Role for Phosphatidylinositol 3-Kinase in the Sorting and Transport of Newly Synthesized Lysosomal Enzymes in Mammalian Cells

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Abstract. Previous work with the yeast *Saccharomyces cerevisiae* has demonstrated a role for a phosphatidylinositol-specific PI 3-kinase, the product of the *VPS34* gene, in the targeting of newly synthesized proteins to the vacuole, an organelle functionally equivalent to mammalian lysosomes (Schu, P. V., K. Takegawa, M. J. Fry, J. H. Stack, M. D. Waterfield, and S. D. Emr. 1993. *Science [Wash. DC].* 260:88–91). The activity of Vps34p kinase is significantly reduced by the PI 3-kinase inhibitors wortmannin, a fungal metabolite, and LY294002, a quercetin analog (Stack, J. H., and S. D. Emr. 1994. *J. Biol. Chem.* 269:31552–31562). We show here that at concentrations which inhibit *VPS34*-encoded PI 3-kinase activity, wortmannin also inhibits the processing and delivery of newly synthesized cathepsin D to lysosomes in mammalian cells with half-maximal inhibition of delivery occurring at 100 nM wortmannin. As a result of wortmannin action, newly synthesized, unprocessed cathepsin D is secreted into the media. Moreover, after accumulation in the *trans*-Golgi network (TGN) at 20°C, cathepsin D was rapidly missorted to the secretory pathway after addition of wortmannin and shifting to 37°C. At concentrations that inhibited lysosomal enzyme delivery, both wortmannin and LY294002 caused a highly specific dilation of mannose 6-phosphate receptor (M6PR)–enriched vesicles of the prelysosome compartment (PLC), which swelled to ~1 μm within 15 min after treatment. With increasing time, the inhibitors caused a significant yet reversible change in M6PR distribution. By 3 h of treatment, the swollen PLC vacuoles were essentially depleted of receptors and, in addition, there was a fourfold loss of receptors from the cell surface. However, M6PRs were still abundant in the TGN. These results are most consistent with the interpretation that PI 3-kinase regulates the trafficking of lysosomal enzymes by interfering with a M6PR-dependent sorting event in the TGN. Moreover, they provide evidence that trafficking of soluble hydrolases to mammalian lysosomes and yeast vacuoles rely on similar regulatory mechanisms.

THE targeting of newly synthesized proteins to lysosomes in mammalian cells and to the functionally equivalent organelle in the yeast, the vacuole, has been studied for a number of years. Lysosome biogenesis in mammalian cells has been analyzed primarily by biochemistry and immunocytochemistry (for reviews see Kornfeld and Mellman, 1989; Kornfeld, 1992), whereas the yeast *Saccharomyces cerevisiae* has been examined through a genetic approach to generate mutants, called *vps* for "vacuolar protein sorting", that are defective in various aspects of vacuole biogenesis (for reviews see Klinosky et al., 1990; Raymond et al., 1992). These different approaches have identified a variety of molecules that mediate the specific targeting and transport of newly synthesized proteins to mammalian lysosomes and yeast vacuoles. Although the vacuole is considered to be equivalent to lysosomes, to date, relatively few regulatory molecules have been identified that are common to both pathways.

In mammalian cells, newly synthesized lysosomal enzymes are specifically diverted from the secretory pathway by the mannose 6-phosphate (M6P)[1] recognition system (for reviews see Kornfeld and Mellman, 1989; Kornfeld, 1992). Soluble lysosomal enzymes, bearing M6P residues on their N-asparagine–linked oligosaccharides, bind to one of two types of M6P receptors (M6PRs) in the *trans*-Golgi network (TGN) where receptor-enzyme complexes are selectively packaged into clathrin-coated vesicles for transport to the prelysosomal compartment (PLC), which also

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1. *Abbreviations used in this paper*: BFA, brefeldin A; CSF, colony stimulating factor; CSS, calf supreme supplemented; endo H, endoglycosidase H; M6P, mannose-6-phosphate; M6PR, mannose-6-phosphate receptors; MLCK, myosin light chain kinase; PDGF, platelet derived growth factor; PI, phosphatidylinositol; PLC, prelysosomal compartment; VSV-G, vesicular stomatitis virus glycoprotein.

functions as a late endosome compartment (Brown et al., 1986; Griffiths et al., 1988; Geuze et al., 1988). In the acidic environment of the PLC, enzymes dissociate from their receptors leaving the receptors free to cycle back to the TGN for multiple rounds of transport (Brown et al., 1986; Geuze et al., 1988; Griffiths et al., 1988; Goda and Pfeffer, 1988). The soluble enzymes are ultimately delivered to lysosomes via either maturation of the PLC or vesicular transport to, or fusion with, preexisting lysosomes (Mullock et al., 1994; Roederer et al., 1990). Failure of newly synthesized lysosomal enzymes to engage the M6PR delivery system results in their secretion (Kornfeld and Mellman, 1989). In addition to their intracellular role, M6PRs are also found on the cell surface, representing ~10% of the total cellular pool, where they participate in receptor-mediated endocytosis. Receptors move freely and rapidly between intracellular and cell surface pools (Braulke et al., 1987; Duncan and Kornfeld, 1988; Jin et al., 1989).

Although a carbohydrate-mediated vacuolar delivery system has yet to be identified in yeast (Klinosky et al., 1990), there are several similarities between delivery of soluble proteins to yeast vacuoles and mammalian lysosomes. For example, at least one soluble vacuolar enzyme, carboxypeptidase Y, appears to be sorted from a late Golgi compartment and specifically delivered to a prevacuole/late endosome-like compartment by a membrane-associated receptor, the product of the VPS10 gene (Graham and Emr, 1991; Vida et al., 1993; Marcusson et al., 1994). Also, delivery to the yeast vacuole requires clathrin, presumably to form coated vesicles (Seeger and Payne, 1992). Therefore, yeast contains a sorting system that is functionally analogous to the M6P system, thus raising the possibility that receptor-mediated delivery of soluble proteins to lysosomes and vacuoles could be subject to similar mechanisms of regulation.

A second VPS gene, VPS34, was found to encode a protein with significant sequence similarity to the p110 catalytic subunit of mammalian phosphatidylinositol (PI) 3-kinase (Herman and Emr, 1990; Hiles et al., 1992). Indeed, yeast cells harboring various point mutations in the lipid kinase domain of Vps34p missort soluble vacuolar proteins into the secretory pathway, and express greatly reduced levels of PI 3-kinase activity (Schu et al., 1993). In addition, Vps34p exists in vivo as a membrane-associated complex with Vps15p (Stack et al., 1993), a soluble enzyme with significant sequence homology to serine/threonine protein kinases (Herman et al., 1991). Interestingly, Vps34p has recently been shown to also exhibit serine/threonine protein kinase activity placing it in a novel class of enzymes capable of phosphorylating both lipid and protein substrates (Stack and Emr, 1994). The only other known example of such an enzyme is the p110 catalytic subunit of mammalian PI 3-kinase (Carpenter et al., 1993; Dhand et al., 1994).

The family of mammalian PI 3-kinases actually represent mulitple biochemically distinct enzymes. The most extensively characterized member is the p110 catalytic subunit. The p110 subunit is complexed with an 85-kD regulatory subunit that has SH2 domains which direct the binding of the heterodimer to tyrosine-phosphorylated proteins (Carpenter et al., 1990; Escobedo et al., 1991; Morgan et al., 1990). Other members include a PI 3-kinase from platelets and myeloid-derived cells that is activated by the $\beta\gamma$ subunits of trimeric G protein (Stephens et al., 1994*a*; Thomason et al., 1994), and a PtdIns-specific 3-kinase from bovine adrenoreticulosa cells (Stephens et al., 1994*b*). PI 3-kinases have been associated with a variety of cellular functions including growth factor-stimulated mitogenesis (Cantley et al., 1991; Whitman and Cantley, 1988), membrane ruffling (Kotani et al., 1994; Wennstrom et al., 1994), generation of the oxygen burst in neutrophils (Arcaro and Wymann, 1993; Okada et al., 1994*a*; Thelen et al., 1994), histamine secretion (Yano et al., 1993), and translocation of glucose transporters to the cell surface (Okada et al., 1994*b*; Hara et al., 1994; Cheatham et al., 1994). Of direct relevance to yeast Vps34p are recent studies showing that the p110/p85 is involved in the regulation of endocytic membrane trafficking events. For example, platelet-derived growth factor (PDGF) receptors are internalized as a complex with p110/p85, and mutant receptors that fail to associate with SH2 domains on the p85 targeting subunit remain at or near the cell surface (Joly et al., 1994). Moreover, work on the receptor for colony stimulating factor (CSF) suggests that its transport from endosomes to lysosomes could also be influenced by interactions with the p110/p85 complex (Carlberg et al., 1991; Shurtleff et al., 1990). Therefore, both Vps34p in yeast and at least one of the PI 3-kinases in mammalian cells appear to function in the regulation of membrane trafficking.

Given these functional similarities, we investigated whether a PI 3-kinase plays an important role in the targeting and transport of lysosomal enzymes in mammalian cells. This line of inquiry is complicated by the existence of multiple PI 3-kinases. However, all are known to be potently inhibited by the membrane permeant reagents wortmannin (Arcaro and Wymann, 1993), a fungal metabolite, and LY294002, an analog of the bioflavinoid quercetin (Vlahos et al., 1994). For example, the well-characterized p110 PI 3-kinase is exquisitely sensitive to wortmannin in the low nanomolar range (Arcaro and Wymann, 1993; Okada et al., 1994*a*; Yano et al., 1993), whereas the yeast PI 3-kinase (Stack and Emr, 1994), and at least one other mammalian PI 3-kinase is sensitive in the high nanomolar to low micromolar range (Stephens et al., 1994*b*). We report here that these PI 3-kinase inhibitors, at concentrations which inhibit the yeast Vps34p PI 3-kinase, rapidly cause the missorting of newly synthesized acid hydrolases from the lysosomal to the secretory pathway and effect the distribution of the M6PR.

