Phosphoinositide 3-kinase regulates maturation of lysosomes in rat hepatocytes

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To obtain information about the role of phosphoinositide 3-kinase (PI3K) in the endocytic pathway in hepatocytes, the uptake and intracellular transport of asialo-orosomucoid (ASOR) was followed in cells treated with wortmannin or LY294002. The two inhibitors, at concentrations known to inhibit the enzyme, did not affect internalization or the number of surface asialoglycoprotein receptors, but they caused a paradoxical increase (approx. 50 % above control values) in the degradation of ASOR labelled with [¹²⁵I]tyramine cellobiose ([¹²⁵I]TC). Wortmannin or LY294002 inhibited the autophagic sequestration of lactate dehydrogenase very effectively, and the enhanced degradation of [¹²⁵I]TC-ASOR could be an indirect effect of reduced autophagy, as an amino acid mixture known to inhibit autophagy also caused increased degradation of [¹²⁵I]TC-ASOR, and its effect was not additive to that of wortmannin or LY294002. Wortmannin or LY294002 had pronounced effects on the late parts of the endocytic pathway in the hepatocytes: first, dense lysosomes disappeared and were replaced by swollen vesicles; secondly,

degradation of [¹²⁵I]TC-ASOR took place in an organelle of lower buoyant density (in a sucrose gradient) than the bulk of lysosomes (identified in the gradient by lysosomal marker enzymes). With increasing length of incubation with wortmannin or LY294002, the density distributions of the lysosomal markers also shifted to lower density and gradually approached that of the labelled degradation products. The labelled degradation products formed from [¹²⁵I]TC-labelled proteins were trapped at the site of formation, because they did not penetrate the vesicle membranes. The results obtained indicate that internalization and intracellular transport of ASOR to lysosomes may take place in the absence of PI3K activity in rat hepatocytes. On the other hand, fusion of late endosomes with lysosomes seems to produce ‘hybrid organelles’ (active lysosomes) that are unable to mature into dense lysosomes.

Key words: asialo-orosomucoid (ASOR), endocytosis, hybrid organelle, multivesicular body, phosphoinositide 3-kinase (PI3K), wortmannin.

INTRODUCTION

Mammalian phosphoinositide 3-kinases (PI3Ks) constitute a family of enzymes whose products are involved in various cellular functions, including the regulation of membrane traffic in the endocytic pathway [1,2]. They can be divided on the basis of their structure and substrate preference into three classes.

PI3Ks belonging to class I link surface-receptor activation to the conversion of the plasma membrane PtdIns(4,5)P₂ into PtdIns(3,4,5)P₃ [3], which in turn elicits a wide range of responses by activating various signalling pathways. Class II PI3Ks use both PtdIns and PtdIns4P as substrates. The class III PI3K hVPS34 produces PtdIns3P through the phosphorylation of PtdIns [4]. The PtdIns3P levels are comparable in resting and stimulated cells [1], suggesting that hVPS34 has a housekeeping role. These enzymes are inhibited by low concentrations of wortmannin [5] and LY294002 [6], and the use of these inhibitors has revealed that 3′-phosphorylated phosphoinositides are involved in several membrane-trafficking pathways: sorting of lysosomal enzymes from the Golgi complex to endosomes [7–10], the transfer of GLUT4 glucose transporters to the plasma membrane [11,12], early endosomal fusion events [13,14] and autophagic sequestration of cytosol [15]. PtdIns3P has also been shown to be a substrate for PtdIns3P 5-kinase to generate PtdIns(3,5)P₂, which has been implicated in the formation of multivesicular bodies (MVBs) [16,17]. Effectors of PtdIns3P are proteins with FYVE

zinc-finger domains. Several FYVE-finger-containing proteins have been identified, including Fab1p, a PtdIns3P 5-kinase that converts PtdIns3P into PtdIns(3,5)P₂, and early endosome antigen 1 (EEA1), which is involved in homotypic early endosomal fusion, and is recruited to early endosomes by Rab5-GTP and PtdIns3P [18].

