Proc. Natl. Acad. Sci. USA
Vol. 92, pp. 10207–10211, October 1995
Cell Biology

Evidence for phosphatidylinositol 3-kinase as a regulator of endocytosis via activation of Rab5

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Communicated by David D. Sabatini, New York University Medical Center, New York, NY, July 28, 1995 (received for review March 31, 1995)

ABSTRACT **Phosphatidylinositol (PI) 3-kinases have been implicated in several aspects of intracellular membrane trafficking, although a detailed mechanism is yet to be established. In this study we demonstrated that wortmannin, a specific inhibitor of PI 3-kinases, inhibited constitutive endocytosis of horseradish peroxidase and transferrin in BHK-21 and TRVb-1 cells. The IC_{50} was ≈40 ng/ml (93 nM). In addition, wortmannin blocked the stimulation of horseradish peroxidase uptake by the small GTPase Rab5 but not the stimulation by the GTPase-defective, constitutively activated Rab5 Gln^{79} → Leu mutant (Rab5:Q79L), providing further evidence that PI 3-kinase activity is essential for the early endocytic process. To further investigate the mechanism, we examined the effect of wortmannin on early endosome fusion *in vitro*. Wortmannin decreased endosome fusion by 80% with an IC_{50} value similar to that in intact cells. Addition of Rab5:Q79L but not wild-type Rab5 reversed the inhibitory effect of wortmannin. Furthermore, addition of a constitutively activated PI 3-kinase but not its inactive counterpart stimulated early endosome fusion *in vitro*. These results strongly indicate that PI 3-kinase plays an important role in regulation of early endosome fusion, probably via activation of Rab5.**

Endocytosis occurs in most eukaryotic cells and is essential for uptake of nutrients, downregulation of cell surface receptors, and maintenance of cell homeostasis (1). Generally there are two types of endocytosis: receptor-mediated endocytosis and fluid-phase endocytosis. Receptor-mediated endocytosis can be either a constitutive process (e.g., the process mediated by transferrin receptor) or a ligand-induced event (e.g., the downregulation of activated platelet-derived growth factor receptor). The internalization of cell surface receptors occurs at specific areas (coated pits) that are coated with clathrin molecules. Fluid-phase endocytosis is a constitutive cellular process that internalizes liquids and solutes from extracellular environments. After internalization, fusion between endosomal vesicles occurs with delivery of endocytosed contents into a population of early endosomes where they are further sorted to other intracellular destinations. Receptor-mediated endocytosis and fluid-phase endocytosis possibly share a common machinery at the early endosome fusion step. For example, the well-characterized small GTPase Rab5 regulates membrane fusion between early endosomes that can be derived from either receptor-mediated endocytosis or fluid-phase endocytosis (2–5).

In the past several years, there has been an explosive discovery of molecules that control distinct membrane trafficking steps. Among the newest additions are phosphatidylinositol (PI) 3-kinases and their lipid products: phosphatidylinositol 3-phosphate (PI-3-*P*), phosphatidylinositol 3,4-bisphosphate, and phosphatidylinositol 3,4,5-trisphosphate. The first suggestion of PI 3-kinase involvement came from the study of VPS34p, a yeast protein essential for protein targeting to the yeast vacuole and vacuole morphogenesis (6). VPS34p shares sequence homology with the catalytic subunit (p110) of a mammalian PI 3-kinase and indeed exhibits PI 3-kinase activity (7). PI 3-kinase is also involved in IgE-mediated histamine secretion (8) and insulin-regulated glucose transport (9–11). PI 3-kinase is necessary for the downregulation (endocytosis) of the activated platelet-derived growth factor receptor (12). Although a detailed mechanism has not been established, these lines of evidence strongly suggest the involvement of PI 3-kinase in several aspects of intracellular membrane trafficking.