Materials and Methods

Materials and Antibodies

Wortmannin, MEM, and DMEM were obtained from Sigma Chem. Co. (St. Louis, MO) and LY294002 was generously provided by Chris Vlahos, Lilly Research Laboratories (Indianapolis, IN). Both were stored as 10-mM stock solutions in DMSO at -20°C. Calf Supreme Supplemented was obtained from GIBCO BRL (Gaithersburg, MD). Tran ^{35}S-label was obtained from ICN (Costa Mesa, CA). Fluorescein-conjugated donkey anti-mouse and rhodamine-conjugated donkey anti-rabbit antibodies were obtained from Jackson Immunoresearch Labs (West Grove, PA). FAb fragments of goat anti-rabbit IgG conjugated with horseradish peroxidase was purchased from Biosys (Compiégne, France). Goat anti-rabbit IgG conjugated with alkaline phosphatase was from Chemicon International, Inc. (Temecula, CA). Rabbit polyclonal antibodies against TGN38 were

kindly provided by K. Howell (University of Colorado School of Medicine). Rabbit polyclonal antibodies against the cation-independent M6PR (Brown and Farquhar, 1987), cathepsin D (Park et al., 1991*a*), and monoclonal antibody 10E6, recognizing a *cis*-Golgi antigen (Wood et al., 1991) were previously characterized.

Metabolic Labeling and Immunoprecipitation

Briefly, CHO or NRK cells grown to near confluency on 35-mm tissue culture dishes were incubated for 30 min in methionine-free labeling medium (Davidson and Balch, 1992) followed by metabolic labeling with 100 μCi of Tran^{35}-S label for 30 min. Cells were washed 3× with PBS and chased in methionine containing media for the indicated times in the presence or absence of wortmannin as described in the figure legends. To determine the extent of processing of intracellular forms of cathepsin D, the media was removed, cells were washed 2× with PBS, and scraped into 0.5 ml of PBS. The recovered cells were pooled with cells obtained from a second 0.5 ml wash. Cells were pelleted and lysed in the presence of 1% Triton X-100 in lysis buffer (20 mM Tris, pH 8.0, 150 mM NaCl, 1 mM EDTA, 1 mM PMSF). Cathepsin D antibody was added and incubated for 1 h at 4°C. Subsequently, the lysate was supplemented with 25 μl of a 50% slurry of protein A–Sepharose beads (Pharmacia, Inc., Piscataway, NJ). After an overnight incubation, beads were washed 2× with lysis buffer containing 0.5% Triton X-100 and released from beads by boiling for 5 min in gel sample buffer as described (Davidson and Balch, 1992). The sample was resolved using SDS-PAGE (Laemmli, 1972), and the distribution of cathepsin D between processed and unprocessed forms were quantitated using a Phosphoimager (Molecular Dynamics, Inc., Sunnyvale, CA). To determine the recovery and processing of secreted forms of cathepsin D, at each time-point the media was harvested from individual tissue culture dishes and processed as described for intracellular forms. Radiolabeling and quantitation of the processing of VSV-G glycoprotein to endo H resistant forms were determined as described (Davidson and Balch, 1993).

Analysis of Phospholipids

NRK cells were prepared for labeling with [^{32}P]phosphate as follows: 80% confluent monolayers of cells were washed twice, phosphate-starved (in either Hepes-buffered DMEM containing 44 mM $NaHCO_3$ and 0.25% BSA, or phosphate-free KHB buffer [116 mM NaCl, 4.6 mM KCl, 1.16 mM KH_2PO_4, 25 mM $NaHCO_3$, 2.5 mM $CaCl_2{\cdot}2H_2O$, 1.16 mM $MgSO_4{\cdot}7H_2O$, 8 mM glucose and 0.2% BSA]) for 45 min, and radiolabeled in phosphate-free DMEM or KHB buffer containing 250 μCi of [^{32}P]phosphate per dish for a total of 2.5 h in 5% CO_2. Wortmannin was added (final concentration of 30 nM to 3 μM) for a total time of 15–90 min before termination of labeling as indicated in the Results section. After transfer to ice, cells were washed twice with cold PBS, removed from the dishes by scraping into PBS, pelleted at 8,000 *g* for 3 min, and frozen as a pellet in liquid N_2. Phospholipid analysis was performed as described (Stack et al., 1995).

Immunocytochemistry

The effects of the PI 3-kinase inhibitors wortmannin and LY294002 were examined on a variety of cells, e.g., rat clone 9 hepatocytes, NRK, CHO, and MDBK cells, with essentially identical results being obtained regardless of cell type. For presenting the data, only the results with Clone 9 hepatocytes will be shown. These cells were routinely maintained in minimal essential media (MEM) containing 10% Calf Supreme Supplemented (CSS). For all of the immunofluorescence experiments described below, cells were grown on coverslips for 2 d to ~50% confluency, washed by quickly dunking the coverslips in a beaker containing ~50 ml of MEM (37°C), and then placed in another dish with MEM (but no CSS) containing the appropriate concentration of inhibitor. The details of each experiment will be given in the Results or Figure Legends. At the end of each treatment, cells were fixed immediately with 3.7% formalin in PBS, pH 7.4, for 10–20 min, washed 3 × 5 in PBS, and then permeabilized with 0.1% Triton X-100 in PBS for 5 min. Cells were further processed for single- or double-label immunofluorescence as described (Park et al., 1991*b*) using the following dilutions of the primary antibodies: rabbit anti-M6PR IgG (1:400), rabbit anti-TGN38 serum (1:200), rabbit anti-cathepsin D (1:400), mouse monoclonal 10E6 ascites fluid (1:400). For double-labeling experiments, rabbit primary antibodies were detected with rhodamine-conjugated donkey anti-rabbit IgG and mouse primary antibodies were detected with fluorescein-conjugated donkey anti-mouse IgG.

For immunoperoxidase labeling and visualization by electron microscopy, cells were grown for 2–3 d in 35-mm petri dishes, rinsed 2× with MEM, and then treated with 1 μM wortmannin in MEM for various periods of time at 37°C. At the end of the incubation period, cells were fixed with periodate-lysine-paraformaldehyde fixative and processed for immunoperoxidase staining as described (Brown and Farquhar, 1989). Rabbit anti-M6PR IgG was used as the primary antibody, and FAb fragments of goat anti-rabbit IgG conjugated with horseradish peroxidase were used as the second antibody.

Acridine Orange Accumulation

Cells were grown on coverslips for 2 d, rinsed in MEM, and then incubated for 3 h in wortmannin (1 μM) or NH_4Cl (25 mM) in MEM at 37°C. Acridine orange was added from a stock solution (5 mg/ml in distilled water) directly to the dishes containing the coverslips to a final concentration of 2.5 μg/ml. Cells were further incubated for 10 min at 37°C, quickly rinsed with MEM, and placed in a 60-mm petri dish containing MEM with or without wortmannin or NH_4Cl as appropriate. Living cells were immediately viewed by fluorescence microscopy as described (Allison and Young, 1969) and photographed using Ektachrome ASA 400 film.

Cell Surface Binding and Internalization of Anti-M6PR IgG

To determine the relative expression of cell surface M6PRs, a cell-associated ELISA assay was performed (Park et al., 1991*b*). Clone 9 cells were grown in 12-well culture plates for 2 d to ~75% confluency, washed 3 × 1 ml with MEM (37°C), and then incubated in the presence or absence of LY294002 (70 μM) or wortmannin (1 μM) in MEM for various periods of time at 37°C. All subsequent steps were done at room temperature. Cells were then immediately fixed in freshly prepared 3.7% formalin in PBS, pH 7.4, for 30 min at room temperature, washed 3 × 5 min with PBS, and incubated with rabbit anti-M6PR IgG (50 μg/ml) for 1 h. Cells were washed 3 × 5 min with PBS and incubated with goat anti-rabbit IgG conjugated with alkaline phosphatase (diluted 1:1,000) for 1 h. Cells were washed 3 × 5 min with PBS, bound antibody was eluted with 100 mM citrate, pH 2.7, and then immediately neutralized with sodium citrate. The solution was clarified in a microfuge for 2 min at the highest setting, and the supernatant was collected. Eluted alkaline phosphatase–conjugated antibody was assayed using 5 mM *p*-nitrophenyl phosphate in 100 mM Tris, 10 mM $MgCl_2$, pH 9.2, for 15 min. The reaction was stopped by adding an equal volume of 5 N NaOH, and the absorbance at 405 nm was measured. Cells incubated with an equal concentration of nonimmune IgG served as a control and the A_{405} of these samples was substracted from the samples incubated with immune IgG.

Results

Wortmannin Inhibits the Normal Processing and Delivery of Newly Synthesized Lysosomal Enzymes

To investigate the role of PI 3-kinases in the targeting of newly synthesized lysosomal enzymes, we examined the effect of wortmannin, a potent PI 3-kinase inhibitor, on cathepsin D transport. Cathepsin D has been shown to be initially synthesized as an ~50-kD unprocessed form which persists as the protein passes through the secretory compartments to the TGN (Hasilik and Neufeld, 1980; Park et al., 1991*a*; Rijnboutt et al., 1992). After M6P-mediated delivery to the PLC (prelysosome compartment), cathepsin D undergoes the first of several proteolytic processing steps to yield an ~44-kD processed form. After reaching lysosomes, cathepsin D undergoes a much slower second proteolytic cleavage to yield the mature form consisting of an ~30-kD polypeptide noncovalently linked to a 14-kD polypeptide (Gieselmann et al., 1983; Rijnboutt et al., 1992). As a measure of the extent of normal processing and trafficking, we determined the fraction of cathepsin D that is found in either the unprocessed form or the PLC-

associated 44-kD processed form after various treatments with wortmannin.

We first examined the normal processing and transport of cathepsin D in NRK cells. Cells were pulse labeled for 30 min and then chased for various periods of time at 37°C. Cathepsin D was efficiently processed from the 50-kD form to the 44-kD PLC-associated form by 180 min of chase (Fig. 1 *A*). However, addition of 1 μM wortmannin to cells during the pulse chase resulted in a dramatic decrease in the processing of the 50-kD form and its loss from the cells. After a 180-min chase in the presence of wortmannin, <10% of the initial 50 kD form was recovered in the cell extracts (Fig. 1 *A*).

To account for the loss of cathepsin D, cells were treated with increasing concentrations of wortmannin, pulse labeled for 30 min, chased for 4 h, and then cathepsin D was immunoprecipitated from both cell extracts and media samples. In the absence of wortmannin, ~90% of all newly synthesized cathepsin D was found to be in the cell-associated, processed form (Fig. 1 *B*). However, inclusion of increasing concentrations of wortmannin during the chase period resulted in a progressively smaller fraction of processed cathepsin D appearing in cells (Fig. 1 *B*, *open bars*) with unprocessed cathepsin D now found to be nearly quantitatively secreted (Fig. 1 *B*, *closed bars*). The effect of wortmannin was observed even as low as 30–40 nM (data not shown). However, at 100 nM wortmannin, a significant amount (40% of the total) of cathepsin D was diverted to the secretory pathway indicating that half-maximal inhibition of sorting occurred at ~100 nM wortmannin. Maximal inhibition of cathepsin D processing and diversion to the extracellular media occurred in the presence of 1–3 μM wortmannin, concentrations which were used in subsequent experiments.

