Phosphatidylinositol 3-Kinase Is Not Required for Recycling of Mannose 6-Phosphate Receptors from Late Endosomes to the Trans-Golgi Network

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Mannose 6-phosphate receptors carry newly synthesized lysosomal hydrolases from the trans-Golgi network to endosomes, then return to the trans-Golgi network for another round of enzyme delivery. Wortmannin, an inhibitor of phosphatidylinositol 3-kinase, interferes with the delivery of newly synthesized lysosomal enzymes to lysosomes. We used two independent assays of mannose 6-phosphate receptor trafficking to determine the precise step that is blocked by wortmannin. Using an assay that monitors resialylation of desialylated cell surface 300-kDa mannose 6-phosphate receptors, we found that receptor endocytosis and transport to the trans-Golgi network were not inhibited by 2 μ M wortmannin. In addition, this concentration of drug had no effect on the transport of the mannose 6-phosphate receptor from late endosomes to the trans-Golgi network using a system that reconstitutes this transport process in cell extracts. Under the same conditions, wortmannin significantly inhibited the generation of mature cathepsin D. In addition, the structurally unrelated phosphatidylinositol 3-kinase inhibitor, LY294002, was also without effect when added to *in vitro* endosome–trans-Golgi network transport reactions. These experiments demonstrate that the interruption in lysosomal enzyme targeting is most likely due to a wortmannin-sensitive process required for the export of these receptors from the trans-Golgi network, consistent with the established role of phosphatidylinositol 3-kinase in the equivalent transport process in *Saccharomyces cerevisiae*.

INTRODUCTION

Mannose 6-phosphate receptors (MPRs)¹ deliver newly synthesized, soluble lysosomal enzymes from the trans-Golgi network (TGN) to prelysosomes (Kornfeld and Mellman, 1989; Kornfeld, 1992). Two types of MPRs have been identified to date. One is a 300-kDa transmembrane glycoprotein that also binds insulin-like growth factor II; the second is a dimer or tetramer of 45-kDa subunits and requires divalent cations for ligand binding *in vitro*. Both MPRs release their ligands upon encountering the acidic lumen of prelysosomes and then return to the Golgi complex to reinitiate another cycle of biosynthetic enzyme transport. MPRs are also present at the cell surface. These

receptors are in rapid equilibrium with their intracellular counterparts, and at least the 300-kDa MPR is capable of endocytosing extracellular lysosomal hydrolases and delivering them to endocytic compartments.

Emr, Stevens, and Jones and their colleagues have characterized a number of gene products required for the delivery of proteins to the yeast vacuole, a compartment that is analogous to a mammalian cell lysosome (Conibear and Stevens, 1995; Stack *et al.*, 1995). More than 40 complementation groups have been identified that are needed for vacuolar protein localization, and mutants in this pathway are called *vps* or vacuolar protein-sorting mutants. Most of the *VPS* genes are not essential, which facilitates an analysis of their distinct functions.

The *VPS15* and *VPS34* genes encode a protein kinase and a phosphatidylinositol 3-kinase (PIP3-kinase), respectively, that are physically associated and

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¹ Abbreviations used: MPR, mannose 6-phosphate receptor; TGN, trans-Golgi network; PIP3-kinase, phosphatidylinositol 3-kinase.

are required for export of vacuolar enzymes from the yeast trans-Golgi (Stack *et al.*, 1993, 1995). Deletion of the *VPS34* gene leads to complete depletion of cellular phosphatidylinositol 3-phosphate, suggesting that the generation of this molecule is associated uniquely with vacuolar protein sorting. Since Vps34p shares homology with a mammalian PI3-kinase, it was of interest to determine whether this class of enzyme was also used by mammalian cells to direct lysosomal enzyme targeting.

Inhibitors of PI3-kinase, such as wortmannin, are potent inhibitors of the processing and delivery of mammalian lysosomal enzymes from the TGN to lysosomes (Brown *et al.*, 1995; Davidson, 1995). Under the same conditions, wortmannin failed to inhibit the endocytosis of cell surface MPRs. These workers concluded that their results were most easily explained by a model in which export of receptors was directly blocked, in accordance with the role of PI3-kinase in vacuolar protein sorting in yeast. However, it was possible that the missorting of lysosomal enzymes was due to a disruption in MPR trafficking (Shepherd *et al.*, 1996). Indeed, Reaves *et al.* (1996) have proposed that wortmannin blocks MPR traffic between late endosomes and the TGN.