In this study, we address the importance of PI 3-kinase activity in constitutive endocytosis and endosome fusion. A very useful tool in the study of PI 3-kinases is the fungal metabolite wortmannin, which covalently binds to the catalytic subunit (p110) of mammalian PI 3-kinase and irreversibly inhibits the enzymatic activity at nanomolar concentrations (8, 13, 14). This inhibition is specific, since at these low concentrations wortmannin has no effect on any other kinase. We therefore examined the effect of wortmannin on fluid-phase endocytosis [horseradish peroxidase (HRP) uptake] and transferrin receptor-mediated endocytosis and demonstrated a wortmannin-sensitive step in these membrane trafficking events. Furthermore, we have found that wortmannin is a strong inhibitor of early endosome fusion *in vitro* and that this inhibition can be reversed by adding the constitutively activated Rab5 Gln^{79} → Leu mutant (Rab5:Q79L), indicating that the wortmannin-sensitive step is upstream of Rab5 function, in preparation for endosomal vesicle docking/fusion. Finally, we provide direct evidence indicating that a constitutively activated PI 3-kinase stimulates early endosome fusion *in vitro*.

MATERIALS AND METHODS

Cells and Wortmannin Treatment. BHK-21 cells (a baby hamster kidney cell line, purchased from the American Type Culture Collection) and TRVb-1 cells (a Chinese hamster ovary cell line, kindly provided by T. McGraw, Dept. of Pathology, Columbia University, New York) were grown to confluence as monolayers and maintained in 35-mm dishes. The growth media for the two cell lines were Eagle's minimal essential medium supplemented with Earle's salts (α-MEM) containing 10% fetal bovine serum and Ham's F12 medium containing 5% fetal bovine serum and 200 μg of G418 per ml, respectively. Wortmannin was purchased from Sigma and dissolved in dimethyl sulfoxide (DMSO) at 1 mg/ml (stock solution). In each experiment, wortmannin was freshly diluted

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Abbreviations: HRP, horseradish peroxidase; PI, phosphatidylinositol; PI-3-*P*, phosphatidylinositol 3-phosphate; α-MEM, Eagle's minimal essential medium containing Earle's salts; DMSO, dimethyl sulfoxide; BSA, bovine serum albumin.

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to desired concentrations with α-MEM and added to cell monolayers (2 ml per dish) that were previously rinsed with α-MEM. The wortmannin treatment was for 15 min in a 37°C incubator and was followed by endocytosis assays (see below). The DMSO concentration was <0.1% after dilution and had no effect on the endocytic activity of the cells (data not shown).

HRP Endocytosis. BHK-21 and TRVb-1 cell monolayers in 35-mm dishes were washed three times with serum-free α-MEM, and HRP uptake was initiated by addition of 1 ml of α-MEM containing 0.5–1.0% (wt/vol) HRP (Sigma) and 0.2% (wt/vol) bovine serum albumin (BSA). The uptake was conducted at 37°C for the indicated times. After uptake, the cells were washed three times with phosphate-buffered saline (PBS) containing 0.2% BSA and then scraped into 1 ml of PBS with a cell scraper (Fisher). The dishes were rinsed once with 1 ml of PBS and the cell suspensions were pooled. Cells were centrifuged at 800 × *g* for 3 min in a Beckman GPR centrifuge. Cell pellets were washed once by resuspension in 2 ml of PBS and recentrifugation. Each cell pellet was lysed in 500 μl of PBS containing 0.1% (vol/vol) Triton X-100. Cell lysates were assayed for HRP activity as described (4).

Transferrin Receptor-Mediated Endocytosis. Confluent TRVb-1 cell monolayers were incubated with serum-free Ham's F12 medium containing 0.2% BSA for 1 hr at 37°C to deplete endogenous transferrin. Iron-loaded human transferrin (Sigma) was labeled with ^{125}I as described (15). ^{125}I-labeled transferrin was bound to cell surface receptors by incubation at 4°C for 90 min in Ham's F12 medium containing 0.2% BSA and a saturating concentration of ^{125}I-transferrin (6 mg/ml), followed by four washes with PBS to remove unbound ligand. Uptake was initiated by adding prewarmed (37°C) Ham's F12 medium containing 0.2% BSA and a 100-fold excess of unlabeled transferrin and the cells were incubated at 37°C for various times as indicated. At each time point, the medium was collected and the cells were washed once with PBS containing 0.2% BSA. The medium and the wash were pooled and the radioactivity was measured in a Packard γ-ray detector, indicating the amount of recycled ^{125}I-transferrin. Cell surface-bound ^{125}I-transferrin was determined by acid stripping. Cell monolayers were washed twice with ice-cold 0.5% acetic acid/0.5 M NaCl, pH 3.0, and then once with PBS. The washes were combined and the radioactivity was determined, indicating the amount of ^{125}I-transferrin that remained on the cell surface. Finally, the cells were solubilized with PBS containing 1% (vol/vol) Triton X-100 and 0.1% (wt/vol) NaOH and the radioactivity in the lysates was determined, indicating the amount of cell-associated ^{125}I-transferrin.