To confirm whether the observed defects in cathepsin D sorting correlated with a defect in PI-3 kinase activity, we measured cellular levels of PtdIns 3-P after addition of varying concentrations of wortmannin. NRK cells were labeled to isotopic steady state with [^{32}P]phosphate and then incubated with various doses and for varying times with wortmannin. Cellular lipids were extracted, deacylated, and then fractionated using anion exchange HPLC. We found that doses of wortmannin (nM to 3 μM) that resulted in a defect in cathepsin D sorting, also resulted in a rapid decrease in cellular levels of PtdIns 3-P. Treatment with wortmannin for as little as 15 min led to a greater than threefold (70%) reduction in the level of PtdIns 3-P. This rapid loss of PtdIns 3-P presumably corresponds to the rapid inactivation of PI 3-kinase activity in these cells. These observations are very consistent with those obtained by Stephens et al. (1994*b*) on a different cell type.

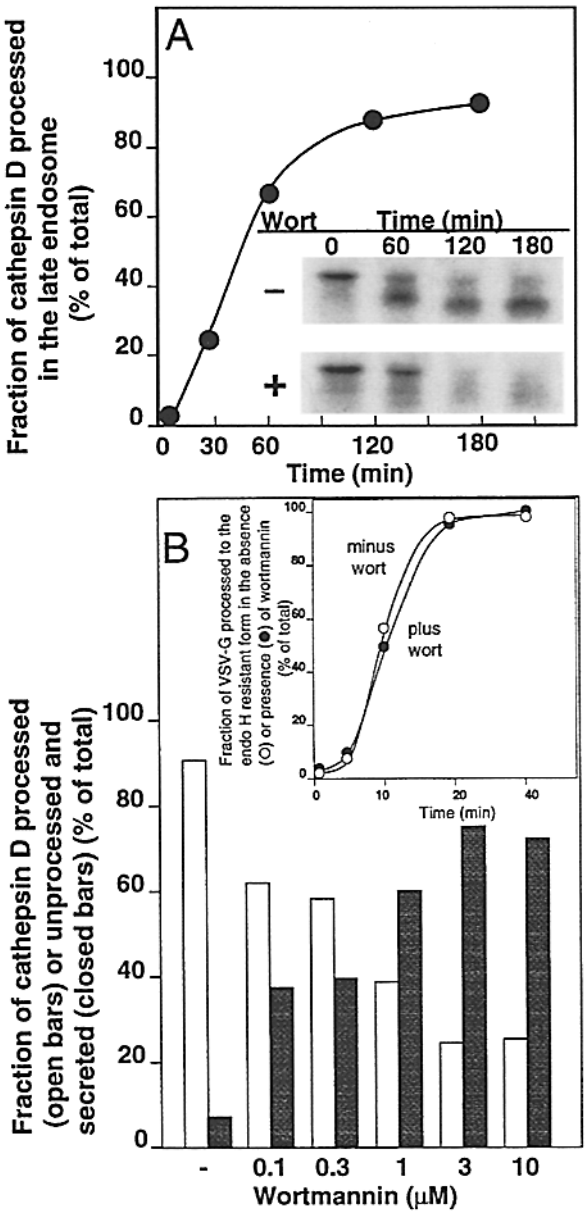

Figure 1. The PI 3-kinase inhibitor, wortmannin, inhibits the processing of newly synthesized cathepsin D. (*A*) NRK cells were pulse labeled for 30 min with [^{35}]methionine, washed, and chased for various periods of time (all at 37°C) as indicated on the graph and above the gel lanes. At these time points, cathepsin D was immunoprecipitated from cell extracts, separated by SDS-PAGE, and analyzed by Phosphoimager. (−) Processing in the absence of wortmannin shows nearly complete conversion of the higher molecular weight 50-kD form to the 44-kD, PLC-associated form by 180 min of chase. (+) In the presence of 1 μM wortmannin, very little 50-kD form is processed and most of it is lost from the cell extracts. The graph shows the amount of the 50-kD form that was processed to the 44-kD form, and therefore the amount that reached the late endosome/PLC in untreated cells. (*B*) Wortmannin diverts newly synthesized cathepsin D from the lysosomal to the secretory pathway. NRK cells were incubated with the indicated concentrations of wortmannin for 15 min, pulse labeled for 30 min with [^{35}]methionine, washed, and chased for 4 h at 37°C in the presence of cold methionine (in the continuous presence of wortmannin as appropriate). Cathepsin D was immunoprecipitated from cell lysates (*open bars*) or media samples (*closed bars*) and the amount of unprocessed (50 kD immature) and processed (44 kD PLC-associated) forms were determined using SDS-PAGE and Phosphoimager analysis as described in Materials and Methods. With increasing concentrations of wortmannin, the recovery of intracellular, processed forms of cathepsin D was markedly inhibited (*open boxes*). Loss of intracellular forms was strongly correlated with the appearance of unprocessed cathepsin D in the media (*closed bars*). (*Inset*) Wortmannin (3 μM) had no effect on the processing of VSV-G to endo H resistant forms. Virus infection, radiolabeling, and quantitation of the processing of VSV-G was determined as described (Davidson and Balch, 1993).

To determine if wortmannin was interfering with a step common to both the constitutive secretory and lysosomal pathways, we examined the effect of wortmannin on the transport of vesicular stomatitis virus glycoprotein (VSV-G), a type 1 membrane protein which is transported to the cell surface in a fashion identical to plasma membrane proteins. As shown in Fig. 1 *B* (*inset*), concentrations up to 3 μM wortmannin had no detectable effect on the processing of VSV-G to the endoglycosidase H (endo H) resistant complex form indicative of normal transport through the Golgi stack. In addition, there was no effect on the delivery of VSV-G to the cell surface as detected by indirect immunofluorescence (data not shown). The combined results suggest that wortmannin is specifically affecting a step related to the sorting of cathepsin D from the TGN or delivery to the PLC.

Wortmannin Rapidly Diverts Newly Synthesized Cathepsin D to the Constitutive Secretory Pathway

To account for the fate of the various forms of cathepsin D in the presence or absence of wortmannin, a time course of processing was more closely examined in pulse-chase experiments. In these experiments, cells were pulse labeled with [^{35}S]methionine for 30 min and then chased in the continuous presence of wortmannin for various periods of time. In the absence of wortmannin, the majority of cathepsin D remained in the intracellular pool as the 44-kD PLC form by 6 h of chase (Fig. 2). In the presence of wortmannin, ~80% of the cathepsin D was secreted into the media, as the 50-kD unprocessed form by 3 h. The cathepsin D remaining in cells at this time point was exclusively recovered in the unprocessed form suggesting a potent defect in the delivery of wortmannin to the PLC.

Lysosomal enzymes are sorted from the secretory pathway in the TGN for delivery to PLC/endosomal compartments (Kornfeld and Mellman, 1989). To more precisely determine the location of the wortmannin-induced diversion of cathepsin D from the lysosomal to the secretory pathway, cells were pulse labeled for 30 min and chased for 3 h at 20°C to accumulate newly synthesized enzyme in the TGN (Griffiths and Simons, 1986). Subsequently, cells were incubated for 15 min at 20°C in the presence of wortmannin, to equilibrate the various trafficking pathways with the drug, and then shifted to 37°C to resume transport (Fig. 3). Before shifting cells to 37°C, 80–90% of the cathepsin D was found in cells in the unprocessed form indicating that it had, for the most part, not passed beyond the TGN (Fig. 3 *A*). After shifting to 37°C in the absence of wortmannin, nearly all of the cathepsin D remained in the cells and by 3 h of chase >60% was processed to the 44-kD PLC/lysosomal form, a value equivalent to that observed in control cells in this experiment which were chased for 4 h at 37°C in the absence of wortmannin and the 20°C block (65% processed to the 44-kD form). In contrast, following shift of cells from 20°C to 37°C in the presence of wortmannin, cathepsin D appeared in the extracellular medium nearly quantitatively without the 1-h lag period observed for transport from the ER (Fig. 2), reflecting its efficient accumulation in the TGN at 20°C. Moreover, it was all secreted in the unprocessed form. None of the residual cell-associated cathepsin D was detectable in the processed forms over the background level observed be-

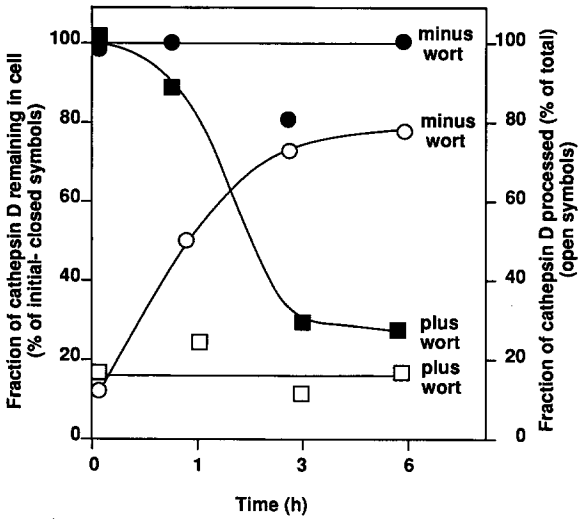

Figure 2. Time course of cathepsin D processing and fate in the presence or absence of wortmannin. Cells were pulse labeled with [^{35}]methionine for 30 min, washed, and chased in the presence of cold methionine and 3 μM wortmannin for the indicated time. Cathepsin D was immunoprecipitated from either cell extracts or media, and the amount of processed and unprocessed cathepsin D determined as described in Materials and Methods.

