Wortmannin-sensitive trafficking steps in the endocytic pathway in rat liver endothelial cells

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Liver endothelial cells (LECs) play an important homoeostatic role by removing potentially harmful macromolecules from blood. The extremely efficient endocytosis in LECs makes these cells an interesting model for the study of the involvement of phosphoinositides in the different steps of the endocytic process. In the present investigation we have studied the effect of wortmannin, an inhibitor of phosphatidylinositol kinases, on uptake, recycling and intracellular transport of ^{125}I-labelled ovalbumin, which is taken up in LECs via mannose-receptor-mediated endocytosis. Wortmannin was found to inhibit both uptake and degradation of ovalbumin. Further studies indicated that the reduced uptake via the mannose receptor was due both to a reduction of the number of surface receptors and a reduction in the rate of receptor–ligand internalization. Transport of ligand from endosomes to lysosomes was prevented, leading to increased recycling of internalized ligand. Wortmannin treatment released the Rab5 effector EEA1 from the endosomes and caused reduced size of early endosomes.

Key words: mannose receptor, PI 3-kinase, ricin.

INTRODUCTION

Liver endothelial cells (LECs) express several receptors that mediate endocytosis of blood constituents, including immune complexes, oxidized lipoproteins, connective-tissue components and various glycoproteins [1]. Studies of the uptake mediated by the mannose receptor and the scavenger receptors in LECs have indicated that the rate of internalization of receptor–ligand complexes is very high. The endocytic rate constant (K_e) for the internalization of ovalbumin (OVA) by the mannose receptor in isolated rat LECs has been estimated to be 4.12 min^{-1}, which corresponds to a half-life of $\approx$ 10 s for the surface pool of ligand–receptor complexes [2]. The high rate of formation of endocytic vesicles as well as the very efficient membrane traffic in the endocytic pathway in LECs [3,4] suggest that these cells may be particularly well equipped with molecules that control membrane traffic. Indeed, earlier studies have demonstrated that the rat LECs express very high concentrations of Rab4, Rab5 and Rab7, members of the Ras superfamily that control endocytosis and recycling [5,6]. Other proteins known to be parts of the endocytic machinery, such as clathrin and adaptor protein 2 (AP-2), are also expressed at high levels in LECs [7].

During the last few years it has become clear that 3-phosphoinositides have important roles in endocytic-pathway membrane traffic [8]. Potential sites of action of phosphoinositide 3-kinases (PI 3-kinases) in endocytic membrane traffic are between plasma membrane and endosomes [9–11], in the homotypic fusion between early endosomes [12,13], between late endosomes and lysosomes [14], and between recycling endosomes and plasma membrane [15]. An understanding of how PI 3-kinases regulate some membrane-traffic steps is now emerging. Activation of type-I PI 3-kinases by various agonists leads to a rapid (10–100-fold) increase in PtdIns(3,4)P_2 and PtdIns(3,4,5)P_3, which regulate the membrane binding and activity of effector proteins containing various pleckstrin homology domains [16–18]. On the other hand, type-III kinases produce PtdIns3P, which recruits FYVE finger proteins that regulate constitutive membrane traffic [19,20], while a newly identified phosphatidyl 3-phosphate 5-kinase further converts PtdIns3P into PtdIns(3,5)P_2, which probably activates as-yet-unidentified effectors involved in the formation of multi-vesicular bodies [21,22].

The best understood effector of phosphinositides so far is EEA1, a cytosolic protein that may associate with early endosome membranes through the combined interaction with activated Rab5 and PtdIns3P through its C-terminus [13]. Cytosolic EEA1 forms a long coiled-coil dimer [23] and, since it also contains a Rab5-binding site at its N-terminus [13], a possible scenario is that it acts by tethering two Rab5-positive membranes prior to SNARE (soluble *N*-ethylmaleimide-sensitive factor attachment protein receptor)-dependent fusion. In accordance with this model, treating cells with the PI 3-kinase inhibitor wortmannin leads to detachment of EEA1 from early endosome membranes [13,24], and inhibition of fusion of early endosomes [13,25].

Several studies investigating the involvement of PI 3-kinases in various endocytic transport steps, using different cell lines, have led to apparently conflicting results. While treatment of BHK cells with wortmannin inhibits fluid-phase endocytosis, suggesting that PI 3-kinase may be involved in the formation of endocytic vesicles [9,10], it had no effect on endocytic rates in HepG2 or K562 cells [11,15,26]. There are also discordant data regarding effects of wortmannin on endocytosis and recycling from early endosomes/sorting endosomes. In Chinese hamster ovary cells wortmannin treatment increases the internalization rate of transferrin while the recycling rate is decreased [27]. Similar studies on transferrin endocytosis in HFS cells showed no effect on either internalization or recycling rates [28].