***In Vitro* Endosome Fusion Assay.** Early endosomes were loaded with either dinitrophenylated β-glucuronidase or mannosylated anti-dinitrophenyl IgG by a 5-min uptake at 37°C in the macrophage cell line J774E (16). Cells were washed and homogenized in 2 ml of homogenization buffer (250 mM sucrose/0.5 mM EGTA/20 mM Hepes·KOH, pH 7.0). The homogenates were centrifuged at 800 × *g* for 5 min to eliminate nuclei and cell debris. The supernatants were centrifuged at 37,000 × *g* for 1 min, followed by another centrifugation at 50,000 *g* for 5 min in a Beckman L-100 ultracentrifuge. The pellets, enriched with early endosomes, were suspended in fusion buffer (250 mM sucrose/0.5 mM EGTA/1 mM dithiothreitol/1.5 mM $MgCl_2$/0.025% dinitrophenyl-BSA/50 mM KCl/20 mM Hepes·KOH, pH 7.0) containing Sephadex G-25-filtered cytosol (1 mg/ml) and an ATP-regeneration system (1 mM ATP/8 mM phosphocreatine with creatine kinase at 31 units/ml). Fusion reactions were conducted at 37°C for 1 hr and quantified by the β-glucuronidase activity in the immunocomplex (16).

RESULTS

Wortmannin Inhibits Fluid-Phase Endocytosis. HRP is one of the best-characterized markers for fluid-phase endocytosis. We first examined HRP uptake in BHK-21 and TRVb-1 cells that were pretreated with various concentrations of wortmannin. Wortmannin inhibited HRP uptake in both cell types in a concentration-dependent manner (Fig. 1 *A* and *B*). At 100 ng/ml, wortmannin blocked HRP uptake by 50%, and higher concentrations of wortmannin (up to 1 mg/ml) showed no further inhibition. The IC_{50} was ≈40 ng/ml (93 nM) in BHK-21 cells. We also tested the effect of wortmannin on macrophages that are well known for their high endocytic activity. Pretreatment of J774E macrophages with wortmannin at 100 ng/ml resulted in 70–80% reduction in HRP uptake (data not shown). To test whether wortmannin affects an early step in endocytosis, we examined the kinetics of HRP uptake, paying particular attention to the early time points. BHK-21 cell monolayers were either treated or not treated with wortmannin at 100 ng/ml and HRP uptake was monitored at various times up to 60 min. Wortmannin inhibited HRP uptake at