The purpose of the present study was to obtain information about the role of PI3Ks in the endocytic pathway in rat hepatocytes, using wortmannin and LY294002 as inhibitors of the enzyme. We have, in an earlier study, found that wortmannin inhibits both uptake and degradation of ligands internalized by receptor-mediated endocytosis in rat liver endothelial cells [19]. The effect on degradation was found to be due to a blockade of transport of ligand from endosomes to lysosomes. The effects of wortmannin and LY294002 on endocytosis of asialo-orosomucoid (ASOR) in hepatocytes differed qualitatively from those observed in the liver endothelial cells. First, the inhibitors, at concentrations that effectively inhibit endocytic uptake of ovalbumin {labelled with [¹²⁵I]tyramine cellobiose ([¹²⁵I]TC)} in liver endothelial cells, did not affect the rate of internalization of [¹²⁵I]TC-ASOR. Secondly, wortmannin, at relatively low concentrations, caused an unexpected increase in degradation of endocytosed [¹²⁵I]TC-ASOR. Using subcellular fractionation together with electron microscopy, we found that degradation of the ligand in wortmannin-treated cells took place in a swollen organelle of low density (in sucrose gradients). We discuss the

Abbreviations used: ASOR, asialo-orosomucoid; β -AGA, β -acetylglucosaminidase; EEA1, early endosome antigen 1; EGFR, epidermal growth factor response; LDH, lactate dehydrogenase; MVB, multivesicular body; PI3K, phosphoinositide 3-kinase; PNS, postnuclear supernatant; TC, tyramine cellobiose.

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possibility that wortmannin does not prevent fusion between late endosomes and lysosomes (and the formation of a 'hybrid organelle'), but inhibits the maturation events that result in the reformation of dense lysosomes.

MATERIALS AND METHODS

Biochemicals

Collagenase, LY294002 and wortmannin were obtained from Sigma Chemical Co. (St. Louis, MO, U.S.A.). Na¹²⁵I was from Amersham Biosciences (Little Chalfont, Bucks., U.K.). All other chemicals were of analytical grade. ASOR labelled with [¹²⁵I]TC, prepared as described in [20], was used as a marker for clathrin-dependent endocytosis. A complete physiologically balanced mixture of amino acids was prepared as described by Kovács et al. [21].

Cell preparation and incubation

Hepatocytes were prepared from 18-h-starved male Wistar rats (250–300 g) by collagenase perfusion [22]. The cells were incubated as suspensions (4 ml aliquots with shaking at 37 °C, usually 50–75 mg wet weight/ml) in incubation buffer (20 mM Hepes, pH 7.5, 145 mM NaCl, 5.4 mM KCl, 0.33 mM Na₂HPO₄, 0.34 mM KH₂PO₄, 0.8 mM MgSO₄ and 2 mM CaCl₂). Dose-response experiments showed that wortmannin at low concentrations (≤ 200 nM) induced enhanced degradation and density shift of endocytic vesicles. After prolonged incubation, the wortmannin-induced density change was reversed because the drug is labile in aquatic milieu. In experiments where the cells were subjected to prolonged incubation, we therefore routinely added 100 nM wortmannin initially and then added 50 nM wortmannin every 30 min to maintain the concentration of the drug.

Enzyme assays

β-Acetylglucosaminidase (β-AGA) activity was determined as described by Barrett [23].

Determination of radioactivity

Radioactivity was measured in a Kontron gamma counter. Degradation of [¹²⁵I]TC-ASOR was followed by measuring the radioactivity soluble in 10 % (w/v) trichloroacetic acid (acid-soluble radioactivity). BSA (0.5 %) was added as a carrier.

Subcellular fractionation

Cell suspensions in homogenization buffer (10 mM Hepes, pH 7.2, 0.25 M sucrose and 1 mM EDTA) were homogenized by passing through a 25-gauge needle 6–8 times. The homogenates were fractionated by isopycnic centrifugation in sucrose gradients. A postnuclear supernatant (PNS) fraction was prepared by centrifuging a 5 ml sample of the homogenate at 2000 g for 2 min, the resulting nuclear fraction (N fraction) was resuspended in 5 ml of homogenization buffer and centrifuged again at 2000 g for 2 min. Homogenization and centrifugation were carried out at 0–4 °C. In isopycnic centrifugation experiments, 4 ml aliquots of the PNS fraction were initially layered on top of preformed 21–54 % (w/w) sucrose gradients and centrifuged at 85 000 g in a Beckman SW 28 rotor at 4 °C for 5.5 h. The

gradients were divided into 18 fractions of 2 ml by upward displacement using Maxidens (Amersham Biosciences) as displacement fluid. The densities of the fractions were calculated from the refractive indices.