In this report we have analyzed, directly, the recycling of the 300-kDa MPR from late endosomes back to the TGN. We have done this quantitatively in intact cells by desialylating the receptors at the surface and monitoring their resialylation due to their return to the TGN as a function of time. We have also used a cell-free system that reconstitutes late endosome to TGN transport *in vitro*. Using both of these approaches, we show that wortmannin does not block MPR recycling under conditions in which lysosomal enzyme targeting is blocked. These experiments provide a direct demonstration that MPR recycling is insensitive to wortmannin, and thus this process is unlikely to require generation of phosphatidylinositol 3-phosphate.

MATERIALS AND METHODS

ATP was purchased from Pharmacia (Piscataway, NJ). *Vibrio cholerae* neuraminidase, Pansorbin, LY294002, and antihuman cathepsin D were purchased from Calbiochem (La Jolla, CA). Cell culture media were obtained from Life Technologies, Inc. and methionine- and cysteine-free medium was made from minimal essential medium (MEM) Select-Amine kit (Life Technologies, Inc., Grand Island, NY) and was supplemented with 10% dialyzed fetal calf serum (FCS). All other chemicals were from purchased Sigma (St. Louis, MO) unless otherwise stated.

Cell Culture

Chinese hamster ovary wild-type and CHO 1021 cell lines were maintained in α -MEM supplemented with 7.5% FCS. K562 cells were maintained in RPMI 1640 supplemented with 10% FCS.

Recycling of MPRs from the Cell Surface to the TGN in Living Cells

The assay was essentially as described (Duncan and Kornfeld, 1988; Riederer *et al.*, 1994). CHO cells were grown in 6-cm dishes to 50% confluence. After washing three times with TD (25 mM Tris-HCl, pH 7.4, 5.4 mM KCl, 137 mM NaCl, 0.3 mM Na_2HPO_4), cells were labeled with 170 μCi of Tran^{35}S label in 1 ml of methionine- and cysteine-free medium overnight. Cells were chased in complete medium for 2 h and then washed once in TD and once in α -MEM containing 1% FCS. Sialic acid residues were removed by addition of 0.02 U *V. cholerae* neuraminidase in 1 ml of α -MEM containing 1% FCS and 20 mM HEPES (pH 7.4). After 2 h at 37°C, cells were washed four times in TD and twice in complete medium and recultured for the indicated times in the presence or absence of 2 μM wortmannin. The reaction was stopped by aspiration of media and the addition of 0.4 ml of detergent stop mix (Goda and Pfeffer, 1988) on ice. After clarification of the lysate by centrifugation at 95,000 rpm for 10 min at 4°C in a TLA 100.2 rotor, supernatants were analyzed for the fraction of MPR which had acquired sialic acid. Proteins were separated by an 8% SDS-polyacrylamide gel and quantitated using a PhosphorImager (Molecular Dynamics, Sunnyvale, CA).

K562 cells (2×10^7) were collected by centrifugation and washed three times with TD. Cells were suspended in 30 ml of methionine- and cysteine-free medium containing 3 mCi of Tran^{35}S label and incubated overnight. Labeled cells were collected by centrifugation and chased in 30 ml of RPMI 1640 containing 10% FCS for 2 h at 37°C. Cells were washed once in TD and one in RPMI 1640 containing 1% FCS. The cells were suspended in 20 ml of RPMI 1640 containing 1% FCS and 20 mM HEPES (pH 7.4) and treated with 0.02 U/ml *V. cholerae* neuraminidase at 37°C for 2 h. Cells were transferred to ice, washed four times with TD and twice with RPMI 1640 containing 1% FCS. Cells (2×10^6) were suspended in RPMI 1640 with 10% FCS and 20 mM HEPES (pH 7.4) and recultured in the presence or absence of 2 μM wortmannin for the indicated times at 37°C. Reactions were stopped by transferring the cells to ice and collecting cells by centrifugation. Detergent stop mix (1 ml) was added and resialylation was measured as described for CHO cells.

Transport of MPRs from Late Endosomes to the TGN In Vitro

In vitro transport was carried out as described (Dirac-Svejstrup *et al.*, 1994); the efficiency of slug lectin (Goda and Pfeffer, 1988) was found to be 50% and transport values were corrected accordingly.