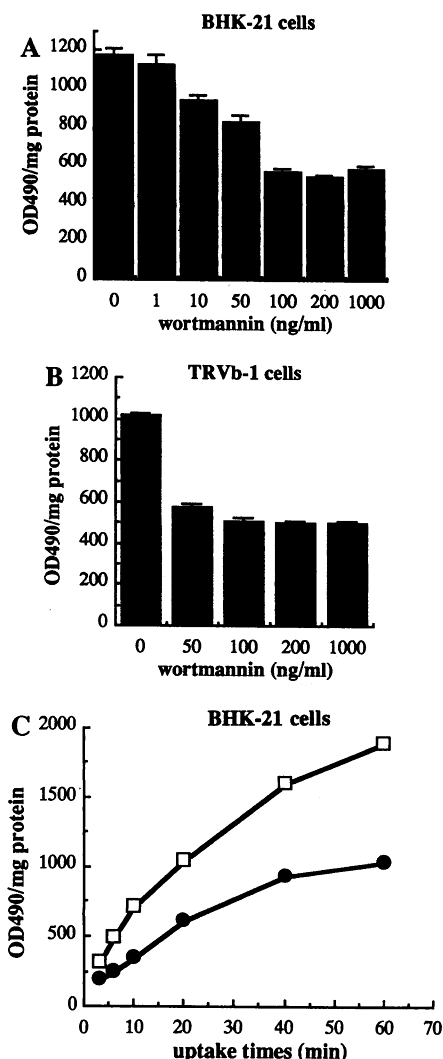

FIG. 1. (*A* and *B*) Inhibition of HRP uptake by wortmannin. Confluent BHK-21 (*A*) and TRVb-1 (*B*) cell monolayers in 35-mm dishes (≈5 × 10^5 cells per dish) were treated for 15 min with the indicated concentrations of wortmannin, and uptake of HRP (5 mg/ml) during 1 hr at 37°C and the cell-associated HRP activities were determined. The bar graphs show the means of triplicate samples and the calculated standard deviations. (*C*) HRP uptake kinetics. Confluent BHK-21 cell monolayers in 35-mm dishes were either treated (●) or not treated (□) with Wortmannin at 100 ng/ml, and HRP (10 mg/ml) uptake at 37°C was determined at the indicated times. The data are presented as the means of triplicate samples. Two experiments gave similar results.

every time point (Fig. 1*C*). Even for a 3-min uptake, the inhibition was evident in wortmannin-treated cells.

Wortmannin Decreases Transferrin Receptor-Mediated Endocytosis. We studied the kinetics of a single cycle of transferrin endocytosis in TRVb-1 cells, a CHO cell line that expresses functional human transferrin receptor. Cells were depleted of endogenous transferrin, ^{125}I-labeled human transferrin was bound to the surface of the cell monolayers at 4°C, and endocytosis at 37°C was determined at various times (Fig. 2). At each time point, the radioactivities in the medium, associated with the cell surface, and within the cells were measured, representing relative quantities of the recycled, cell surface bound, and intracellular transferrins, respectively. Internalization of ^{125}I-transferrin was slowed by wortmannin (Fig. 2*A*), and intracellular accumulation of ^{125}I-transferrin was reduced (Fig. 2*B*). The release of ^{125}I-transferrin into the extracellular medium was slowed by wortmannin, indicating that the drug delayed recycling (Fig. 2*C*). Wortmannin also blocked the constitutive endocytosis mediated by the mannose receptor in J774E macrophages (data not shown).

Effect of Wortmannin on Rab5- and Rab5:Q79L-Stimulated HRP Uptake. Rab5 is a well-characterized Ras-like small GTPase whose activated, GTP-bound form promotes early endosome fusion (3, 18, 19) which in turn stimulates HRP uptake and transferrin endocytosis (2, 4). To address whether this stimulation requires PI 3-kinase activity, we examined the effect of wortmannin on HRP uptake in BHK-21 cells overexpressing wild-type Rab5 or the GTPase-defective, constitutively activated mutant Rab5:Q79L (4, 20). Confluent BHK-21 cell monolayers were infected with recombinant Sindbis viruses expressing Rab5 or Rab:Q79L or with the vector Sindbis virus as a control. At 4 hr postinfection, the cells were treated with wortmannin at 100 ng/ml and then HRP uptake during 1 hr was assayed. In control cells that were not treated with wortmannin, overexpression of Rab5 and Rab5:Q79L showed a 2-fold stimulatory effect on HRP uptake (Fig. 3). In cells treated with wortmannin, however, the Rab5-mediated stimulation of HRP uptake was completely blocked (Fig. 3). Interestingly, the Rab5:Q79L-mediated stimulation was not affected by wortmannin treatment. Because wild-type Rab5 requires activation steps (e.g., guanine nucleotide exchange) to become GTP-bound and biologically active whereas Rab5:Q79L is constitutively activated and will bypass the activation steps, our data suggest that PI 3-kinase activity is required for activation of Rab5. These results also strengthen the notion that the inhibitory effects of wortmannin on cellular functions are due to its specific inhibition of PI 3-kinases rather than general cytotoxicity (8, 14).

