) |

Wortmannin 0.42 M desferoxamine in the absence or presence of wortmannin (a representative experiment is shown in Fig. 2D). In data pooled from seven experiments, we observed a 33% decrease in the rate of \(^{125}\)I-labeled transferrin efflux from the wortmannin-treated cells (Table I).

The steady-state distribution of transferrin receptors between the interior and surface of cells is determined by both the rates of endocytosis (\(k_e\)) and recycling (\(k_r\)). Changes in either parameter will be reflected in a new steady-state distribution. The increase in \(k_e\) and the decrease in \(k_r\) observed in wortmannin-treated cells predict a redistribution of transferrin receptors from the cell surface to the cell interior, resulting in a decrease in surface binding. The magnitude of the predicted change in surface binding, based on the ratio of the changes in \(k_e\) and \(k_r\), was similar to the observed decrease in cell-surface \(^{125}\)I-labeled transferrin binding (42% as opposed to 45%) (Table I).

100 \(\mu\)M desferoxamine in the absence or presence of wortmannin. We also measured the internalization of \(^{125}\)I-labeled insulin in CHO/IR cells, which overexpress the human insulin receptor. Unlike transferrin receptors, which internalize constitutively, insulin receptors enter coated pits in a ligand-stimulated manner (46, 47). Moreover, changes in the insulin receptor membrane-spanning domain that increase the rate of receptor lateral diffusion on the cell surface increase the rate of receptor endocytosis (48), suggesting that events prior to entry into coated pits are rate-limiting for insulin receptor endocytosis. We found that the rate constant for \(^{125}\)I-labeled insulin uptake in CHO/IR cells was unaffected by wortmannin (\(k_e = 0.116 ± 0.006\) versus \(0.125 ± 0.03\) in the absence and presence of wortmannin) (data not shown). Thus, the ligand-stimulated events that precede entry of insulin receptors into coated pits appear to be independent of wortmannin-sensitive enzymes.

Morphology of the Recycling Compartment—We used Cy3-transferrin and chased in wortmannin for 2 min, we saw an accumulation of ligand in numerous peripheral punctate structures similar to those seen in control cells (Fig. 3C). The intensity of labeling was decreased in wortmannin-treated cells relative to control cells, reflecting the decrease in cell-surface transferrin receptors caused by preincubation with wortmannin (Fig. 2B). After 12 min of chase, the distribution of labeled transferrin in wortmannin-treated cells was significantly different than in control cells, with a persistence of label in peripheral punctate structures (Fig. 3D). After 15 min of chase, pericentriolar concentration could be seen in some wortmannin-treated cells (data not shown). However, the labeled structures in these cells were more vesiculated and less uniform in appearance that those in control cells (data not shown; but see Fig. 4, A and B).

These data suggest that wortmannin reduces the rate of delivery of internalized transferrin from endosomes to the recycling compartment. Alternatively, the altered morphology of the labeled compartment in wortmannin-treated cells could reflect a change in the fidelity of sorting, with delivery of internalized transferrin to a different compartment. To differentiate these possibilities, we examined the degree to which transferrin internalized prior to wortmannin treatment mixed with transferrin internalized in the presence of wortmannin. Cells were preincubated to equilibrium by a 45-min incubation with Cy3-transferrin in the absence of wortmannin; under these conditions, label accumulates in the pericentriolar recycling compartment. The cells were then washed and chased in medium containing FITC-transferrin in the absence or presence of 100 nM wortmannin. During this incubation, Cy3-transferrin effluxes from the cells, whereas FITC-transferrin accumulates.

In untreated cells labeled with Cy3-transferrin and then chased with FITC-transferrin for 15 min, residual Cy3 labeling of the recycling compartment was easily observable (Fig. 4A). Moreover, FITC-transferrin could be seen to accumulate in the same intracellular compartment that contains Cy3-transferrin (Fig. 4C). In cells chased with FITC-transferrin in the presence of wortmannin for 15 min, the residual Cy3 labeling of the recycling compartment is much brighter than in control cells (Fig. 4B), indicating that wortmannin slowed the recycling of transferrin receptors back to the cell surface. Moreover, the vesication of the pericentriolar compartment in wortmannin-treated cells is evident (Fig. 4B). However, the altered compartment is still clearly labeled with FITC-transferrin (Fig. 4D), indicating that this compartment is in communication with early endosomal structures containing newly internalized transferrin.