Maturation of Cathepsin D

K562 cells (1×10^6 cells/ml) were collected by centrifugation (250 $\times$ g, 5 min) and washed twice with TD. After suspension in methionine- and cysteine-free medium (10^7 cells/3 ml), they were incubated for 30 min at 37°C to deplete endogenous methionine and cysteine. Cells (1×10^7) were collected by centrifugation and suspended in 1 ml of the same medium containing 20 mM HEPES (pH 7.4) and 0.5 mCi of Tran^{35}S label for 15 min at 37°C. After centrifugation, cells were suspended in RPMI 1640 containing 20 mM HEPES (pH 7.4), 10% FCS, 5 mM mannose 6-phosphate (sodium salt). Cells (2×10^6 /reaction) were chased at 37°C for the indicated time in the presence or absence of 2 μM wortmannin. Reactions were transferred to ice and medium was removed by centrifugation. RIPA buffer (20 mM sodium phosphate, pH 7.5, 150 mM NaCl, 1% Triton X-100, 0.5% deoxycholate, 0.1% SDS; 0.5 ml) was added and lysates were transferred to a new tube. Another 0.5 ml of RIPA was used to wash the tube and combined with the lysate. Pansorbin prewashed with RIPA buffer (30 μl) was added to each lysate and incubated on ice for 15 min. After preclearing the lysate by centrifugation at 95K rpm for 10 min in TLA 100.2 rotor, 10 μl of antihuman cathepsin D antibody was added, and incubated at 4°C

overnight. Immune complexes were collected using 30 μ l of pre-washed Pansorbin after incubation for 15 min at room temperature. Complexes were washed three times with RIPA buffer and once with TD and 30 μ l of sample buffer were added. Samples were analyzed by 10% SDS-polyacrylamide gels under nonreducing conditions, and radioactivity was determined by PhosphorImager.

RESULTS

To test whether PI3-kinase is required for the recycling of MPRs from late endosomes to the TGN, we examined the effect of the PI3-kinase inhibitor, wortmannin, on MPR recycling using two different assays. The first assay measures the transport of cell surface MPRs back to the TGN. Metabolically labeled CHO cells are treated with neuraminidase to remove sialic acid from surface MPRs. These receptors are endocytosed and traffic through the endosomal pathway to the TGN (Duncan and Kornfeld, 1988). Upon reaching the TGN, the receptors encounter sialyltransferase and their oligosaccharide chains are modified. By following the reacquisition of sialic acid residues by MPRs over time, we can monitor the recycling of MPRs to the TGN in living cells.

As shown in Figure 1, MPRs reacquired sialic acid with linear kinetics over a period of at least 4 h of incubation. This was true in both CHO cells (top panel) or human K562 cells (bottom panel). As reported previously (Duncan and Kornfeld, 1988), the extent of resialylation was greater in K562 cells than in CHO cells, which is likely due to a difference in the abundance of sialyltransferase activity in the Golgi complexes of these cell types. The presence of 2 μ M wortmannin did not alter significantly the extent of sialic acid readdition in either of these cell lines.

The best characterized PI3-kinase in mammalian cells is inhibited by nanomolar concentrations of wortmannin (Arcaro and Wyman, 1993; Yano *et al.*, 1993; Okada *et al.*, 1994), but one PI3-kinase is only inhibited by submicromolar concentrations of wortmannin (Stephens *et al.*, 1994). Nevertheless, 2 μ M wortmannin should have been sufficient to inhibit either class of enzyme. Thus, wortmannin had no effect on MPR recycling from the cell surface to the TGN in living cells.

Wortmannin Does Not Block MPR Recycling In Vitro

The assay described above measures multiple steps, including transport from the cell surface to early endosomes, from early endosomes to late endosomes, and from late endosomes to the TGN. Davidson (1995) has shown that MPR endocytosis was not inhibited by wortmannin, consistent with our findings. To test directly whether the final step of late endosome to TGN transport requires PI3-kinase, we used an *in vitro* assay that reconstitutes this transport event (Goda and

Figure 1. Wortmannin fails to inhibit recycling of MPRs to the TGN in living cells. CHO cells (top panel) or K562 cells (bottom panel) were metabolically labeled, treated with neuraminidase, and then recultured for the indicated times in the presence (●) or absence (○) of 2 μ M wortmannin. Resialylated MPRs were determined as described in MATERIALS AND METHODS. Values are the average of duplicates.