In this study we have examined the role of PI 3-kinase in endocytosis in isolated rat LECs by studying the effects of wortmannin on several steps in the endocytic process in these cells. Treatment with 100 nM wortmannin reduced internalization of [^{125}I]TC-OVA (where TC is tyramine cellobiose) by more than 80 %. This reduction was caused both by a reduction in the number of cell-surface receptors and a decrease in the

Abbreviations used: HRP, horseradish peroxidase; LEC, liver endothelial cell; OVA, ovalbumin; PI 3-kinase, phosphoinositide 3-kinase; TC, tyramine cellobiose; AP-2, adaptor protein 2.

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internalization rate. Wortmannin treatment also perturbed transport from endosomes to lysosomes and thereby blocked degradation of ligand while at the same time causing an increase in recycling of internalized ligand.

MATERIALS AND METHODS

Experimental animals

Male Wistar rats, weighing 200–250 g, were used in all experiments. The animals were fed standard laboratory pellets *ad libitum*.

Chemicals and equipment

OVA, albumin, collagenase (type IV), yeast mannan, PMSF, *p*-nitrophenyl-*N*-acetyl-β-D-glucosaminide and wortmannin were obtained from Sigma (St. Louis, MO, U.S.A.). $Na^{125}I$ was obtained from Nycomed Amersham Isopharma (Kjeller, Norway). Iodixanol and Maxidens were obtained from Nycomed A/S (Oslo, Norway). Eagle's minimal essential medium and L-glutamine were obtained from Flow Laboratories (Irvine, Ayrshire, U.K.). Gentamicin was obtained from Gibco BRL (Uxbridge, Middx, U.K.). Human fibronectin was a gift from Dr Bård Smedsrød (Institute for Medical Biology, University of Tromsø, Norway), and TC was a gift from Dr Helge Tolleshaug (Nycomed A/S). Mannosylated BSA–fluorescein conjugate and mannosylated BSA–Texas Red conjugate were purchased from Molecular Probes (Eugene, OR, U.S.A.). Rabbit antisera against EEA1 were kindly donated by Dr Harald Stenmark (The Norwegian Radium Hospital, Montebello, Oslo, Norway). Sephadex PD-10 columns were obtained from Pharmacia LKB (Uppsala, Sweden). Vectorstain ABC kit was purchased from Vector Laboratories (Burlingame, CA, U.S.A.). All additional chemicals were of analytical grade.

Preparation of radioactive ligands

OVA was labelled with ^{125}I, with sodium hypochlorite as the oxidizing agent [29]. [^{125}I]TC-OVA was prepared by derivatization with radio-iodinated TC ([^{125}I]TC) according to Pittman and co-workers [30]. Briefly, 100 nM TC in 20 mM phosphate buffer, pH 7.4, was reacted with 1 mCi of ^{125}I in an Iodogen-coated tube for 45 min. The iodine-labelled TC was transferred to another tube containing 6 μl of 0.2 M KI and 6 μl of cyanuric chloride (2,4,6-trichloro-1,3,5-triazine; 10 mM in acetonitrile) and was incubated for 3 min. To this reaction mixture was added 1.0 mg (20 nmol) of OVA in 250 μl of 0.2 M Na_2CO_3, pH 9.5. After incubating at room temperature for 1 h, free ^{125}I or [^{125}I]TC was removed from the labelled protein by gel filtration on a Sephadex PD-10 column.

Preparation of isolated LECs

Isolated liver cells were prepared by two-step collagenase perfusion according to Seglen [31]. A non-parenchymal liver cell fraction was prepared by differential centrifugation and LECs were isolated by centrifugal elutriation (2500 rev./min, flow rate 22 ml/min) according to Blomhoff et al. [32]. For further purification, the LECs were resuspended in 20 % (w/v) Nycodenz in incubation buffer [33] (final density, 1.12 g/ml), carefully overlaid with incubation buffer containing 1 % BSA, and centrifuged for 15 min at 1450 ***g***. The LECs were collected from the interface, washed and resuspended in incubation buffer containing 1 % BSA. The yield of purified LECs was typically $(5–10) \times 10^6$ cells/g of liver (wet weight). Cell viability, as assessed by the Trypan Blue exclusion test, was routinely essentially 100 %.

For microscopy, elutriated LECs were suspended in Eagle's minimal essential medium supplemented with 2 mM L-glutamine and 50 μg/ml gentamicin and plated on to glass coverslips coated with fibronectin (1 μg/cm^2). After 1.5 h at 37 °C, non-adherent cells were removed by washing with PBS, the cultures incubated further for 30 min at 37 °C and then used immediately for experiments. The cultures were essentially free from contamination by other liver cell types.