The wortmannin-induced slowing of efflux from the recycling compartment is even more striking after a 45-min chase. In control cells chased for 45 min, Cy3-transferrin in the pericentriolar region of the cell (Fig. 3B) (38).
triolar structures has been substantially washed out (Fig. 4E), replaced by FITC-transferrin that entered during the chase (Fig. 4G). In contrast, the punctate pericentriolar structures in wortmannin-treated cells are still brightly labeled with Cy3-transferrin after 45 min (Fig. 4F). Again, Cy3-transferrin colocalizes with newly internalized FITC-transferrin (Fig. 4H), suggesting that the punctate pericentriolar structures in wortmannin-treated cells are in communication with early endosomal structures. The increased labeling of the recycling compartment during the FITC-transferrin chase in wortmannin-treated cells (Fig. 4, compare C with D and G with H) reflects the wortmannin-induced redistribution of transferrin-receptor complexes into the cell interior (Fig. 2A). Taken together, the data in Figs. 3 and 4 show that wortmannin has a pronounced inhibitory effect on both entry into and efflux from the recycling compartment.

Effects of Wortmannin on Lysosomal Delivery and Endosomal Acidification—The endocytic system internalizes both ligands and receptors by a common pathway, subsequently sorting receptors for recycling and ligands for lysosomal degradation. To determine whether wortmannin affects the sorting of ligands into a degradative pathway, we measured the degradation of endocytosed 35S-labeled SFV, which is sorted from endosomes to the lysosome (49). In control cells, we observed a characteristic 10–15-min lag period before the appearance of viral degradation products in the medium, which reached a maximum at ~90 min (Fig. 5). In contrast, the time lag before the detection of degraded SFV in the medium was longer (20–30 min) in wortmannin-treated cells, and degraded SFV in the medium of wortmannin-treated cells was half that of control cells by 90 min. The differences in the rates of SFV degradation could not be explained by differences in the rates of internalization of surface-bound SFV, which were not affected by wortmannin (data not shown).

Although these data suggest that wortmannin had significant effects on the kinetics of late endosomal sorting, the altered kinetics of SFV degradation could be due to effects on the acidification of the endosomal lumen. We therefore examined the endocytic uptake of 35S-labeled SFV using a conformation-specific antibody to the SFV E1 spike protein. The E1 spike protein undergoes a conformational change when exposed to the acidic conditions of the early endosome (pH 6.2 or lower), exposing an epitope that is recognized by monoclonal antibody E1a-1 (40).

Untreated (Fig. 6A) or wortmannin-treated (Fig. 6B) cells were allowed to bind 35S-labeled SFV at 4°C and then warmed rapidly to 37°C. At various times, the cells were lysed, and proteins were precipitated with either anti-spike antibody or conformation-specific antibody E1a-1. No difference in the total amount of cell-associated virus was detected in control versus wortmannin-treated cells (Fig. 6, A and B, lanes a). In both treated and untreated cells, a decrease in cell-associated virus due to lysosomal degradation was apparent by 15–30 min (Fig. 6, A and B, lanes d and e). Similarly, in both untreated and wortmannin-treated cells, the E1 spike protein was first recognized by antibody E1a-1 after 5 min at 37°C (Fig. 6, A and B, lanes j), with levels of E1a-1-precipitable protein reaching maximal levels by 15 min (lanes l). In control experiments, treatment of cells with monensin completely blocked the conversion of E1 to the acidic conformation (Fig. 6, A and B, lanes o), whereas brief treatment of bound virus with acidic buffer (pH 5.5) caused maximal conversion of the E1 protein to its acidic conformation (lanes p). We conclude that endosomal acidification is not grossly affected by wortmannin.