Pfeffer, 1988; Dirac-Svejstrup *et al.*, 1994). The assay uses a cell line in which oligosaccharide chains fail to acquire sialic acid due to the absence of the Golgi CMP-sialic acid translocator (Deutscher *et al.*, 1984). Such cells are metabolically labeled and broken, and extracts are mixed with purified rat liver Golgi membranes, cytosol, ATP, and an ATP-regenerating system. Transport is measured by isolating the MPRs and determining the extent of sialic acid addition as before.

Figure 2 shows the results of *in vitro* transport reactions carried out using three different concentrations of cytosolic proteins in the presence or absence of wortmannin. Cytosol-stimulated transport is likely to reflect events seen in living cells, because both pro-

Figure 2. Wortmannin does not inhibit recycling of MPRs from late endosomes to the TGN in vitro. Transport assays were carried out in the presence of the indicated amounts of cytosol, in the presence or absence of 2 μ M wortmannin. Values represent the average of duplicates.

cesses depend on functional Rab9 GTPase (Lombardi *et al.*, 1993; Riederer *et al.*, 1994). As expected, transport was stimulated by cytosol addition. In all cases, wortmannin failed to inhibit transport; at high cytosol, the drug actually stimulated transport to a very small extent. Thus, wortmannin had no influence on MPR recycling from late endosomes to the TGN.

LY294002 is another inhibitor of PI3-kinase that is structurally unrelated to wortmannin (Vlahos *et al.*, 1994). Brown *et al.* (1995) showed that lysosomal enzyme sorting was also defective in cells treated with this compound. To confirm that MPR transport did not require PI3-kinase, we tested the effect of LY294002 at 100 μ M, a concentration shown by others to inhibit in vitro early endosome fusion by at least 80% (Jones and Clague, 1995). At these concentrations, MPRs are found in the same swollen organelles observed for wortmannin-treated cells (Brown *et al.*, 1995). As shown in Figure 3, like wortmannin, LY294002 failed to inhibit the transport of MPRs from late endosomes to the TGN in vitro.

Wortmannin Inhibits Maturation of Cathepsin D

Since wortmannin failed to inhibit MPR trafficking, it was essential that we confirm that our preparation of wortmannin was, in fact, active in the cell lines studied. For this reason, we examined the maturation of cathepsin D, a process shown by Brown *et al.* (1995) and Davidson (1995) to be inhibited by wortmannin. Cathepsin D is synthesized as a 53-kDa proenzyme. After delivery by MPRs to late endosomes, it is processed to a 47-kDa form by removal of the propeptide portion. Once in lysosomes, it is further cleaved to generate noncovalently associated 31-kDa and 14-kDa polypeptides. For these experiments, we used K562 cells and commercial antihuman cathepsin D anti-

Figure 3. LY294002 does not inhibit recycling of MPRs from late endosomes to the TGN in vitro. Transport assays were carried out in the presence of the indicated amounts of cytosol, in the presence or absence of 100 μ M LY294002. Values represent the average of duplicates.

body. As an indicator of cathepsin D transport, we monitored the appearance of the mature 31-kDa species.

As previously reported, treatment with wortmannin effectively inhibited the maturation of cathepsin D (Figure 4). After 2 h of treatment, cells contained only 45% of the normal level of newly synthesized and processed cathepsin D. Thus, as reported previously, wortmannin interferes with the delivery of proteins to lysosomes. [It should be noted that Davidson (1995) observed an even greater block in cathepsin D processing in wortmannin-treated K562 cells than that reported here. It is important to note that lysosomal

Figure 4. Wortmannin inhibits maturation of cathepsin D. K562 cells were labeled and chased in the presence (●) or absence (○) of 2 μ M wortmannin. Values shown represent the amount of PhosphorImager radioactivity detected as the 31-kDa mature cathepsin D.

enzyme sorting is exquisitely sensitive to the precise growth conditions used; indeed, small differences in ammonia ion concentration in the media can lead to significant differences in sorting efficiency and enzyme production. Since the experiments reported here were carried out under identical conditions with identical media, these variables are less of a factor within a given set of experiments, but could easily explain differences between the two laboratories.]