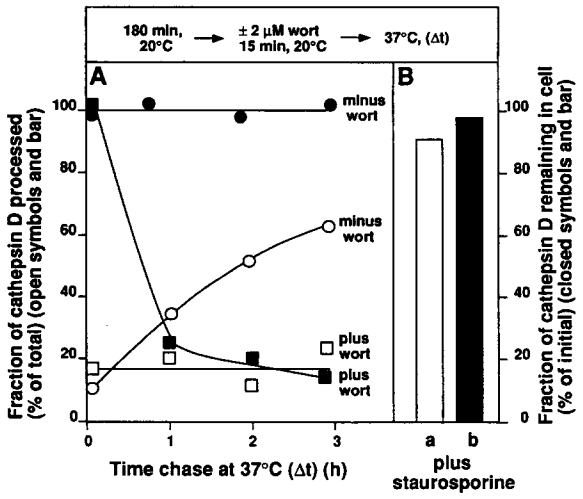

Figure 3. Targeting and processing of newly synthesized cathepsin D from the TGN to the PLC is inhibited by wortmannin. (*A*) Cells were labeled for 30 min with [^{35}]methionine and chased for 3 h at 20°C to accumulate unprocessed cathepsin D in the TGN (Griffiths and Simon, 1986). Wortmannin (3 μM final concentration) was added where indicated for 15 min at 20°C. Cells were then shifted to 37°C for the indicated time. Cathepsin D was immunoprecipitated from cell extracts or media, and the amount of processed and unprocessed forms determined as described in Materials and Methods. (*B*) Staurosporine does not affect the sorting and processing of cathepsin D. After a 3-h accumulation of cathepsin D at 20°C in the TGN, cells were incubated in the presence of staurosporine (10 μM) for 15 min at 20°C. Cells were then shifted to 37°C and the distribution of cathepsin D in the cells and media determined after a 4-h chase at 37°C as described in Materials and Methods.

fore shift of cells from 20°C to 37°C. These results indicate that wortmannin acts rapidly and virtually quantitatively, a result which is most consistent with a site of action at the TGN.

To confirm the specificity of wortmannin on lysosomal enzyme targeting, we examined the effects of staurosporine, a general inhibitor of protein kinases, including myosin light chain kinase (MLCK) (Watson et al., 1988) which is a potential target of wortmannin at the concentrations being used in our studies. As shown in Fig. 3 *B*, the addition of 10 μM staurosporine 15 min before shift from 20°C to 37°C had no effect on the processing of cathepsin D to the PLC associated 44-kD form, nor resulted in the diversion of cathepsin D to the secretory pathway. This result indicates that MLCK or a broad spectrum of other protein kinases (sensitive to staurosporine) are not responsible for the observed effects of wortmannin on lysosomal enzyme targeting.

Effect of PI 3-Kinase Inhibitors on the M6PR and Its Intracellular Compartments

One explanation for the effect of wortmannin on the targeting of lysosomal enzymes is that it causes a defect in the M6PR transport cycle. Typically, M6PRs are found by immunofluorescence microscopy to be localized in small vesicular and tubular structures in the region of the Golgi complex near the nucleus (Fig. 4, 0 μM), corresponding to the TGN and the PLC/late endosome compartments. However, addition of wortmannin for just 15 min at concentrations which inhibited cathepsin D processing and delivery resulted in the formation of large M6PR-stained vacuoles (Fig. 4, 0.1, 1.0, 10 μM). After a 15-min incubation, swollen vacuoles were just detectable in 0.1 μM wortmannin, and they reached maximal diameters in 5–10 μM of the inhibitor. Incubation of cells with 50–100 nM wortmannin also resulted in vacuole formation, but over a longer period of time (data not shown). The Golgi complex, as shown by staining with the *cis*-Golgi specific monoclonal antibody, 10E6, was not affected by wortmannin over this concentration range (Fig. 4).

A more extended time course of treatment with 1 μM wortmannin revealed a striking phenotype of the drug's effect. Dilated M6PR-stained vacuoles were first detectable even after only 5 min of treatment (data not shown), and by 15 min, the vacuoles had reached maximum size (Fig. 5, 15 min). Notably, there was a high correlation between the M6PR-stained vacuoles and the vacuoles visualized by phase contrast microscopy (Fig. 5), as seldom were phase lucent vacuoles seen that did not also stain for the receptor after 15 min of treatment. Gradually, however, the large phase lucent vacuoles stained less intensely for the receptor such that by 3 h of treatment, M6PRs were not detectable in the vacuoles by immunofluorescence (Fig. 5, 3 h). Instead, M6PRs were found primarily in the juxtanuclear Golgi region, an area adjacent to but essentially lacking the large vacuoles (Fig. 5, 3 h). These results suggest that in the presence of wortmannin, M6PRs cycle out of the swollen PLC/late endosome vacuoles and accumulate in a compartmentally distinct structure, perhaps the TGN.

To obtain independent evidence for a role of PI 3-kinase in mammalian lysosomal enzyme targeting, we examined if the bioflavinoid quercetin analog, LY294002, which has recently been shown to be a highly selective and reversible inhibitor of PI 3-kinase activity (Vlahos et al., 1994), had an identical effect on the M6PR. We found that treatment of cells with LY294002 for 15 min also caused the specific dilation of M6PR-stained vacuoles (Fig. 6 *B*). In addition, washing cells free of the drug for 1 h allowed the vacuoles to revert to control morphology. Likewise, when cells were treated with LY294002 for 3 h, and then washed free of the drug, M6PRs resumed their normal distribution in the TGN and vesicles of the PLC (data not shown). Similar recovery experiments were tried with wortmannin but with inconclusive results. In fact, recent studies report conflicting results concerning whether or not wortmannin is a reversible inhibitor (Clarke et al., 1994; Thelen et al., 1994; Woscholshi et al., 1994). In all other aspects so far tested, LY294002 was identical to wortmannin except that LY294002 was effective at higher concentrations (50–70 μM), consistent with the relative potencies of these inhibitors on PI 3-kinase activities (Stephens et al., 1994*b*; Stack and Emr, 1994).

The wortmannin- and LY294002-induced PLC vacuoles resembled those produced after neutralization of acidic compartments by the weak bases chloroquine and NH_4Cl (Brown et al., 1984, 1986), treatments which also result in the secretion of lysosomal enzymes (Gonzalez-Noriega et al., 1980). To determine if the PI 3-kinase inhibitors were acting via a similar "weak base" effect on the PLC, living cells were incubated with the vital fluorescent dye, acridine orange, which accumulates in acidic organelles: at low concentrations acridine orange fluoresces green; at high concentrations after accumulation in acidic compartments it changes to orange-red fluorescence (Allison and Young, 1969). In control cells, acridine orange accumulated in numerous small vesicles corresponding to the PLC, other endosomes, and lysosomes which showed a bright orange-red fluorescence (Fig. 7 *A*). In cells treated with 1 μM wortmannin for 3 h, the typical large PLC vacuoles formed in which acridine orange also accumulated to produce a bright orange-red fluorescence (Fig. 7 *B*). Similar results were obtained with LY294002-treated cells (data not shown). In contrast, when cells were treated with the weak base NH_4Cl for 3 h to neutralize the intralumenal pH of acidic compartments, numerous vacuoles were formed, however these were unstained by acridine orange (Fig. 7 *C*). In addition, acridine orange staining was completely lost from the wortmannin-induced vacuoles by subsequent addition of NH_4Cl (data not shown). These results suggest that wortmannin and LY294002 do not significantly alter the pH of the PLC vacuoles.

The above results show that after 3 h of wortmannin treatment, M6PRs are substantially depleted from the dilated vacuoles but are still found in the juxtanuclear Golgi region suggesting that receptors may have returned to the TGN. To confirm this, cells were first treated with wortmannin for 3 h and then subsequently with brefeldin A (BFA), a fungal metabolite which has previously been shown to induce the formation of membrane tubules from the TGN but not PLC/late endosomes (Tooze and Hollingshead, 1992; Wood et al., 1991; Wood and Brown, 1992). Just as in cells treated with BFA alone (Fig. 8 *B*), in

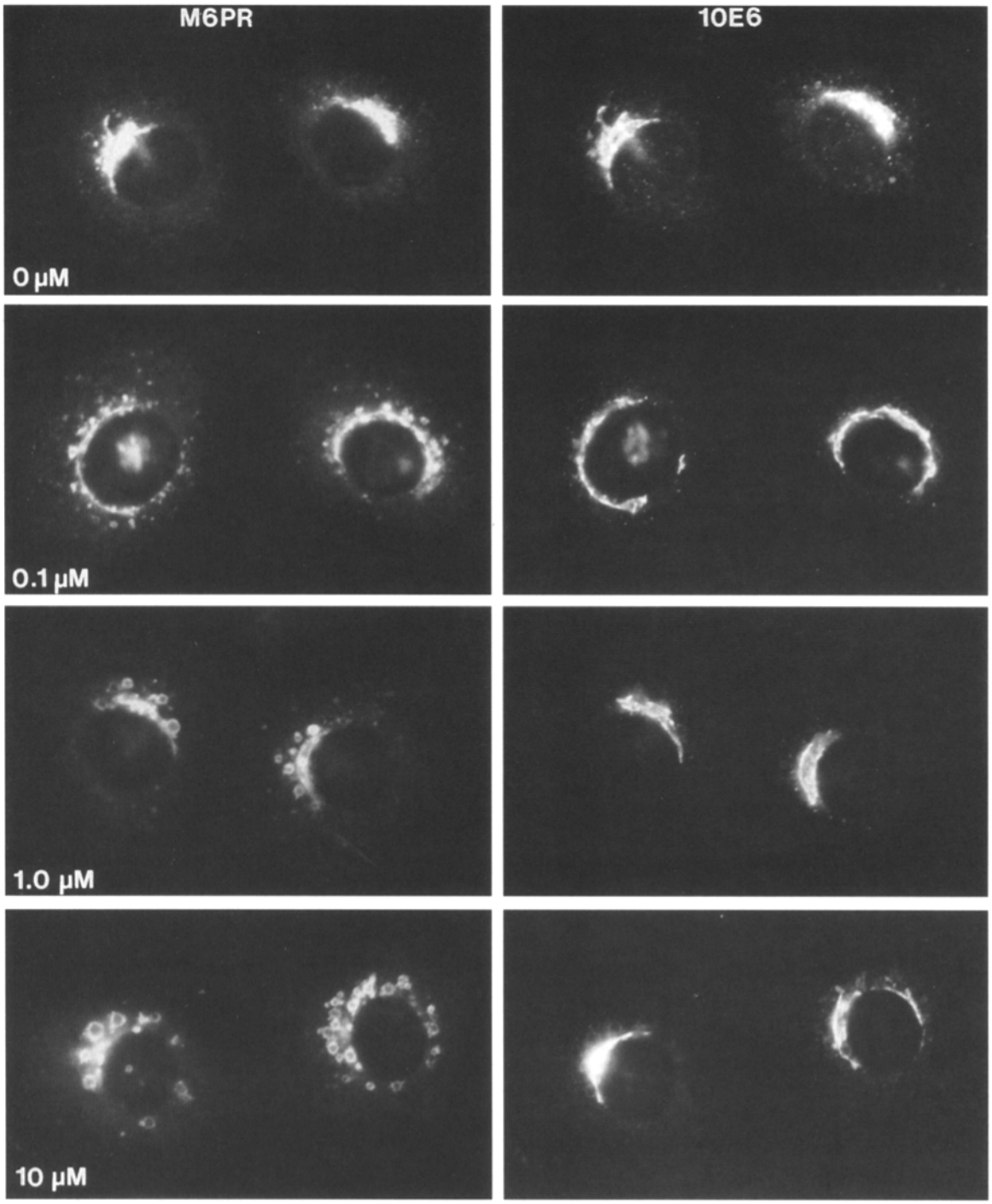

Figure 4. Effect of wortmannin on the distribution of M6PRs. Cells were incubated for 15 min at 37°C with the concentrations of wortmannin indicated on the left hand micrographs. Cells were fixed and stained by double-label immunofluorescence using polyclonal anti-M6PR and monoclonal 10E6 anti-Golgi antibodies. The left hand column shows the M6PR staining and the corresponding 10E6 localizations are on the right.

wortmannin-pretreated cells, BFA induced the formation of long M6PR-stained tubules from the juxtanuclear region, thus showing that receptors are located in the TGN even after 3 h of wortmannin treatment (Fig. 8 *C*).