Effect of Wortmannin on Biosynthetic Trafficking—We examined the transit and processing of VSV-G and the SFV E2 glycoprotein to determine the potential effects of wortmannin on biosynthetic vesicular trafficking. To measure the net rate of
delivery of newly synthesized membrane glycoprotein to the plasma membrane in the absence or presence of wortmannin, we infected CHO cells with vesicular stomatitis virus, pulse-labeled the cells with $[^{35}\text{S}]$methionine, and specifically labeled plasma membrane VSV-G by a cell-surface biotinylation protocol at 4 °C. The kinetics of VSV-G arrival at the plasma membrane was consistent with previous studies (50) and was unaffected by 100 nM wortmannin (data not shown). We also examined the processing of the SFV E1 and E2 spike proteins, which occurs in a post-Golgi compartment (40, 51). SFV-infected cells were incubated in the absence or presence of 100 nM wortmannin and pulse-labeled with $[^{35}\text{S}]$methionine. Processing of the SFV spike proteins was then analyzed by immunoprecipitation and SDS-polyacrylamide gel electrophoresis. Wortmannin had no effect on the cleavage of the E2 glycoprotein from its 62-kDa precursor form to its mature form (data not shown). Secretion of assembled virions was also unaffected by wortmannin (data not shown). Taken together, the experiments with VSV and SFV indicate that wortmannin does not affect transit of newly synthesized glycoproteins from the endoplasmic reticulum, through the Golgi apparatus, and to the plasma membrane.

**Effect of Wortmannin on Lysosomal Sorting**—Lysosomal hydrolases are sorted to the lysosome in CHO cells by virtue of the binding of mannose 6-phosphate residues to the mannose 6-phosphate receptor in the Golgi apparatus (52). A small proportion of lysosomal hydrolases are also secreted, and this alternative pathway is augmented during transformation of a number of cell types (53). In yeast, mutation of VPS-34 causes the mis-sorting of vacuolar hydrolases to the secretory pathway (20). Moreover, two recent studies have suggested that wortmannin diverts newly synthesized cathepsin D into the secretory pathway (54, 55). To determine if treatment of CHO cells with wortmannin caused a similar perturbation of lysosomal...
enzyme sorting, we measured the secretion of cathepsin D into the medium of cells treated with 100 nM wortmannin. Fig. 7A shows total intracellular cathepsin D and secreted cathepsin D from control or wortmannin-treated cells; the two gels were intentionally overexposed for identical lengths of time to permit visualization of secreted cathepsin D. 100 nM wortmannin had no effect on either the synthesis or stability of intracellular cathepsin D (Fig. 7A, upper panel), and secretion of cathepsin D was extremely low in both untreated and wortmannin-treated cells (lower panel). In five separate experiments, we detected no significant increase in cathepsin D secretion in cells treated with 100 nM wortmannin.

In contrast to the data obtained with 100 nM wortmannin, we saw a significant effect on the secretion of cathepsin D after treatment of cells with 2 µM wortmannin. This concentration of drug is 20-fold higher than that required for maximal effects on pH5/p110 PI 3-kinase activity or transferrin receptor redistribution. Fig. 7B shows identical exposures of intracellular and secreted cathepsin D from control cells, cells treated with 2 µM wortmannin, or cells treated with 50 µM chloroquine. Treatment of cells with 2 µM wortmannin had no significant effect on the synthesis or stability of intracellular cathepsin D, whereas treatment of cells with chloroquine increased the intracellular half-life of newly synthesized cathepsin D, presumably by inhibiting lysosomal degradation (Fig. 7B, upper panel). Notably, both 2 µM wortmannin and 50 µM chloroquine had pronounced effects on the secretion of newly synthesized cathepsin D. Secreted cathepsin D was undetectable in control cells (Fig. 7B, lower panel) using a fluorographic exposure time one-third that used in Fig. 7A. In contrast, secreted cathepsin D was easily seen after 3 h in cells treated with 2 µM wortmannin (Fig. 7B, lower panel). 2 µM wortmannin induced a level of cathepsin D secretion similar to that seen in cells treated with chloroquine, a drug that is known to divert lysosomal enzymes into the secretory pathway (56).

FIG. 6. Effect of wortmannin on endosomal acidification. TRVb-1 cells pretreated with carrier (A) or 100 nM wortmannin (B) were allowed to bind 35S-labeled SFV on ice for 90 min. After washes to remove unbound virus, the cells were warmed to 37 °C in the continued absence or presence of wortmannin. At various times, the cells were lysed, and 35S-labeled viral proteins were immunoprecipitated with anti-spike antibody (A and B, lanes a–h) or antibody E1a-1 (lanes i–p), which recognizes the E1 spike protein after it has undergone an acid-dependent conformational change. Lanes g and o show immunoprecipitates from incubations that included monensin (10 µM) to block acidification. Lanes h and p show cells treated with pH 5.5 buffer for 1 min prior to lysis.
wortmannin induce changes distinct from those observed at concentrations that maximally inhibit the p85/p110 PI 3'-kinase.