Measurements of binding, uptake and degradation of radiolabelled ligands by LECs

Suspensions of purified LECs in incubation buffer containing 1 % BSA were preincubated at 37 °C for 15 min in the presence or absence of wortmannin, and further incubated in the presence of radiolabelled ligand. Binding studies were carried out at 4 °C: samples of cells, taken at various time points, were sedimented and washed three times by centrifugation (5 min at 250 ***g***) in incubation medium containing 1 % BSA. Cell-associated radioactivities were subsequently measured. To determine surface-bound and internalized ligand in cells incubated with [^{125}I]TC-OVA at 37 °C, samples of cells (250 μl) were first washed three times in ice-cold incubation medium by centrifugation (5 min at 250 ***g***). The washed cells were suspended in ice-cold incubation medium containing 5 mM EGTA, and subsequently sedimented for 5 min at 250 ***g***. Radioactivity associated with the sedimented cells was assumed to represent internalized ligand, whereas surface-bound ligand was calculated from the amounts of radioactivity released by the EGTA treatment. Binding of [^{125}I]TC-OVA to the mannose receptor in LECs is calcium-dependent, and bound [^{125}I]TC-OVA will therefore be released by EGTA treatment [2]. To measure uptake and degradation of [^{125}I]TC-OVA in cells incubated at 37 °C, samples of cells were first washed three times by centrifugation in incubation medium containing 5 mM EGTA, treated with ice-cold 10 % trichloroacetic acid and then sedimented by centrifugation (5 min at 250 ***g***). Acid-soluble and acid-precipitable radioactivities were measured in the cell samples.

Subcellular fractionation

LECs were incubated with [^{125}I]TC-OVA in Eagle's minimal essential medium for various time periods. The cells were washed three times at 4 °C in 0.25 M sucrose containing 10 mM Hepes and 1 mM EDTA, pH 7.25 (homogenization buffer). After washing, the cells were homogenized by sonication (MSE Soniprep 150 Ultrasonic Disintegrator) at maximum force for 4 s. Sonication disrupts the plasma membrane, but does not disrupt internal membranes to the same extent. About 70 % of the lysosomal enzyme β-acetylglucosaminidase remained sedimentable after the treatment, indicating that most of the lysosomes were not disrupted. Post-nuclear supernatants (4 ml), prepared by centrifugation for 2 min at 2000 ***g***, were layered on top of linear sucrose gradients (32 ml, density range, 1.05–1.25 g/ml) and centrifuged for 7 h at 85000 ***g*** in a Sorvall Combi-Plus centrifuge with a Beckman SW-28 rotor. Gradients were divided into 2 ml fractions by upward displacement using Maxidens as displacement fluid. Aliquots of the fractions were precipitated in the presence of 0.5 % BSA in 10 % trichloroacetic acid. Acid-soluble and acid-precipitable radioactivities were measured. β-Acetylglucosaminidase was assayed according to Barrett [34], and the density of each fraction was determined from its refractive index using the formula $d = n \times 3.41 - 3.555$ (where n = refractive index).

Detection of EEA1 by Western blotting

Endothelial cells (5×10^6 cells/ml) were treated with or without 100 nM wortmannin for 30 min and washed twice. Then the cells were homogenized as described in the previous section, and postnuclear fractions were centrifuged at 50000 ***g*** for 60 min. After protein determination, the pellets and supernatants were analysed by SDS/PAGE followed by immunoblotting with anti-EEA1 antibodies.

Preparation of specimens for fluorescence microscopy

Isolated rat endothelial cells were seeded on 10 mm-diameter fibronectin-coated coverslips for 2 h and washed four times with PBS. In some experiments cells were incubated with OVA Alexa dye (0.25 mg/ml) for 5–10 min in order to label early endosomes. The cells were washed three times with ice-cold PBS containing 10 mM EGTA and fixed with 2 % paraformaldehyde in PBS (pH 7.4) for 15 min at room temperature. Following fixation, cells were washed three times in PBS and then incubated for 30 min at room temperature with the rabbit anti-EEA1 antibody diluted 1:1000 in 0.5 % saponin in PBS. After rinsing three times with PBS, coverslips were incubated further with Alexa-dye-labelled goat anti-rabbit sera diluted 1:200 in 0.5 % saponin in PBS for 20 min at room temperature, washed three times in PBS, and mounted on glass slides in Vectashield mounting medium (Vector Laboratories, Burlingame, CA, U.S.A.). The cells were examined under a Leica confocal microscope equipped with a 100× objective, using identical settings for control and wortmannin-treated cells.