To confirm these immunofluorescence results, and to more precisely localize M6PRs after wortmannin treatment, cells were also processed for immunoperoxidase localization by electron microscopy. In control cells, M6PRs are found in tubular elements of the TGN, clathrin-coated buds and vesicles, and in larger vesicles (0.2–0.4 μm in diameter) of the PLC (Fig. 9 *A*). After treatment of cells with 1 μM wortmannin for just 15 min, few of the normally sized, M6PR-enriched PLC vesicles, typically found near the Golgi region, were observed. Instead, large dilated vacuoles that stained with anti-M6PR antibodies were found in the central regions of the cytoplasm near the

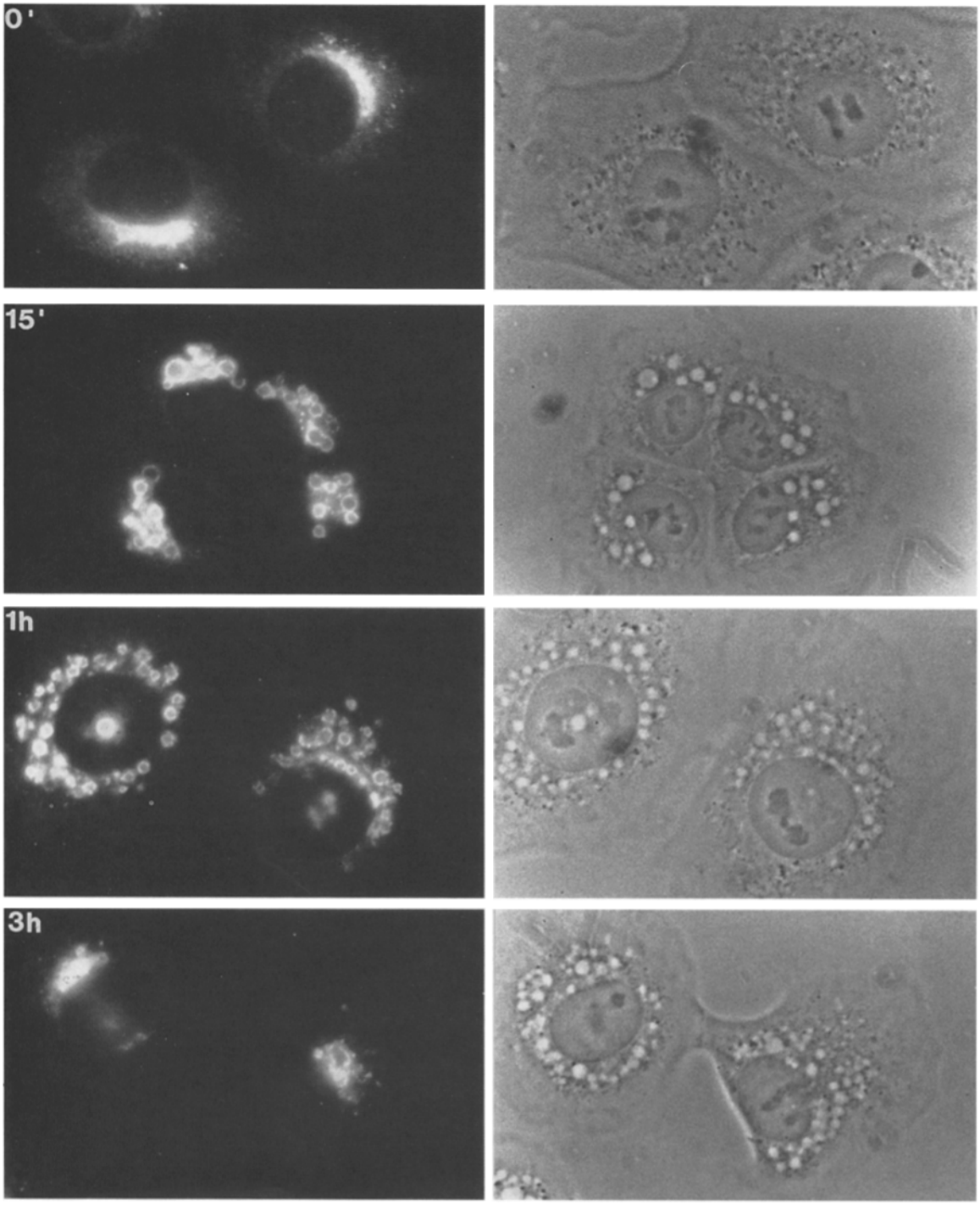

Figure 5. Time course of wortmannin-induced dilation of M6PR-positive vacuoles. Cells were incubated with 1 μM wortmannin at 37°C for the times indicated on the micrographs, fixed, and stained with anti-M6PR antibodies. Left hand column shows the M6PR staining and on the right are the corresponding phase contrast micrographs for each time point. Between 15 min and 1 h of treatment, there is a one-to-one correspondence between the M6PR-stained vacuoles and those seen by phase contrast microscopy. In the 15 min and 1 h micrographs, the plane of focus was centered on the vacuoles, and therefore, the juxtanuclear, Golgi signal was obscured (see Fig. 1, 1.0 for vacuole and Golgi region staining). By 3 h of treatment, however, the phase lucent vacuoles are still present but are no longer brightly stained. Instead, staining was primarily in juxtanuclear tubules and vesicles.

Golgi complex (Fig. 9 *B*). These M6PR-enriched vacuoles reached diameters of ~1 μm. In contrast to this distribution, after 3 h of wortmannin treatment, the large vacuoles were still present, however, they stained much less intensely for M6PRs (Fig. 9 *C*). M6PRs were, however, easily detected in the TGN throughout the period in which receptors became depleted from the large vacuoles (Fig. 9 *C*). Similar results were obtained with LY294002.

The above results strongly suggest that the dilated vacuoles produced by both wortmannin and LY294002, which

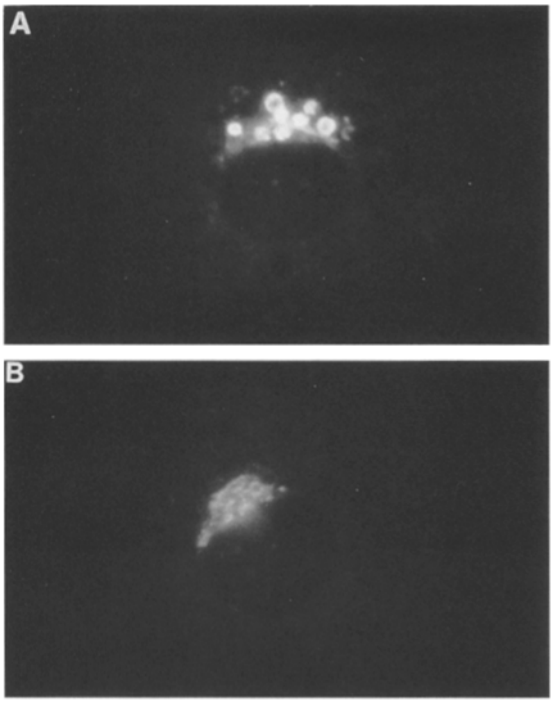

Figure 6. The PI 3-kinase inhibitor, LY294002, induces the reversible formation of M6PR-enriched vacuoles. (*A*) Cells were incubated with 70 μM LY294002 for 15 min at 37°C and then fixed with formalin or (*B*) washed with MEM (but without LY294002), and incubated in MEM plus 10% CSS for 1 h at 37°C.

initially stained brightly for the M6PR but much less so by 3 h of treatment, are derived from the PLC. However, it is possible that other compartments contribute as well. To determine if the vacuoles were derived from elements of the TGN, cells were treated with wortmannin for 15 min and then fixed and stained by immunofluorescence with antibodies against TGN38. Wortmannin was found to have no effect on TGN38 staining (Fig. 10, *TGN38*). Likewise, the lysosomes in wortmannin-treated cells, as revealed by staining with anti-cathepsin D antibodies (Fig. 10, *CD*), were not swollen and looked identical to those in normal cells (Wood and Brown, 1992).