Wortmannin Inhibits Early Endosome Fusion *in Vitro*. Because the inhibition pattern of wortmannin on HRP uptake and transferrin endocytosis is similar to that of the dominant negative Rab5 mutants (2, 4) and because Rab5 is a known regulator of early endosome fusion (3, 18), our data suggest the involvement of PI 3-kinase in early endosome fusion. To confirm this contention, we tested directly whether wortmannin would affect early endosome fusion *in vitro*. Early endosome and cytosol preparations were preincubated with various concentrations of wortmannin for 10 min at room temperature before the initiation of the fusion reaction at 37°C. Wortmannin inhibited early endosome fusion in a concentration-dependent manner with an IC_{50} of 30–40 ng/ml (Fig 4*A*), similar to the inhibitory effect of wortmannin on HRP uptake in intact cells (Fig. 1). Wortmannin at 100 ng/ml inhibited the fusion reaction by ≈80% (Fig. 4*A*). Addition of Rab5:Q79L, but not wild-type Rab5, reversed the inhibitory effect of wortmannin and stimulated endosome fusion (Fig. 4*A*). This result made it unlikely that the inhibition by wortmannin was due to nonspecific damaging of endosomal membranes. Boiled

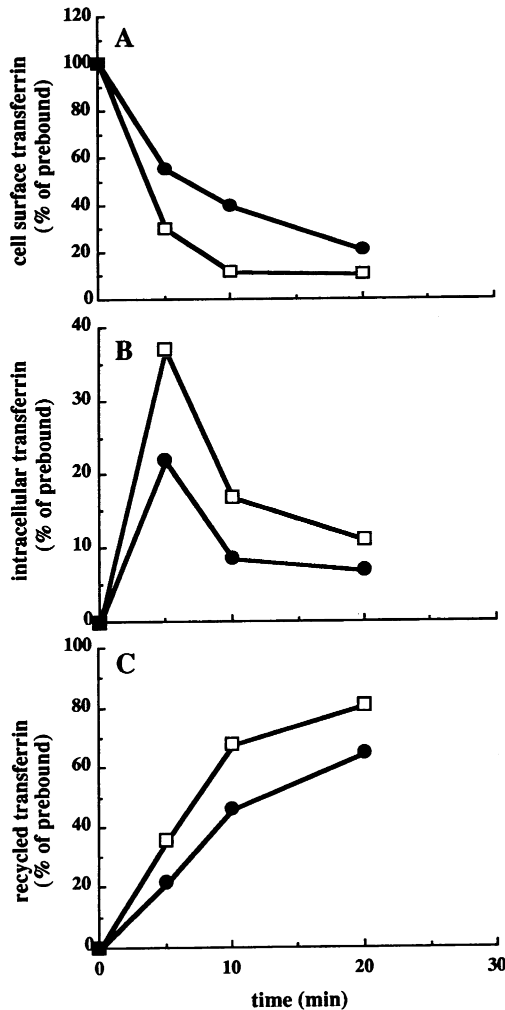

FIG. 2. Inhibition of transferrin endocytosis by wortmannin. Confluent TRVb-1 cell monolayers were either treated (●) or not treated (□) with wortmannin at 100 ng/ml for 15 min and then incubated with ^{125}I-transferrin for a single cycle of transferrin endocytosis (17). Shown are the data from a typical experiment indicating the amounts of ^{125}I-transferrin on the cell surface (*A*), within the cell (*B*), and released into the medium (*C*) after various times of internalization. The experiment was repeated three times and the results were reproducible.

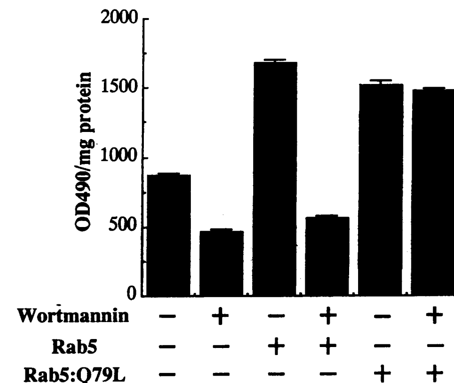

FIG. 3. Effect of wortmannin on Rab5- and Rab5:Q79L-stimulated HRP uptake. Confluent BHK-21 cell monolayers in 35-mm dishes were infected with either the vector virus as a control or the recombinant Sindbis viruses expressing Rab5 or Rab5:Q79L. At 4 hr postinfection, cells were either treated or not treated with wortmannin at 100 ng/ml for 15 min and 1-hr HRP uptake was determined. The bar graph shows the amount of cell-associated HRP activity and the calculated standard deviations.