DISCUSSION

At steady state, in many cell types, most MPRs are localized to a prelysosomal compartment and a smaller fraction are found in the TGN and at the cell surface (Griffiths *et al.*, 1988). Nevertheless, these pools of receptor are in constant equilibrium (Sahagian and Neufeld, 1983). Several laboratories have shown that wortmannin interferes with the delivery of newly synthesized lysosomal enzymes to lysosomes (Brown *et al.*, 1995; Davidson, 1995). Although the drug does not block MPR endocytosis, the steady-state level of surface MPRs was decreased in cells in which PI3-kinase was blocked (Brown *et al.*, 1995). Together these results led Brown *et al.* (1995) and Davidson (1995) to conclude that the export of MPRs from the TGN likely requires PI3-kinase activity.

Addition of wortmannin at >100 nM leads to the generation of large MPR-containing vacuolar structures (Brown *et al.*, 1995; Reaves *et al.*, 1996; Spiro *et al.*, 1996). The morphological appearance of these structures led Reaves *et al.* (1996) to conclude that MPRs became trapped in such structures upon drug addition. An alternative and equally plausible possibility is that the compartment occupied by most MPRs at steady-state changes its morphology upon wortmannin addition, without losing its capacity to export MPRs.

We have shown here that wortmannin, a relatively specific inhibitor of PI3-kinase, does not influence the recycling of MPRs from late endosomes to the TGN. This conclusion was based on an *in vivo* assay that monitors a combination of endocytosis and endosome to TGN transport in living cells, and also from experiments which used an *in vitro* assay that reconstitutes only the latter process. The failure of wortmannin to inhibit the *in vitro* reaction was probably not due to a decreased potency of this drug in *in vitro* reactions, because homotypic early endosome fusion is inhibited by nanomolar concentrations of wortmannin *in vitro* (Jones and Clague, 1995; Li *et al.*, 1995; Spiro *et al.*, 1996). Moreover, the processing of a newly synthesized lysosomal enzyme was inhibited significantly, consistent with previous reports (Brown *et al.*, 1995; Davidson, 1995). In addition, we used wortmannin at concentrations (2 μ M) well above the IC₅₀ for this agent (50 nM), and similar results were obtained using

the structurally unrelated PI3-kinase inhibitor LY294002.

Other laboratories have shown that wortmannin also has significant effects on the endosomal system. Wortmannin increases transferrin receptor internalization while decreasing the rate with which this receptor recycles back to the cell surface (Li *et al.*, 1995; Spiro *et al.*, 1996). The net result is a decrease in cell surface transferrin receptors, which parallels the loss of cell surface MPRs seen in wortmannin-treated cells (Brown *et al.*, 1995). The block in transferrin receptor recycling seems to be due to blocked events at the early endosome/recycling endosome compartment(s) because wortmannin is a potent inhibitor of early endosome fusion *in vitro* (Jones and Clague, 1995; Li *et al.*, 1995; Spiro *et al.*, 1996). The wortmannin-induced increase in endocytosis rates may be due to the generation of a relative excess of transport factors that normally function in both endocytosis and endosome fusion, such as the Rab5 GTPase. Other endosomal events inhibited by wortmannin include degradation of activated platelet-derived growth factor receptors (Joly *et al.*, 1994, 1995), insulin-stimulated translocation of GLUT1 and GLUT4 glucose transporters (Clark *et al.*, 1994) and transferrin receptors (Shepherd *et al.*, 1995) to the cell surface, and transcytosis (Hansen *et al.*, 1995).

The inability of wortmannin to block the transport of MPRs from late endosomes to the TGN uncovers a mechanistic distinction between events which underlie this process and events responsible for early endosome fusion and endosome to cell surface recycling. Since MPRs appear to be able to traverse from the cell surface to the TGN in the presence of wortmannin, it appears that this overall transport process does not require a wortmannin-sensitive factor. In this regard, our results are in agreement with those of Reaves *et al.* (1996) who reported that wortmannin-treated cells were perfectly competent to internalize antibodies to TGN38, and this protein, with antibodies bound, appeared to return to the TGN. It therefore seems likely that traffic from late endosomes to lysosomes and from recycling endosomes to the cell surface are both blocked by wortmannin, whereas traffic from early to late endosomes and from late endosomes to the TGN are not. This conclusion would be consistent with all of the data published from multiple laboratories to date.