To determine if, indeed, endocytic material could be delivered to the LY294002-induced vacuoles, we examined the endocytosis of antibodies bound to cell surface M6PRs. Cells were incubated with or without LY294002 for 3 h, and then subsequently in the continuous presence of LY294002 with anti-M6PR IgG, which binds to cell surface receptors, for 30 min to allow the endocytic delivery of receptor-bound antibodies to the PLC/late endosome compartment. Internalized antibodies were detected in fixed and permeabilized cells with rhodamine-conjugated anti–rabbit IgG antibodies. In untreated cells, surface bound anti-M6PR IgG was delivered after 30 min of uptake to a juxtanuclear cluster of vesicles, consistent with delivery to the PLC (Fig. 11 *A*). In cells pretreated with LY294002 for 3 h, anti-M6PR IgG was also internalized but was instead found in a juxtanuclear cluster of large vacuoles (Fig. 11 *B*). Identical labeling of dilated vacuoles was obtained when cells were pretreated with LY294002 for only 15 min before uptake of antibodies (data not shown). Endocytosis of anti-M6PR antibodies was primarily due to receptor-bound antibodies, and not fluid-phase uptake, because incubation of either untreated or treated cells with an equivalent concentration of preimmune IgG did not result in a detectable intracellular signal (data not shown). These results show that the LY294002-induced vacuoles are derived from the PLC/late endoso-

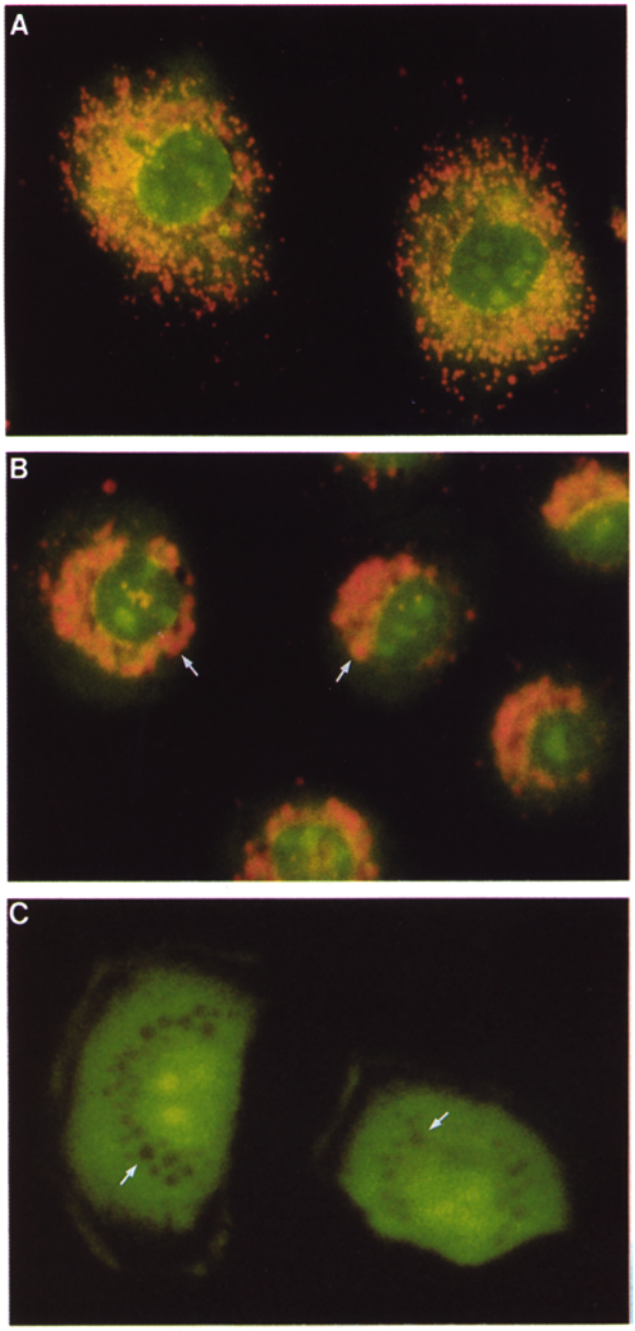

Figure 7. Wortmannin does not significantly affect the accumulation of the pH-sensitive dye, acridine orange, into the lumen of the PLC vacuoles. (*A*) Control cells showing small, bright orange-red vesicles corresponding to acidic organelles. (*B*) Cells incubated with wortmannin (1 μM for 3 h) before incubation with acridine orange produce swollen PLC vacuoles (*arrows*) that still fluoresce a bright orange-red. (*C*) Cells incubated with NH_4Cl (25 mM for 3 h) also produced swollen vacuoles (*arrows*) but these do not accumulate acridine orange.

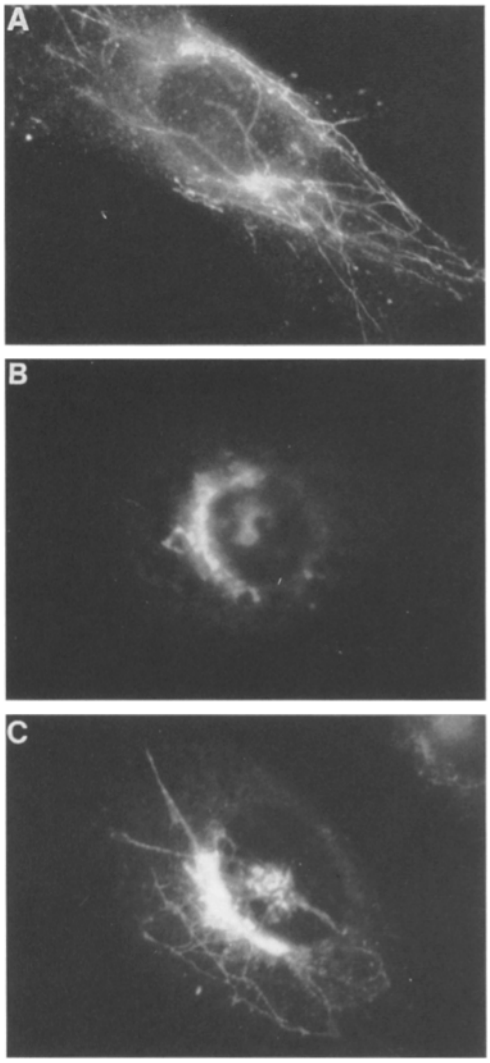

Figure 8. After long term wortmannin treatment, M6PRs are still located in the TGN. Cells were treated as indicated below and then stained by immunofluorescence for the M6PR. (*A*) Cells treated with BFA (10 μg/ml) for 5 min at 37°C. (*B*) Cells treated with wortmannin (1 μM) for 3 h at 37°C. The large swollen vacuoles, which no longer stain intensely for the M6PR can be seen as "ghosts" in the cytoplasm. (*C*) Cells treated with wortmannin (1 μM) for 3 h and then with BFA (10 μg/ml) for 5 min at 37°C in the continuous presence of wortmannin.

mal vesicles. Moreover, they demonstrate that whether or not intracellular M6PRs are present in the dilated PLC vacuoles in normal amounts (i.e., 15 min vs 3 h of LY294002 treatment; see Fig. 5), receptors originating from the cell surface could nevertheless be internalized and delivered to the PLC/late endosomal compartment.

Although antibodies that were bound to cell surface M6PRs could be endocytosed, we noticed that the intensity of the intracellular signal was less in cells pretreated with LY294002 for 3 h than in untreated cells. This result could be due to a decrease in the number of cell surface receptors, the rate of internalization, or both. Using a cell-associated ELISA assay, we found that treatment of cells with 70 μM LY294002 for ~2.5 h leads to a fourfold loss of surface receptors (Fig. 12). Similar results were obtained using wortmannin (data not shown). After removal of LY294002 for 2 h, surface receptors returned to ~70% of the pretreated values. These results suggest that as M6PRs are endocytosed at the cell surface, they are not replenished by intracellular stores.

Discussion

We find that the potent PI 3-kinase inhibitor, wortmannin, substantially inhibits the processing and intracellular retention of newly synthesized cathepsin D, and therefore, its specific delivery to lysosomes. Half-maximal inhibition of cathepsin D sorting occurred at ~100 nM wortmannin and correlated with a threefold decrease in cellular levels of PtdIns 3-P. The effect of wortmannin on cathepsin D trafficking was shown to involve a step specific to the lysosomal pathway and not to some common step early in the constitutive secretory pathway because the processing and transport of newly synthesized VSV-G was unaffected by wortmannin. Moreover, later steps of the constitutive secretory pathway were likewise unaffected because: (*a*) VSV-G efficiently reached the cell surface; and, (*b*) cathepsin D was not retained within cells but was constitutively secreted from cells. Thus, wortmannin completely abolished sorting of cathepsin D from the secretory pathway by affecting some transport step either at the level of the TGN or the delivery site in the PLC, but it had no observable effect on transport from the TGN to the cell surface.

These results strongly suggest that proper trafficking of acid hydrolases to lysosomes in mammalian cells requires the activity of a PI 3-kinase. The impetus for this work came from previous studies showing that the targeting and delivery of newly synthesized proteins to the yeast vacuole depend on the product of the *VPS34* gene, a protein known to have significant homology to the p110 catalytic subunit of mammalian PI 3-kinase (Herman and Emr, 1990; Hiles et al., 1992) and which has been directly demonstrated to have PI 3-kinase activity (Schu et al., 1993). In addition, Stack and Emr (1994) have recently shown that Vps34p enzymatic activity is sensitive to the PI 3-kinase inhibitors, wortmannin, and LY294002. Finally, the idea that wortmannin inhibits lysosomal enzyme sorting in mammalian cells is consistent with studies in yeast demonstrating that soluble vacuolar hydrolases appear to be sorted in the late Golgi for delivery to a late endosome-like compartment (Vida et al., 1993; Graham and Emr, 1991). Fractionation studies further indicate that Vps34p functions in a late Golgi compartment (Stack and Emr, 1993). Therefore, our separate studies provide evidence that a common PI 3-kinase–mediated regulatory mechanism is used for the specific targeting and transport of proteins to both mammalian lysosomes and yeast vacuoles.

One possible complication in our studies is that at the concentrations needed to induce missorting of lysosomal enzymes, wortmannin has been reported to inhibit some protein kinases, such as myosin light chain kinase (Nakanishi et al., 1993), and PI 4-kinase (Cheatham et al., 1994). However, our results are strengthened by finding that an unrelated PI 3-kinase inhibitor, LY294002, also alters the distribution of M6PRs and the morphology of the PLC, but has little effect on a variety of protein kinases (Vlahos et al., 1994). We also found that staurosporine, a general