Electron microscopy

Electron microscopy was performed essentially as described elsewhere [35,36]. In short, cells preincubated with or without 100 nM wortmannin for 15 min were given a pulse of 10 mg/ml horseradish peroxidase (HRP; type VI, Sigma) for 10 min at 37 °C. The cells were then washed thrice in ice-cold 0.1 M phosphate buffer and fixed in 2.0 % glutaraldehyde in 0.1 M cacodylate buffer (pH 7.2). Subsequently they were incubated with diaminobenzidine (2.0 mg/ml) for 15 min, followed by a 45 min incubation with diaminobenzidine and 0.02 % H_2O_2. The reaction was stopped by washing with cacodylate buffer and the cells were postfixed in 1.5 % OsO_4 and processed for Epon embedding without further staining. Sections of 100–200 nm were cut on an LKB microtome and examined at 80 kV under a Phillips CM100 microscope.

Quantification of coated pits in cultured sinusoidal endothelial cells

LECs grown in Costar dishes were fixed with 2.0 % glutaraldehyde in 0.1 M cacodylate buffer (pH 7.2). Postfixation was performed with 2 % OsO_4 in the same buffer, followed by an overnight staining with 0.5 % tannic acid (dissolved in water). Cells were then dehydrated and embedded in Epon. Sections (60–70 nm thick) were cut vertically to the substratum. Micrographs were taken at a primary magnification of ×6600 under a Philips CM100 microscope at 80 kV. Counting of coated pits was performed directly on enlarged negatives at a final magnification of ×30000. Only coated pits in direct connection with the plasma membrane were scored.

RESULTS

Effects of wortmannin on mannose-receptor-mediated endocytosis of [^{125}I]TC-OVA

Uptake and degradation of [^{125}I]TC-OVA in suspensions of LECs incubated in the absence and presence of increasing concentrations of wortmannin are shown in Figure 1(A). Wortmannin inhibited both uptake and degradation of the labelled ligand, with half-maximal inhibition at 15–20 nM. The reduced degradation of [^{125}I]TC-OVA in the presence of wortmannin was not just an indirect effect of its reduced uptake, as the amount of acid-soluble degradation products, measured as a percentage of cell-associated radioactivity, decreased with increasing wortmannin concentration.

Wortmannin reduces both the surface pool of surface mannose receptors and the rate of ligand internalization

The reduced rate of uptake of [^{125}I]TC-OVA in the presence of wortmannin could be caused by a reduced rate of internalization of the receptor–ligand complex and/or the receptor could be

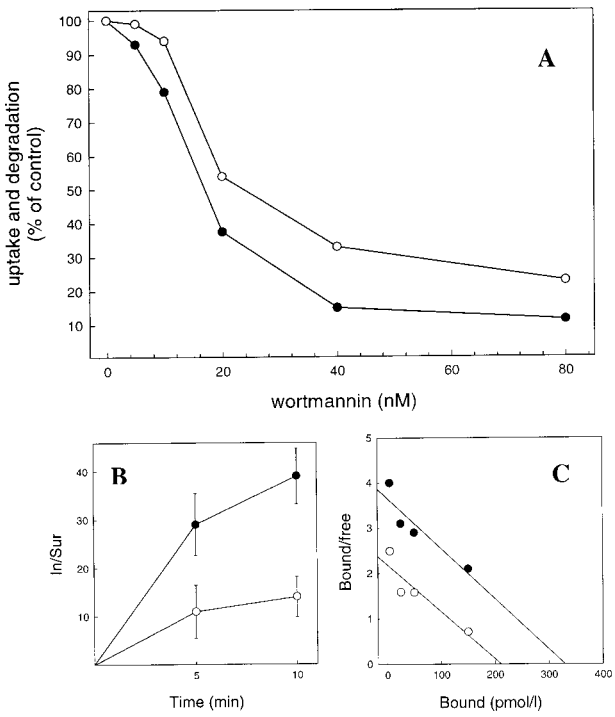

Figure 1 Effects of wortmannin on mannose-receptor-mediated endocytosis and degradation of OVA

(**A**) Effects of wortmannin on uptake and degradation of [^{125}I]TC-OVA: LECs (2.5×10^6/ml) were preincubated at 37 °C for 15 min in incubation medium containing 1 % BSA with the indicated concentrations of wortmannin. Then, 40 min after addition of 12 nM [^{125}I]TC-OVA, cell samples (250 μl) were taken from the flasks and washed in incubation medium containing 1 % BSA and 5 mM EGTA, and acid-precipitable (●) and acid-soluble (○) radioactivities were assayed as described in the Materials and methods section. Uptake is presented as the percentage of cell-associated radioactivity in control cells. Degradation of [^{125}I]TC-OVA was measured as the proportion of cell-associated radioactivity that was acid soluble, and the proportions of degraded (acid-soluble) ligand in the different cell samples are presented as percentages of the control values. Results from a representative experiment are shown. Each point represents the mean of duplicate assays. (**B**) Effect of wortmannin on the ratio between internalized and surface-bound ligand (In/Sur) for internalization of OVA. LECs (2.5×10^6/ml) were preincubated for 15 min at 37 °C in the absence (●) or presence (○) of 100 nM wortmannin. Upon addition of 5 nM [^{125}I]TC-OVA, cell samples were taken at the indicated time points, and internalized and surface-bound radioactivities were measured as described in the Materials and methods section. Each point represents the mean ± S.E.M. from triplicate assays. (**C**) Wortmannin reduces surface binding of [^{125}I]TC-OVA. LECs (2.5×10^6/ml), preincubated for 15 min at 37 °C in the absence (●) or presence (○) of 100 nM wortmannin, were further incubated for 120 min at 4 °C in incubation buffer containing 1 % BSA with increasing concentrations of [^{125}I]TC-OVA. Binding was assayed as described in the Materials and methods section and is presented as a Scatchard plot. Each point represents the mean ± S.E.M. from triplicate assays.