Stahl and coworkers (Li *et al.*, 1995) have found that a GTP hydrolysis-defective, constitutively activated Rab5 mutant protein could overcome wortmannin inhibition, and, from this, they concluded that Rab5 acts downstream of a wortmannin-sensitive process. Transport of MPRs from late endosomes to the TGN requires the Rab9 GTPase (Lombardi *et al.*, 1994; Riederer *et al.*, 1994), but appears to be insensitive to wortmannin. Thus, despite the common requirement

for a Rab GTPase, these processes have different requirements. This is not surprising since endosome fusion is a homotypic fusion event, whereas endosome to TGN transport is not. In any case, it will be important to identify the wortmannin-sensitive component that distinguishes endosome fusion from endosome to TGN transport.

Our data strongly support the conclusion that PI3-kinase is required for the export of MPRs from the TGN, rather than export from late endosomes. Thus, as first shown by Emr and colleagues (Stack *et al.*, 1995), PI3-kinase is likely to function in the same transport events in both *S. cerevisiae* and mammalian cells.

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REFERENCES

- Arcaro, A., and Wymann, M.P. (1993). Wortmannin is a potent phosphatidylinositol kinase inhibitor: the role of PI 3,4,5 trisphosphate in neutrophil responses. *Biochem. J.* 296, 297–301.
- Brown, W.J., DeWald, D.B., Emr, S.D., Plutner, H., and Balch, W.E. (1995). Role for phosphatidyl inositol 3-kinase in the sorting and transport of newly synthesized lysosomal enzymes in mammalian cells. *J. Cell Biol.* 130, 781–796.
- Clark, J.F., Young, P.W., Yonezawa, K., Kasuga, M., and Holman, G.D. (1994). Inhibition of the translocation of GLUT1 and GLUT4 in 3T3-L1 cells by the PI 3-kinase inhibitor, wortmannin. *Biochem. J.* 300, 631–635.
- Conibear, E., and Stevens, T.H. (1995). Vacuolar biogenesis in yeast: sorting out the sorting proteins. *Cell* 83, 513–516.
- Davidson, H.W. (1995). Wortmannin causes mis-targeting of procathepsin D. Evidence for the involvement of a PI 3-kinase in vesicular transport to lysosomes. *J. Cell Biol.* 130, 797–805.
- Deutscher, S.L., Nuwayhid, N., Stanley, P., Briles E.I.B., and Hirschberg, C.B. (1984). Translocation across Golgi membranes: a CHO glycosylation mutant deficient in CMP-sialic acid transport. *Cell* 39, 295–299.
- Dirac-Svestrup, A.B., Soldati, T., Shapiro, A.D., and Pfeffer, S.R. (1994). Rab-GDI presents functional rab9 to the intracellular transport machinery and contributes selectivity to rab9 membrane recruitment. *J. Biol. Chem.* 269, 15427–15430.
- Duncan, J., and Kornfeld, S. (1988). Intracellular movement of two mannose 6-phosphate receptors: return to the Golgi apparatus. *J. Cell Biol.* 106, 617–628.
- Goda, Y., and Pfeffer, S.R. (1988) Selective recycling of the mannose 6-phosphate receptor to the trans Golgi network *in vitro*. *Cell* 55, 309–320.
- Griffiths, G., Hoflack, B., Simons, K., Mellman, I., and Kornfeld, S. (1988). The mannose 6-phosphate receptor and the biogenesis of lysosomes. *Cell* 52, 329–341.
- Hansen, S.H., Olsson, A., and Casanova, J.E. (1995). Wortmannin, an inhibitor of phosphoinositide 3-kinase, inhibits transcytosis in polarized epithelial cells. *J. Biol. Chem.* 270, 28425–28432.
- Joly, M., Kazlauskas, A., and Corvera, S. (1995). Phosphatidylinositol 3-kinase activity is required at a potendocytotic step in platelet-

derived growth factor receptor trafficking. *J. Biol. Chem.* 270, 13225–13230.

Joly, M., Kazlauskas, A., Fay, F.S., and Corvera, S. (1994). Disruption of PDGF receptor trafficking by mutation of its PI 3-kinase binding sites. *Science* 263, 684–687.