Degradation of [¹²⁵I]TC-ASOR in a cell-free system

To determine whether or not lysosomes from wortmannin-treated cells degraded endocytosed [¹²⁵I]TC-ASOR at a higher rate than control lysosomes, cells that had been incubated with [¹²⁵I]TC-ASOR in the presence or absence of wortmannin for 10 min at 37 °C were washed and re-incubated with or without wortmannin for 30 min to allow the ligand to enter lysosomes. Lysosome-containing fractions were then prepared by centrifuging a PNS fraction for 25 min at 10 000 g. The fractions were resuspended in homogenization buffer in the presence or absence of an ATP-regenerating system consisting of 1.8 mM ATP, 14.7 mM creatine phosphate and 73.3 µg/ml creatine phosphokinase. Degradation of [¹²⁵I]TC-ASOR was followed by measuring the radioactivity soluble in 10 % (w/v) trichloroacetic acid.

Electron microscopy

Electron microscopy was performed essentially as follows: hepatocytes were given a pulse of ASOR-coated 20 nm gold particles ($A_{520} = 5$, final concentration) for 270 min at 37 °C in order to label lysosomes. The cells were then washed and re-incubated in ligand-free medium with or without wortmannin. At various time points, samples were taken and processed for electron microscopy. The cells were washed three times in ice-cold 0.1 M phosphate buffer (14.96 g Na₂HPO₄ · 2H₂O and 2.21 g NaH₂PO₄ · H₂O in 1 litre of water, pH 7.2), fixed in 2.0 % (w/v) glutaraldehyde in 0.1 M cacodylate buffer (21.4 g C₂H₆AsNaO₂ · 3H₂O in 1 litre of water, pH 7.2), followed by postfixation in 2 % (w/v) OsO₄ and processed for Epon embedding. Sections of 60–90 nm were cut on a LKB microtome, stained with uranyl acetate and lead citrate, and examined at 80 kV in a Phillips CM100 microscope.

Measurement of autophagic sequestration of lactate dehydrogenase (LDH)

Autophagic sequestration of LDH was measured as described previously in [24], using the percentage of total cellular LDH present in cell corpses as the basic measurement parameter.

RESULTS

Effects of wortmannin on uptake and degradation of [¹²⁵I]TC-ASOR

Wortmannin in low concentrations does not inhibit the uptake of [¹²⁵I]TC-ASOR

In initial experiments, we determined the dose dependency of wortmannin on the endocytic uptake and degradation of [¹²⁵I]TC-ASOR. The results are shown in Figures 1(A) and 1(C). Wortmannin at concentrations > 10 µM had only minor effects on the uptake of [¹²⁵I]TC-ASOR. At 30 µM wortmannin, the uptake was reduced by approx. 40 %.

Wortmannin enhances degradation of [¹²⁵I]TC-ASOR in hepatocytes

Whereas uptake of [¹²⁵I]TC-ASOR was unaffected by wortmannin at low concentrations, the drug unexpectedly enhanced

Figure 1 Effects of wortmannin on uptake, surface binding and degradation of [^{125}I]TC-ASOR, and on autophagic sequestration of LDH

(A) and (C) Suspensions of hepatocytes ($3 \times 10^6/\text{ml}$) were pre-incubated with increasing concentrations of wortmannin for 15 min at 37°C , and were then incubated further at 37°C with saturating concentrations of [^{125}I]TC-ASOR ($0.1 \mu\text{M}$). Uptake of ligand was determined in cell aliquots taken after 15 min, whereas degradation of ligand was measured as acid-soluble radioactivity in cell aliquots taken after 60 min. Each point is the mean $\pm$ S.D. of four different experiments. For the experiment shown in (C), cell-associated radioactivity and degraded [^{125}I]TC-ASOR were measured after 1 h of incubation at 37°C . Degradation products in the cells were calculated as the proportion of total cell-associated radioactivity and the results obtained for wortmannin-treated cells are presented as percentage of control values. Uptake (B) and degradation (D) of [^{125}I]TC-ASOR in control cells (filled circles) and cells treated with $0.1 \mu\text{M}$ wortmannin (open circles) are presented as a function of time. Suspensions of hepatocytes ($3 \times 10^6/\text{ml}$) were pre-incubated with wortmannin for 15 min at 37°C , and were then incubated further at 37°C with saturating concentrations of [^{125}I]TC-ASOR (100 nM). (E) Effect of wortmannin on the autophagic sequestration of LDH in hepatocytes. Hepatocytes were incubated for 90 min with increasing concentrations of wortmannin. The activity of LDH in sedimentable cell corpses was measured and is expressed as a percentage of the total cell-associated activity. Each point is the mean $\pm$ S.D. of triplicate samples from a single typical experiment. (F) Effects of wortmannin on the binding of [^{125}I]TC-ASOR. Suspensions of hepatocytes were pre-incubated with increasing concentrations of wortmannin for 30 min at 37°C . The cells were then incubated at 4°C in the presence of 5 nM [^{125}I]TC-ASOR. Cell-associated radioactivity was determined in triplicate samples after 2 h of incubation. Results are means $\pm$ S.D. of five experiments, expressed as percentages of control values at each time point.