Table 1 Effect of wortmannin on the number of coated pits in LECs

LECs were incubated with 100 nM wortmannin for 30 min and prepared for electron microscopy as described in the Materials and methods section. Coated pits were identified morphologically and counted only when in direct connection with the plasma membrane. Each value represents the mean ± S.E.M. from the number of micrographs indicated. The results of two experiments were combined.

Treatment	Σ coated pits	Total membrane length (μm)	Coated pits/μm of membrane	Number of micrographs
Control	79	615	0.114 ± 0.022	24
Wortmannin (100 nM)	69	944	0.064 ± 0.019[a]	29

[a] $P < 0.05$ versus control according to Student's t test.

sequestered intracellularly, leading to a reduced number of surface receptors available for binding. To determine whether the pool of surface receptors and/or the rate of internalization of receptor–ligand complexes were altered by wortmannin treatment, two sets of experiments were performed. First, surface-bound and internalized ligand was measured in cells incubated at 37 °C with [^{125}I]TC-OVA in the presence and absence of wortmannin. Secondly, the number of active surface mannose receptors was measured by Scatchard analysis. Figure 1(B) shows the ratio between internalized and surface-bound ligand at 5 and 10 min following the addition of [^{125}I]TC-OVA to cells incubated in the absence and presence of wortmannin (100 nM). These data indicate that the rate of internalization of receptor–ligand complexes is lowered by wortmannin. Furthermore, the number of active surface receptors was reduced by approx. 50 %, as indicated by the Scatchard analysis of [^{125}I]TC-OVA binding at 4 °C shown in Figure 1(C). This reduction in surface receptors represents a redistribution to an intracellular compartment, since the total cellular binding capacity of [^{125}I]TC-OVA measured in digitonin-treated cells was not altered by wortmannin (results not shown). Compatible with the lowered number of surface receptors, the number of coated pits on the cell surface (assessed by counting of coated pits on electron micrographs) was also reduced by approx. 50 % following treatment with 100 nM wortmannin (Table 1). The present results indicate that the reduced uptake of [^{125}I]TC-OVA in wortmannin-treated cells is due both to a lowered number of surface mannose receptors and to a reduced rate of internalization of the ligand–receptor complexes.

Wortmannin inhibits transport of [^{125}I]TC-OVA from endosomes to lysosomes

To determine whether the reduced degradation of [^{125}I]TC-OVA in the presence of wortmannin was due to a block in transport between endosomes and lysosomes, postnuclear fractions from control and wortmannin-treated cells were subjected to subcellular fractionation on sucrose gradients at increasing times after addition of [^{125}I]TC-OVA. The density distributions of acid-precipitable and acid-soluble radioactivities as well as the activity of β-acetylglucosaminidase for control and wortmannin-treated cells are shown in Figure 2. The results demonstrate that wortmannin inhibits transport from endosomes to lysosomes. After 2 h of incubation of cells with [^{125}I]TC-OVA the bulk of the radioactivity in control cells had reached the lysosomes and was present as acid-soluble radioactivity. In the wortmannin-treated cells, on the other hand, most of the labelled ligand appears in the gradient at a position typical of endosomes. SDS/PAGE and Western blotting of fractions showed, in accordance with earlier data [4], that the distribution of the labelled ligand coincided with that of Rab5. We observed a small but consistent increase in the buoyant density of the lysosomes in the LECs. This is in contrast with reports indicating that wortmannin reduces lysosomal density [14].