Jones, A.T., and Clague, M.J. (1995). PI 3-kinase activity is required for early endosomes fusion. *Biochem. J.* 311, 31–34.

Kornfeld, S. (1992). Mannose 6-phosphate receptors. *Annu. Rev. Biochem.* 61, 307–330.

Kornfeld, S., and Mellman, I. (1989). The biogenesis of lysosomes. *Annu. Rev. Cell Biol.* 5, 483–525.

Li, G., D'Souza-Schorey, C., Barbieri, M.A., Roberts, R.L., Klippel, A., Williams, L.T., and Stahl, P.D. (1995). Evidence for PI 3-kinase as a regulator of endocytosis via activation of Rab5. *Proc. Natl. Acad. Sci. USA* 92, 10207–10211.

Lombardi, D., Soldati, T., Riederer, M.A., Goda, Y., Zerial, M., and Pfeffer, S.R. (1993). Rab9 functions in transport between late endosomes and the trans-Golgi network. *EMBO J.* 12, 677–682.

Okada, T., Sakuma, L., Fukui, Y., Hazeki, O., and Ui, M. (1994). Blockade of chemotactic peptide-induced stimulation of neutrophils by wortmannin as a result of selective inhibition of PI 3-kinase. *J. Biol. Chem.* 269, 3564–3567.

Reaves, B.J., Bright, N.A., Mullock, B.M., and Luzio, J.P. (1996). The effect of wortmannin on the localisation of lysosomal type I integral membrane glycoproteins suggests a role of PI 3-kinase activity in regulating membrane traffic late in the endocytic pathway. *J. Cell Sci.* 109, 749–762.

Riederer, M.A., Soldati, T., Shapiro, A.D., Lin, J., and Pfeffer, S.R. (1994). Lysosome biogenesis requires mannose 6-phosphate receptor recycling from endosomes to the trans-Golgi network. *J. Cell Biol.* 125, 573–582.

Sahagian, G.G., and Neufeld, E.F. (1983). Biosynthesis and turnover of the mannose 6-phosphate receptor in cultured Chinese hamster ovary cells. *J. Biol. Chem.* 258, 7121–7128.

Shepherd, P.R., Reaves, B.J., and Davidson, H.W. (1996). PI 3-kinases and membrane traffic. *Trends Cell Biol.* 6, 92–97.

Shepherd, P.R., Soos, M.A., and Siddle, K. (1995). Inhibitors of PI 3-kinase block exocytosis but not endocytosis of transferrin receptors in 3T3-L1 adipocytes. *Biochem. Biophys. Res. Commun.* 211, 533–539.

Spiro, D.J., Boll, W., Kirchhausen, T., and Wessling-Resnick, M. (1996). Wortmannin alters the transferrin receptor endocytic pathway *in vivo* and *in vitro*. *Mol. Biol. Cell* 7, 355–367.

Stack, J.H., Herman, P.K., Schu, P.V., and Emr, S.D. (1993). A membrane associated complex containing the Vps15 protein kinase and the Vps34 PI 3-kinase is essential for protein sorting to the yeast lysosome-like vacuole. *EMBO J.* 12, 2195–2204.

Stack, J.H., Horazdovsky, B., and Emr, S.D. (1995). Receptor-mediated protein sorting to the vacuole in yeast: roles for a protein kinase, a lipid kinase and GTP binding proteins. *Annu. Rev. Cell Dev. Biol.* 11, 1–33.

Stephens, L., Cooke, F.T., Walters, R., Jackson, T.R., Volina, S., Gout, I., Waterfield, M.D., and Hawkins, P.T. (1994). Characterization of a PI specific PI 3-kinase from mammalian cells. *Curr. Biol.* 4, 203–214.

Vlahos, C.J., Matter, W.F., Hui, K.Y., and Brown, R.F. (1994) A specific inhibitor of PI 3-kinase, 2-(4-morpholinyl)-8-phenyl-4, H-1-benzopyran-4-one (LY294002). *J. Biol. Chem.* 269, 5241–5248.

Yano, H., Nakanishi, S., Kimura, K., Hanai, H., Saitoh, Y., Fukui, Y., Nonomura, Y., and Matsuda, Y. (1993). Inhibition of histamine secretion by wortmannin through the blockade of PI 3-kinase in RBL-2H3 cells. *J. Biol. Chem.* 268, 25846–25856.