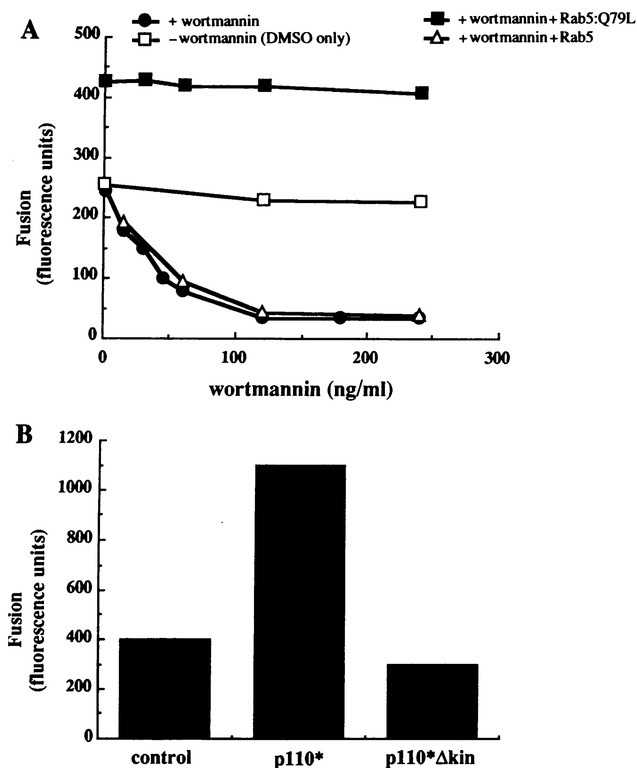

FIG. 4. Involvement of PI 3-kinase in early endosome fusion. The *in vitro* endosome fusion assay was conducted as described (16). (*A*) Effect of wortmannin on *in vitro* endosome fusion. Before the initiation of the fusion reactions, the endosome and cytosol preparations were combined and preincubated for 10 min at room temperature with various concentrations of wortmannin before the fusion reaction was conducted in the absence (●) or presence of Rab5 (△) or Rab5:Q79L (■) at 18 μg/ml. [Rab5 and Rab5:Q79L were produced as glutathione *S*-transferase fusion proteins in *Escherichia coli*, purified, and prenylated *in vitro* (5).] Because wortmannin was dissolved in DMSO, the preincubation step was also conducted with corresponding concentrations of DMSO as controls (□). Shown are the means of duplicate samples from an *in vitro* endosome fusion experiment. The experiment was repeated three times and the results were reproducible. (*B*) *In vitro* endosome fusion experiment supplemented with Sf9 cytosol (0.8 mg/ml) containing the constitutively activated PI 3-kinase (p110*) or its inactive counterpart (p110*Δkin) or neither of the components (control). The results were reproducible in independent experiments.

Rab5:Q79L as a control had no effect (data not shown). In other control experiments, early endosome and cytosol preparations were preincubated with DMSO (the solvent for wortmannin), and within the concentration range (up to 0.1%) DMSO showed no effect on endosome fusion (Fig. 4*A*). Although wortmannin also inhibits the myosin light-chain kinase, this inhibition is 100-fold less effective and requires millimolar concentrations of the drug (8). Furthermore, the myosin light chain kinase inhibitor ML-7 (Calbiochem) did not have any effect in the endosome fusion assay described above (data not shown). Taken together, the data suggest that wortmannin inhibits early endosome fusion by blocking PI 3-kinase activity.

PI 3-Kinase Stimulates Early Endosome Fusion *in Vitro*. We then tested the effect of a constitutively activated PI 3-kinase (p110*) (21) and its inactive counterpart (p110*Δkin) (21) on *in vitro* endosome fusion. The p110* and p110*Δkin proteins were produced in Sf9 cells using a baculovirus vector. Cells were lysed by hypotonic shock and postnuclear supernatants were centrifuged at 100,000 × *g* and separated into pellet and supernatant fractions. The supernatant fractions, containing p110* or p110*Δkin, were equilibrated with the endosome fusion buffer by dialysis and used as the cytosol sources for the *in vitro* endosome fusion assay. In comparison to the control cytosol containing neither p110* nor p110*Δkin, the cytosol containing the constitutively activated p110* showed almost 3-fold stimulation of endosome fusion, whereas the cytosol containing the inactive form p110*Δkin had no such effect (Fig. 4*B*).