degradation of the ligand at concentrations between 20 nM and $1 \mu\text{M}$ (Figure 1C). At concentrations $> 10 \mu\text{M}$, wortmannin inhibited degradation. LY294002 (at concentrations ranging from 10 to $100 \mu\text{M}$) caused increased degradation of endocytosed [^{125}I]TC-ASOR in a similar dose-dependent manner to wortmannin (results not shown). As shown in Figure 1(C), cell-associated radioactivity and degraded [^{125}I]TC-ASOR were measured after incubation for 1 h at 37°C . Degradation products in the cells were calculated as the proportion of total cell-associated radioactivity and the results obtained for the wortmannin-treated cells are presented as percentages of control values. In Figure 1, uptake (Figure 1B) and degradation (Figure 1D) of [^{125}I]TC-ASOR in control cells (filled circles) and cells treated with $0.1 \mu\text{M}$ wortmannin (open circles) are presented as a function of time. The results show that degradation starts simultaneously in control cells and wortmannin-treated cells, approx. 10–15 min after initiation of uptake.

High concentrations of wortmannin increase the number of surface asialoglycoprotein receptors

Figure 1(F) shows binding of [^{125}I]TC-ASOR (5 nM) at 4°C to cells that had been pre-incubated at 37°C with or without increasing concentrations of wortmannin for 15 min. The results show that binding of the ligand decreased slightly at low wortmannin concentrations, but increased in cells that were incubated at high concentrations of the drug. To determine the number of surface asialoglycoprotein receptors following wortmannin treatment, Scatchard analysis was employed. The results obtained showed that the number of surface receptors/cell at $20 \mu\text{M}$ wortmannin was $(2.8 \pm 0.3) \times 10^5$, compared with $(1.8 \pm 0.2) \times 10^5$ in control cells. The affinity values, expressed as K_d values, were roughly equal for treated and control cells: $(2.1 \pm 0.2) \times 10^{-8} \text{ M}$ and $(1.8 \pm 0.2) \times 10^{-8} \text{ M}$ respectively. The explanation of the rise in the number of surface receptors

may be that the rate of receptor internalization is diminished, whereas recycling remains at control values (results not shown). Wortmannin at high concentrations may possibly interfere with the basal activity of PI3K C2 α , which is necessary for normal coated vesicle cycle [25].

Does wortmannin enhance the degradation of [125 I]TC-ASOR by inhibiting autophagy in hepatocytes?

Both wortmannin and LY294002 in relatively low concentrations were found to inhibit autophagy in rat hepatocytes. Figure 1(E) shows a dose-response curve for the effect of wortmannin on the autophagic sequestration of LDH in suspensions of rat hepatocytes. Half maximal effect was seen at 50 nM wortmannin. These observations are in agreement with those in [15]. The wortmannin-induced increase in degradation of [125 I]TC-ASOR could conceivably be due to a decreased input of autophaged substrate to the lysosomes. To test this possibility, we examined the effect of a mixture of amino acids, known to inhibit autophagic sequestration [26,27], on the degradation of [125 I]TC-ASOR. Inclusion of amino acids in the incubation medium increased degradation to roughly the same extent as that achieved using wortmannin or LY294002. Treatment of cells with a combination of amino acids and wortmannin had the same effect as amino acids alone (results not shown). These observations may suggest that wortmannin enhances [125 I]TC-ASOR degradation indirectly by preventing autophagic influx of substrates into the lysosomes. An alternative explanation of the enhanced degradation in the presence of wortmannin could possibly be that [125 I]TC-ASOR reaches the degradative compartment at a higher rate in these cells. This does not, however, seem to be the case, as seen in Figure 1(D).