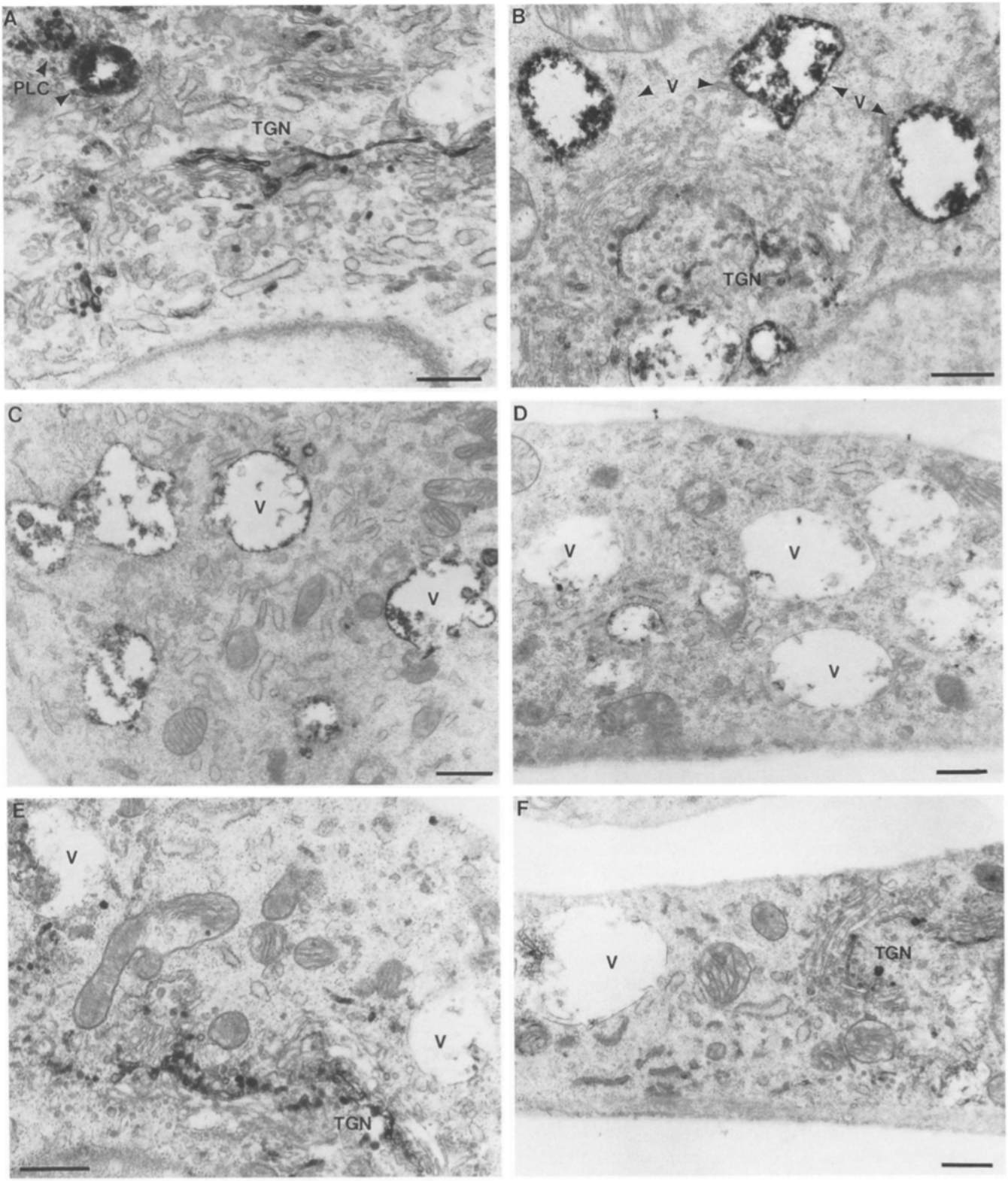

Figure 9. M6PRs become depleted from the swollen PLC vacuoles during wortmannin treatment: immunoperoxidase observations at the electron microscopic level. (*A*) In control cells, M6PRs are located in tubular elements of the TGN, clathrin-coated buds and vesicles, and in larger vesicles of the PLC. (*B* and *C*) After treatment with 1 μM wortmannin for 15 min, large dilated vacuoles (*V*) of the PLC form which contain significant amounts of immunoperoxidase reaction product. (*D–F*) After treatment with 1 μM wortmannin for 3 h, large dilated vacuoles (*V*) are still found in the Golgi region, however, these vacuoles have very little reaction product. Conversely, tubular elements of the TGN still contain detectable levels of M6PRs. Bars, 0.5 μm.

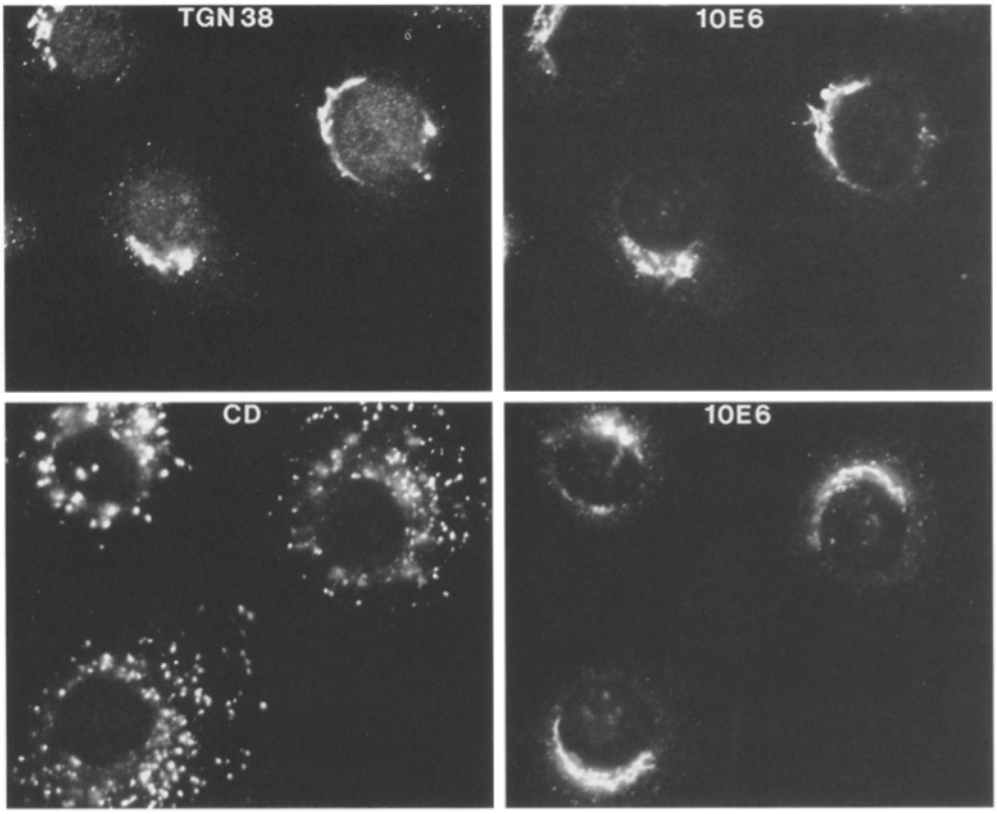

Figure 10. Wortmannin does not affect the TGN or lysosomes. Cells were treated with 1 μM wortmannin for 15 min at 37°C and then fixed and stained by double-immunofluorescence with anti-TGN38 and 10E6 antibodies or anti-cathepsin D (*CD*) and 10E6 antibodies as indicated on the micrographs.

inhibitor of many protein kinases including MCLK, and which blocks early steps in Golgi transport (Davidson et al., 1992), had no observable effect on the sorting of cathepsin D from the TGN and subsequent processing in the PLC/late endosome compartments. Similarly, it seems unlikely that wortmannin exerts its effects on lysosomal enzyme trafficking through PI 4-kinase because concentrations as low as 100 nM wortmannin, which have little effect on PI 4-kinase (Stephens et al., 1994*b*), resulted in ~40% of newly synthesized cathepsin D being missorted to the secretory pathway. Finally, in the accompanying manuscript, Davidson has independently found that wortmannin inhibits lysosomal enzyme sorting, and furthermore, he reports that the concentration of wortmannin required to inhibit lysosomal enzyme delivery varies considerably between different cell types, but can be in the 20–40 nm range (Davidson, 1995). The reasons for differences in sensitivity could be related to the presence of multiple PI 3-kinases or differential metabolism of the drug in different cells. Thus, while a small role for PI 4-kinase cannot be completely ruled out, our studies and those of Davidson are most consistent with wortmannin inhibiting a PI 3-kinase that is involved in the trafficking of newly synthesized lysosomal enzymes. Davidson's studies further complement ours by showing that wortmannin does not inhibit the formation of M6P residues nor the M6P-dependent uptake of lysosomal enzymes by cell surface M6PRs (Davidson, 1995).

Which of the PI 3-kinase isoforms might be involved in lysosomal enzyme trafficking? One possibility is the p110/p85 PI 3-kinase that has already been shown to be involved in the endocytosis and trafficking of the PDGF receptor (Joly et al., 1994). However, we consider this unlikely given that this enzyme is exquisitely sensitive to inhibition by wortmannin (IC_{50} = 1–5 nM). The differential sensitivity of distinct membrane trafficking pathways in mammalian cells to wortmannin is intriguing in light of recent studies demonstrating that yeast Vps34p PI 3-kinase activity is also less sensitive to wortmannin (IC_{50} ~4 μM) (Stack and Emr, 1994). Recently, a PtdIns-specific 3-kinase was discovered in bovine adrenoreticulosa cells that exhibits sensitivity to wortmannin (Stephens et al., 1994*b*) similar to that observed for Vps34 PI 3-kinase (Stack and Emr, 1994), and to the level required to block lysosomal enzyme sorting. Therefore, it is possible that the enzyme discovered by Stephens et al., (1994*b*) may be a *VPS34* homolog, or it is also possible that yet another PI 3-kinase awaits discovery.

Several experiments suggest that wortmannin is acting at the level of the TGN to inhibit proper sorting of lysosomal enzymes from the constitutive secretory pathway. The conclusions from these data are summarized in a model presented in Fig. 13. First, we found that when newly synthesized cathepsin D was allowed to accumulate in the TGN at 20°C, a 15-min incubation with wortmannin was sufficient to quantitatively missort cathepsin D to the secretory pathway following shift to 37°C. This rapid action of wortmannin is faster by a factor of 3–4 fold than the apparent time it takes lysosomal enzymes to get from the TGN to the PLC (Rosenfeld et al., 1982; Rijnboutt et al., 1992), and is therefore not compatible with an effect on the M6P-dependent delivery site at the PLC.