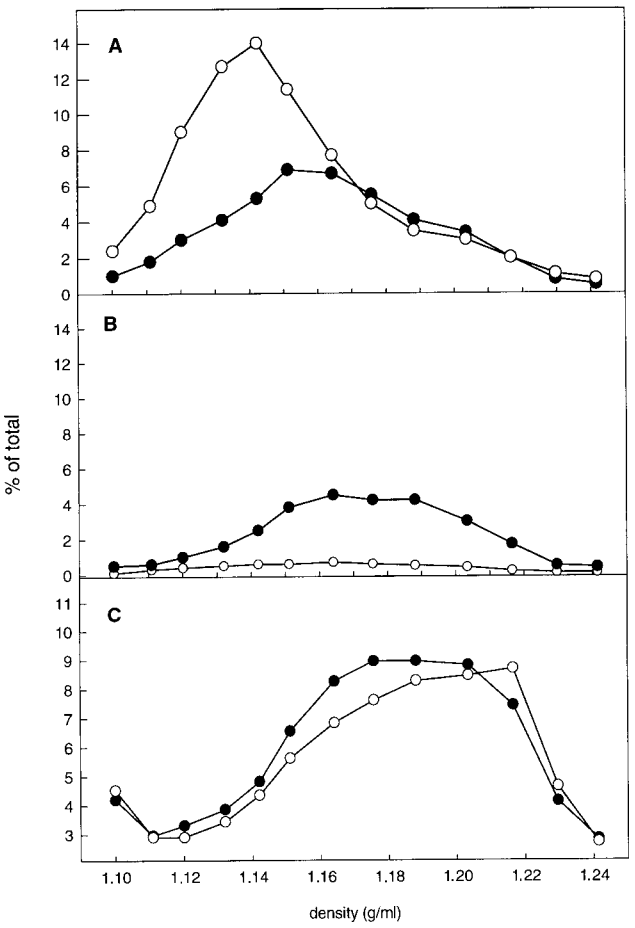

Figure 2 Effect of wortmannin on the intracellular transport of [^{125}I]TC-OVA

LECs (2.0×10^6/ml) were preincubated for 15 min at 37 °C in the absence (●) or presence (○) of 100 nM wortmannin, pulsed with 5 nM [^{125}I]TC-OVA for 15 min, washed three times in incubation medium and incubated further for 45 min in the presence or absence of 100 nM wortmannin. The cells were homogenized, and post-nuclear supernatants prepared and fractionated following centrifugation in sucrose gradients as described in the Materials and methods section. Acid-precipitable (**A**) and acid-soluble (**B**) radioactivities were measured in each fraction and expressed as the percentage of total activity in the gradient. (**C**) Density distribution of the lysosomal enzyme β-acetylglucosaminidase. The recoveries of activities in the gradients were about 80% of the total activities placed on top of the gradients.

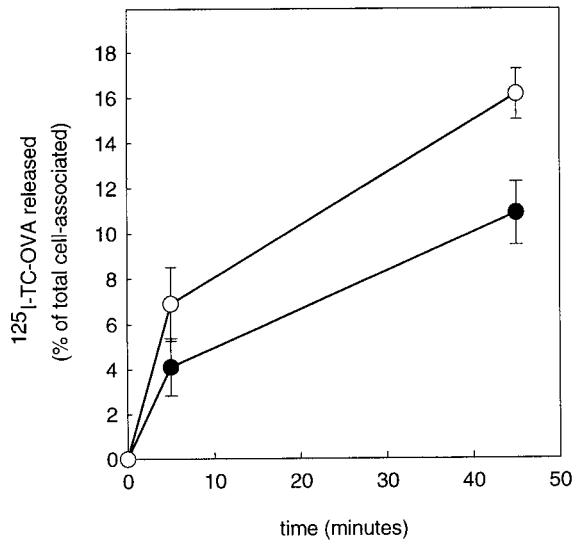

Figure 3 Effect of wortmannin on recycling of [^{125}I]TC-OVA

LECs (2.0×10^6/ml), preincubated for 15 min at 37 °C in the absence (●) or presence (○) of 100 nM wortmannin, were pulsed with 5 nM [^{125}I]TC-OVA for 10 min, washed three times in ice-cold incubation buffer containing 1% albumin, and incubated further in the absence or presence of 5 mM EGTA. Samples were removed at the indicated time points. Acid-precipitable [^{125}I]TC-OVA released by EGTA was assayed and is expressed as a percentage of the amount of cell-associated [^{125}I]TC-OVA at the beginning of the re-incubation period. Each point represents the mean ± S.E.M. from triplicate assays.

Wortmannin increases recycling of ligand from early endosomes

Since transport of ligand to lysosomes was impaired by wortmannin we wanted to examine whether this treatment would lead to increased recycling of ligand from endosomes. It has been shown [37] that a considerable amount of ligand internalized via the mannose receptor in LECs recycles back to the cell surface in association with the receptor. To measure recycling of ligand in association with the mannose receptor from early endosomes, cells that had been given a 10 min pulse of [^{125}I]TC-OVA were washed and re-incubated without ligand in the presence of EGTA. Under these conditions recycling ligand will dissociate from the receptor since binding of ligand is calcium-dependent. To assess the effect of wortmannin on the recycling of ligand the drug was added during the pulse period as well as during the chase period in the presence of EGTA. Figure 3 indicates that wortmannin enhances the release of [^{125}I]TC-OVA. Thus, after 5 min of chase the wortmannin-treated cells released about twice as much internalized ligand as did the control cells. Wortmannin treatment therefore seems to cause increased recycling of ligand from endosomes.