DISCUSSION

Studies from several different laboratories suggest that PI 3'-kinase plays a role in endocytic or other trafficking events in mammalian cells. In this paper, we examined trafficking events in CHO cells treated with 100 nM wortmannin (27, 57, 58). Both α- and β- isoforms of the p110 PI 3'-kinase are half-maximally inhibited by low nanomolar concentrations of wortmannin, as is the recently cloned PI-specific PI 3'-kinase δ; the sensitivity of the δ-stimulated PI 3'-kinase is not yet clear (16, 18, 59). In addition, a PI 4-kinase important in the regulation of intracellular PI(4)P and PI(4,5)P₂ levels is half-maximally inhibited by 100 nM wortmannin and completely inhibited by 1 μM wortmannin (32). This finding is particularly important; PI(4,5)P₂ has been implicated in the regulation of AP-2 adaptin complexes and cytoskeletal components such as profilin and plays a role in regulated secretion (60–62). Moreover, PI(3,4,5)P₃ has been shown to bind to pleckstrin homology domains, suggesting that disruption of PI(3,4,5)P₃ synthesis could affect numerous cellular functions (63). The existence of multiple wortmannin-inhibited enzymes, which are regulated by different mechanisms and may exist in distinct intracellular locations, compromises the use of wortmannin as a putatively specific inhibitor of PI 3'-kinase. Studies currently in progress, involving the microinjection of inhibitory anti-p110 antibodies into TRVb-1 cells, will address the question of whether the wortmannin-sensitive enzyme involved in transferrin trafficking is the p85/p110 PI 3'-kinase.

The kinetic analysis of transferrin receptor trafficking and Semliki Forest virus degradation in wortmannin-treated cells demonstrates that three distinct steps in endocytic trafficking are affected by the drug. First, we see an increase in the rates of 125I-labeled transferrin endocytosis and 55Fe accumulation, both of which measure the rate of constitutive coated pit-mediated internalization. It is therefore possible that a wortmannin-sensitive enzyme is important in regulating the early rate-limiting steps governing coated pits. We did not see changes in the rates of internalization of either 125I-labeled insulin or surface-bound 35S-labeled SFV. However, both insulin and SFV initially bind to receptors located in the microwe- lous regions of the cell membrane and then move to coated pits (47, 49). Furthermore, the rate of insulin receptor endocytosis appears to be limited by the rate of receptor diffusion through the plasma membrane (48) and therefore is probably not a reflection of the absolute rate of coated pit endocytosis.

A second site of wortmannin action may be the sorting endosome. In CHO cells, recycled ligands are sorted from material destined for degradation by an iterative fractionation process in which the sorting endosome matures into a late endosome (38). Consequently, if wortmannin affects the maturation of the sorting endosome, then both transport of the recycling membrane to the pericentriolar recycling compartment and delivery of material to lysosomes could be affected. Consistent with this possibility, wortmannin slows the movement of internalized receptor-bound transferrin from endosomes to the pericentriolar recycling compartment as well as the degradation of internalized SFV, which requires delivery to the lysosome. These changes do not appear to be due to defects in luminal acidification, which is known to affect intracellular trafficking of the transferrin receptor (64, 65), as the acid-dependent conformational changes in the SFV E1 spike protein are normal in wortmannin-treated cells. Although our data could be explained by a common wortmannin-sensitive step in the sorting compartment, we cannot rule out the possibility of independent wortmannin-sensitive steps involved in transit of ligands between the sorting endosome and the recycling compartment or the lysosome. These steps could involve Rab5 or another Rab isoform, as has been recently suggested by Li et al. (66).

A third potential site of action is the recycling compartment, defined as the pericentriolar structure that is labeled by fluo-
resistant transferrin, but not by ligands like low density lipoprotein that are destined for lysosomal degradation (38). Transport from the pericentriolar recycling compartment is the rate-limiting step in transferrin recycling (38). The morphology of the recycling compartment is significantly altered by 100 nM wortmannin, and efflux of transferrin out of the compartment is slowed in wortmannin-treated cells. Although the mechanism by which wortmannin affects the recycling compartment is not yet clear, there is precedent for the regulation of transit is slowed in wortmannin-treated cells. Although the mechanism by which wortmannin affects the recycling compartment is not yet clear, there is precedent for the regulation of transit is not yet clear, there is precedent for the regulation of transit is not yet clear, there is precedent for the regulation of transit...
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