Second, in addition to inhibiting lysosomal enzyme sorting and delivery, both wortmannin and LY294002 altered the distribution of the M6PR. Within 15 min after addition of the inhibitors, M6PR-enriched vesicles of the PLC/late endosome compartment were found to be significantly di-

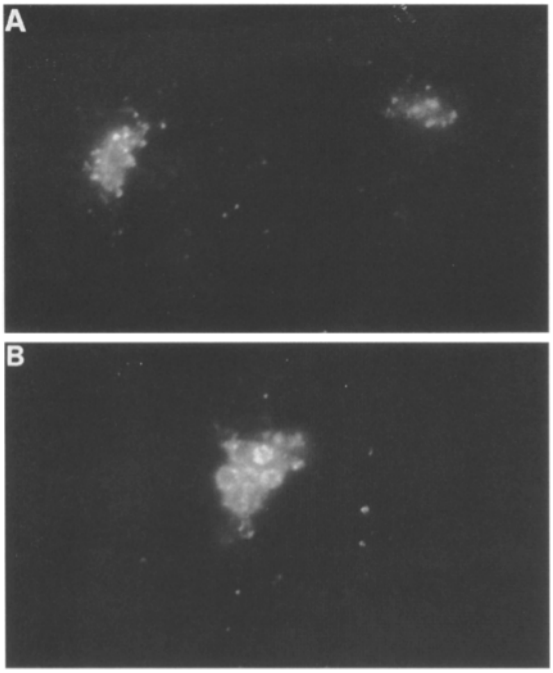

Figure 11. Anti-M6PR antibodies bind to the cells surface and are delivered by endocytosis to the LY294002-induced vacuoles. Control cells (*A*) or cells preincubated with 70 μM LY294002 for 3 h (*B*), followed by incubation with anti-M6PR IgG for 30 min at 37°C. Cells were then fixed, permeabilized, and incubated with rhodamine-conjugated anti–rabbit antibodies to detect the internalized anti-M6PR antibodies.

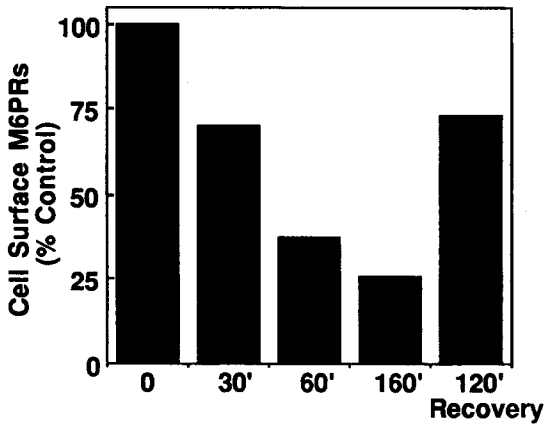

Figure 12. LY294002 treatment causes a reduction in cell surface M6PRs. Cells were incubated in the presence or absence of 70 μM LY294002 for various periods of time (as indicated below the graph) at 37°C. In another case, cells were incubated in the presence of LY294002 as above for 3 h, cells were then washed extensively with MEM containing 10% CSS and incubated in the same for 2 h (120′ recovery on the graph). At the end of all incubations, cells were fixed and incubated with anti-M6PR IgG to detect cell surface receptors. Goat anti–rabbit IgG conjugated with alkaline phosphatase was used to generate a colorimetry assay for surface receptors. Each time point represents the average of triplicate samples and the results are expressed as the percentage of control (untreated) values.

lated. Gradually, however, these same dilated PLC vacuoles stained less intensely for M6PRs such that by 3 h of treatment, the vacuoles contained almost no detectable receptors. Instead, receptors were abundant in the TGN. In the case of LY294002, this redistribution was reversible. In addition to these changes, the level of cell surface M6PRs decreased approximately fourfold over the same time period. Because cell surface M6PRs were capable of being endocytosed and delivered to the PLC in the presence of wortmannin, inhibition of receptor delivery to the cell surface must have occurred. The simplest explanation to account for all these results is that wortmannin inhibits forward movement out of the TGN, but does not effect the recycling of M6PRs from the cell suface or the PLC back to the TGN (Fig. 13).

Although wortmannin had a profound effect on transport of M6PRs from the TGN to the PLC, we and Davidson (Davidson, 1995) independently found that wortmannin had no apparent effect on the endocytic trafficking of M6PRs indicating that PI 3-kinases are not involved in these M6PR trafficking events. However, others have found that the endocytic trafficking of mutant PDGF and CSF receptors lacking the p85/p110 PI 3-kinase–binding sites is significantly impaired. Thus, it would appear that regulation of the endocytic trafficking of tyrosine kinase-type receptors (PDGF, CSF) and the M6PR differs greatly.

Apart from its effect on lysosomal enzyme and M6PR trafficking, we also found that wortmannin (and LY294002) induced a very specific swelling of PLC/late endosome vesicles within 15 min (Fig. 13). The acidotropic weak bases NH_4Cl and chloroquine produce a similar but less organelle-specific swelling (Brown et al., 1984, 1986), and in addition, also cause the missorting of lysosomal enzymes because neutralization of the PLC delivery site prevents receptor-ligand dissociation (Gonzalez-Noreiga et al., 1980). However, the swelling of the PLC/late endosome vesicles by wortmannin was not consistent with a simple weak base-neutralization effect, and also did not appear to be directly coupled to changes in M6PR distribution nor to the missorting of lysosomal enzymes. First, we found that accumulation of the pH-sensitive vital dye acridine orange into the wortmannin-induced PLC vacuoles was apparently unaltered indicating that the pH of the PLC was not significantly changed. Second, wortmannin rapidly (within 15 min) caused the missorting of cathepsin D that had previously accumulated in the TGN by the 20°C block. And third, in the presence of weak bases, swollen PLC vacuoles remain heavily stained for M6PRs (Brown et al., 1984, 1986; Geuze et al., 1985), whereas in wortmannin (or LY294002), PLC vacuoles became reversibly (in the case of LY294002) depleted of receptors which were instead easily detected in the TGN. It is curious that the swelling of the PLC occurred so rapidly and that depletion of receptors took longer. The "apparent" slow depletion of receptors over a 3-h period is likely due in part to the nonquantitive nature of immunofluorescence, but also to the time it takes the entire pool of receptors to recycle back to the TGN (Brown et al., 1986). Although the swelling of the PLC does not appear to inhibit M6PR recycling back to the TGN, we cannot rule out the possibility that wortmannin inhibits an activity in the PLC that is required to "reactivate" M6PRs, so that in the presence of

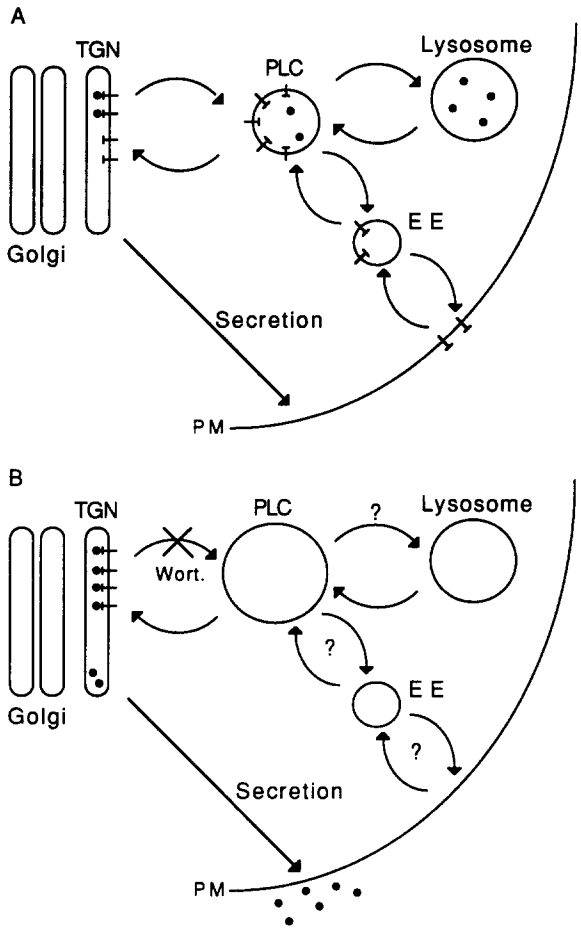

Figure 13. Summary of the effect of PI 3-kinase inhibitors, wortmannin and LY294002, on lysosomal enzyme and M6PR trafficking. (*A*) In untreated cells, M6PRs (●) transport newly synthesized lysosomal enzymes (**T**) from the TGN to the PLC where the enzymes dissociate from the receptors for ultimate delivery to lysosomes. M6PRs can recycle back to the TGN or they can continue on to the plasma membrane (*PM*) to engage in receptor-mediated endocytosis to early endosomes (*EE*). (*B*) In cells treated with wortmannin or LY294002, our results are most consistent with a model in which sorting of M6PR-enzymes from the TGN for delivery to the PLC is inhibited resulting in the secretion of newly synthesized lysosomal enzymes. In addition, vesicles of the PLC become swollen, however, it is unclear if this morphological change is related in any way to the missorting that most likely occurs in the TGN. Question marks are placed at several steps to indicate that (*a*) the extent to which wortmannin affects forward movement out of the PLC is unknown, and (*b*) the endocytic trafficking of receptors may be subjected to different levels of PI 3-kinase regulation. The symbols for M6PRs are not meant to accurately represent the proportion of receptors in each compartment but only to schematically demonstrate changes that occur after treatment with PI 3-kinase inhibitors, and not all possible M6PR pathways are shown.

wortmannin, M6PRs reach the PLC, dissociate their ligands, but return to the TGN in a nonfunctional form.

Taken together, both the biochemical studies of cathepsin D trafficking and the immunocytochemical observations of the M6PR are most consistent with the PI 3-kinase inhibitors affecting sorting of lysosomal enzymes within the TGN and not downstream at the delivery site in the PLC. Apparently, in the presence of wortmannin, both ligand (cathepsin D) and receptor are located together in the TGN, however, they are unable to productively interact to induce sorting. How any particular PI 3-kinase could influence the sorting, packaging, and delivery of vesicular cargo is unclear. In the case of lysosomal enzyme and vacuolar protein trafficking, phosphorylated forms of phosphatidylinositol generated by a PI 3-kinase, could influence receptor-mediated transport in several ways. For example, PI 3-kinase and/or its products could promote the deformation of membrane into vesicle buds (Sheetz and Singer, 1974) or the formation of clathrin-coated vesicles. Our preliminary studies suggest that wortmannin does not inhibit the formation of AP-1 type clathrin-coated buds and vesicles on TGN membranes (Fasano, M. and W. J. Brown, unpublished data). Alternatively, accumulation of PI(3)P in the membrane could influence the ability of M6PRs to bind ligand or become productively localized to clathrin-coated buds in the TGN.

In conclusion, we have provided evidence that a PI 3-kinase is involved in the sorting and delivery of newly synthesized lysosomal enzymes in mammalian cells. These studies provide the first evidence for a molecular link between the regulation of intracellular trafficking to the mammalian lysosome and the yeast vacuole. We expect that in addition to identifying a *VPS34* homolog, other mammalian homologs to *VPS* genes will be identified in the near future, for example, the *VPS15* encoded protein kinase that regulates the activity of Vps34 PI 3-kinase.

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