Wortmannin releases EEA1 from endosomal membranes and reduces the size of early endosomes

It has been shown that EEA1 is a Rab5 effector that is required for endosome fusion. Since EEA1 binds to PtdIns3*P* we con-

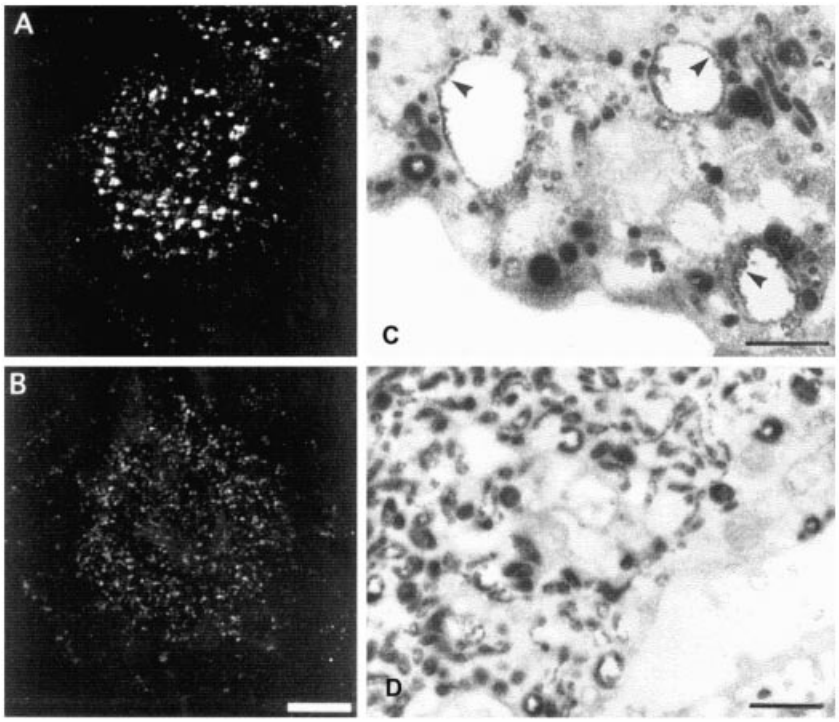

Figure 4 Effect of wortmannin on the intracellular distribution of EEA1

(**A**, **B**) Confocal immunofluorescence micrographs showing the intracellular localization of EEA1. Cultured endothelial cells were incubated for 30 min at 37 °C in the absence (**A**) or presence (**B**) of 100 nM wortmannin, fixed with 3% paraformaldehyde and permeabilized with saponin. EEA1 was visualized by incubation with anti-EEA1 antiserum followed by Alexa-dye-labelled goat anti-rabbit secondary antibody as described in the Materials and methods section. Scale bar, 10 μm. (**C**, **D**) Effects of wortmannin on endosome morphology. Electron micrographs showing endosome morphology of control (**C**) and wortmannin-treated (**D**) cells. The cells were pulsed with HRP for 10 min and prepared for electron microscopy as described in the Materials and methods section. Scale bars, 500 nm.

sidered the possibility that the inhibitory effect of wortmannin on the intracellular transport of endosomes could be a consequence of reduced binding of EEA1 to endosomal membranes. To determine whether wortmannin interferes with EEA1 binding to endosomal membranes and/or affects the morphology of endosomes, we performed immunofluorescence studies in which EEA1 was visualized in cultured LECs. Control cells showed a punctuate pattern with bright staining in rather large structures as well as in smaller spots spread throughout the cytoplasm (Figure 4A). In contrast, cells treated with 100 nM wortmannin for 30 min showed a weaker and more dispersed labelling, suggesting reduced association of EEA1 with endosomal membranes and a reduced size of the early endosomes (Figure 4B). The notion that wortmannin causes dissociation of EEA1 from endosomal membranes was supported by the finding that less EEA1 was membrane-bound (and sedimentable by centrifugation at 50 000 ***g***) following treatment of cells with the drug (100 nM) for 30 min (results not shown).

In order to visualize the effects of wortmannin on early endosomes at the ultrastructural level, control and wortmannin-treated cells were incubated with HRP (10 mg/ml) for 10 min and prepared for electron microscopy as described in the Materials and methods section. Wortmannin treatment had a marked effect on the morphology of the HRP-labelled early endosomes. Whereas in control cells the diaminobenzidine reaction product was found at the rim of large endosomes as well as in numerous small tubular and vesicular structures spread throughout the cytoplasm (Figure 4C, arrowheads), most tracer in the wortmannin-treated cells was distributed in small tubular and vesicular structures (Figure 4D). Large endosomes were found only rarely.