DISCUSSION

PI 3-kinase activity has been implicated in vesicular trafficking in several studies. However, the strongest evidence to date is in yeast, where deletion of the VPS34 gene (encoding the yeast PI 3-kinase) results in a defect in targeting of soluble proteins to the yeast vacuole (7), a process equivalent to vesicular transport from the trans-Golgi network to lysosomes in mammalian cells. PI-3-*P* is the only PI 3-kinase product in yeast and its level is dependent on the presence of functional VPS34 product (22). It is therefore suggested that PI-3-*P* is an essential membrane component in this constitutive membrane trafficking process, probably involved in membrane budding, vesicle fusion, or both. In this context, it is interesting that the yeast homolog of Rab5 (VPS21) is also involved in the transport process from Golgi network to yeast vacuole (23).

The inhibition of constitutive endocytosis by wortmannin in our study indicates a requirement for PI 3-kinase activity in this process. Fluid-phase endocytosis and receptor-mediated endocytosis were inhibited but not abolished by wortmannin, and *in vitro* endosome fusion was strongly inhibited by the drug. The lack of complete inhibition of endocytosis by wortmannin might be due to the presence of one or more wortmannin-resistant PI 3-kinase activities (24) which are involved in the early endocytic pathway. In fact, it has been shown that in intact cells wortmannin is unable to abolish all PI 3-kinase activity (8, 24). Furthermore, by inhibiting Rab5 function and endosome fusion, wortmannin may block endocytosis by slowing internalization rather than by abolishing it. This explanation is supported by the observation that dominant negative mutants of Rab5 (Rab5:S34N and Rab5:N133I) block internalization of the transferrin receptor and the fluid-phase marker HRP in a manner very similar to wortmannin. For example, the Rab5 mutants block endosome fusion in cultured cells, leading to slower internalization of transferrin and HRP (2, 4, 19). An explanation advanced by several groups is that factors required for internalization remain membrane-bound until they are released following vesicle docking and fusion. Since dominant negative mutants of Rab5 impair endosome fusion, they prevent recycling of needed factors and thus slow or block internalization. Impairment of endosome fusion by wortmannin may produce an effect similar to the dominant negative mutants of Rab5 by preventing or slowing release of factors necessary for internalization. Our data show that *in vitro* endosome fusion is strongly inhibited by wortmannin. This inhibition can be reversed by the constitutively activated GTP-bound Rab5 mutant Rab5:Q79L, suggesting that PI 3-kinase activity is involved in a step upstream of Rab5 function. The importance of PI 3-kinase in early endosome fusion is further supported by the gain-of-function evidence (Fig. 4*B*) showing that constitutively activated PI 3-kinase, but not the inactive form of the enzyme, can stimulate early endosome fusion *in vitro*. It now appears that multiple forms of wortmannin-sensitive PI 3-kinase exist (24–27) and it cannot be concluded at this point whether these particular variants of the enzyme are involved in endosome fusion *in vivo*. However, the simplest explanation is that one or more PI 3-kinase activities, perhaps via the lipid products in the membranes, activate Rab5 in preparation for early endosome docking and fusion. We and others have previously shown that the GTP-bound form of Rab5 is the active form in promoting endosome fusion (4, 18, 19). It is possible that the lipid products of PI 3-kinase favor the guanine nucleotide exchange

reaction from Rab5-GDP to Rab5-GTP, leading to endosome fusion. Further work will be required to identify specific PI 3-kinases regulating the endocytic pathway and to elucidate any direct effects of these activities on internalization and regurgitation of membrane proteins.

We thank Rita Boshans, Cheryl Adles, and Marilyn Levy for excellent technical assistance. This work was supported in part by a grant from the National Institutes of Health (to P.D.S.). G.L. is supported by a fellowship from the Parker B. Francis Foundation. C.D.S. is a Lucille P. Markey fellow.

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