DISCUSSION

The present data show that wortmannin, at relatively low concentrations, reduces internalization, increases recycling and blocks transport from early endosomes to degradative compartments of a ligand taken up in LECs by mannose-receptor-mediated, clathrin-dependent, endocytosis. The drug also causes a redistribution of mannose receptors such that fewer receptors are expressed at the cell surface.

Wortmannin inhibited uptake of mannose-receptor ligands both by lowering the number of surface receptors and by reducing the rate of receptor–ligand internalization. The inhibition of mannose-receptor-mediated endocytosis by wortmannin supports the notion that effectors of wortmannin-sensitive PI kinases are involved in clathrin-dependent endocytosis. Two potential effectors are AP-2 and dynamin [38,39]. Dynamin contains a pleckstrin homology domain that binds phosphoinositides [38,40,41], and brings about scission at the neck of clathrin-coated invaginations at the plasma membrane [42,43]. However, dynamin preferentially binds to PtdIns(4,5)P_2, a product of PI 4-kinase that has been reported to be reduced at higher wortmannin concentrations than those used in our experiments [44]. The clathrin adaptor AP-2 is present in high concentrations in LECs [7]. It recognizes the cytoplasmic tails of many membrane receptors, including the mannose receptor, and through its interaction with clathrin serves to recruit the receptors to clathrin-coated pits at the plasma membrane [45,46]. It has been shown that the interaction between the μ2 chain of AP-2 and a peptide mimicking an endocytic receptor tail is enhanced in the presence of PtdIns3P, suggesting that this interaction increases its affinity for receptor tails [47]. It is therefore likely that the observed inhibition of receptor-mediated endocytosis was caused either by impaired recruitment of receptors to clathrin-coated pits or that the overall process of coated-pit formation was inhibited.

The observed reduction of cell-surface receptors can only be explained if the recycling rate of receptors back to the cell surface is reduced even more than the internalization rate by wortmannin treatment. On the other hand, it was found that the proportion of internalized ligand recycled to the cell surface was increased in wortmannin-treated cells. An obvious explanation of this apparent paradox is that more ligand is available for recycling from early endosomes in the wortmannin-treated cells, because the transport of ligand further along the endocytic pathway is blocked, as indicated by the subcellular fractionation experiments. The finding that transport of ligand from endosomes to lysosomes was inhibited by wortmannin also explains why wortmannin reduces degradation of endocytosed ligand. A comparison of the dose–response curves of the effects of wortmannin on internalization and degradation of ligand shows that degradation is nearly as sensitive to the drug as is internalization. The effects of wortmannin on the late endocytic compartments seen in this study are compatible with results obtained in other studies [26,27,48,49] indicating a role for PI 3-kinase in the transport of endocytosed material from endosomes to lysosomes.

In normal rat kidney fibroblasts, terminal dense lysosomes have been shown to fuse with late endosomes and to re-form from the resultant hybrid organelles [14]. While the re-formation of the terminal dense lysosomes is blocked by wortmannin the initial fusion is unaffected, leading to an accumulation of hybrid organelles and a gradual disappearance of the dense lysosomes [14]. In accordance with these observations we have found a similar disappearance of dense lysosomes upon wortmannin treatment in rat hepatocytes (S. A. Mousavi and A. Brech, unpublished work). Wortmannin seems to act at a different transport step in LECs. First, subcellular fractionation experiments showed that the distribution of lysosomal enzymes was not shifted to lower densities, as would be expected if accumulation of a hybrid organelle took place. Secondly, degradation of ligand was almost totally blocked, indicating that endocytosed ligand did not reach degradative compartments.

The mechanisms whereby wortmannin inhibits transport from early endosomes to lysosomes (or fusion between endosomes and lysosomes) are not known, but the high sensitivity of these transport step(s) to wortmannin in LECs suggests that the enzymes affected that lead to the block are PI 3-kinases, possibly PI 3-kinase type III. Wortmannin induced the reduction in size of endosomes in LECs and has been shown in other cell types to inhibit homotypic fusion between early endosomes [12]. Homotypic fusion between endosomes is likely to be a prerequisite for the formation of multivesicular bodies, and, indeed, Neefjes and co-workers have demonstrated that this process is wortmannin-sensitive [48]. Wortmannin may also affect other, not yet well characterized FYVE-finger-containing proteins, some of which may function at a later stage in the endocytic pathway [19,20,50,51].

In conclusion, we have evaluated the effects of the PI 3-kinase inhibitor wortmannin on various stages of the endocytic pathway in LECs. Wortmannin affected three distinct steps in the endocytic pathway: receptor–ligand internalization, recycling and transit from endosomes to degradative compartments. These results fit into a pattern showing that these enzymes are crucial regulators of membrane interactions at several sites in the cell.

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