Wortmannin enhances the degradation of [125 I]TC-ASOR in a cell-free preparation

If wortmannin enhances the degradation of endocytosed [125 I]TC-ASOR by reducing the input of autophaged material into lysosomes, then the degradation of the ligand in lysosomal fractions from wortmannin-treated cells should also be more effective than in control fractions. To test whether or not wortmannin influences the degradation of [125 I]TC-ASOR in an *in vitro* system, control cells and cells treated with wortmannin were incubated with [125 I]TC-ASOR (10 nM) for 10 min and were then incubated in the absence of ligand for 30 min and homogenized. PNS fractions were centrifuged at 10000 *g* for 25 min and the pellets obtained were resuspended in homogenizing buffer. The particulate fractions were incubated in the presence or absence of an ATP-regenerating system as described above. Fractions incubated in the presence of the ATP-regenerating system were also incubated in the presence or absence of concanamycin A (a specific inhibitor of the lysosomal H $^{+}$ -ATPases [28–30]). Figure 2 shows that ATP-dependent, as well as proton-pump-dependent, degradation of [125 I]TC-ASOR was approx. 80% higher in fractions from wortmannin-treated cells than in fractions from control cells.

Wortmannin alters the subcellular distribution of [125 I]TC-ASOR

To characterize further the degradative compartment(s) in wortmannin-treated cells, we employed sucrose-density-gradient centrifugation and electron microscopy. We first examined the effect of the drug on the subcellular distribution of [125 I]TC-ASOR. After 30 min of pre-incubation at 37 °C (for up-regulation

Figure 2 Effect of wortmannin on *in vitro* degradation of [125 I]TC-ASOR

Hepatocytes were incubated at 37 °C with or without wortmannin (0.1 μ M) for 30 min. [125 I]TC-ASOR was then added and the incubation was continued for 10 min. The cells were washed and incubated in the absence of ligand for 30 min. Granule fractions, prepared as described in the Materials and methods section, were incubated in the absence or presence of an ATP-regenerating system. Cells incubated with the ATP-regenerating system were also incubated in the absence or presence of 0.5 μ M concanamycin A (conc). Black bars (ATP dep) show ATP-dependent increase in acid-soluble radioactivity during 60 min of incubation. White bars (conc dep) show the difference in acid-soluble radioactivity after 60 min of incubation of particles in the absence or presence of concanamycin A. Results are means $\pm$ S.D. of three experiments, expressed as percentages of the total acid-precipitable radioactivity that was present initially in the fractions.

of the asialoglycoprotein receptor [31]), cells were incubated with or without wortmannin for 15 min, then [125 I]TC-ASOR (5 nM) was added, and the incubation was continued at 37 °C. The concentration of added ligand was so low that it would be removed from the medium within 5 min. The ligand would therefore be treated as a pulse by the cells. PNS was subsequently prepared and subjected to subcellular fractionation by isopycnic centrifugation in sucrose density gradients. Results of a representative experiment are shown in Figure 3. In both control cells and wortmannin-treated cells, the labelled ligand was banding at 1.12 g/ml, 5 min after initiation of uptake in endosomes (Figures 3A and 3B). At later time points, the distribution of the ligand in wortmannin-treated and control cells differed: in control cells, the labelled ligand was seen sequentially in organelles banding at 1.12 g/ml, 1.15 g/ml and 1.19 g/ml. These results are consonant with several earlier publications [32–34]. The low-density endosomes (at 1.12 g/ml) are early 'sorting' endosomes, the endocytic organelles of medium density are later maturing endosomes, whereas the organelles at 1.19 g/ml are lysosomes, as indicated by the fact that the radioactivity at this position is mainly acid-soluble (Figure 3C). A main effect of wortmannin was to bring about a shift in the density distribution of labelled ligand to lower densities (Figure 3B). In particular, acid-soluble radioactivity in wortmannin-treated cells peaked at 1.16 g/ml, whereas in control cells, peak activity was at 1.19 g/ml (Figure 3C). In control cells, acid-soluble radioactivity coincided with the lysosomal marker enzyme, β -AGA, at all time points tested (results not shown). In wortmannin-treated cells, on the other hand, acid-soluble radioactivity started to appear at a distinctly lower density than that of β -AGA (Figure 3D). With increasing length of incubation, however, the distribution of the enzyme was shifted gradually to lower densities, and after 270 min, its distribution approached that of the acid-soluble activity. Similar results were obtained when two other lysosomal enzymes, cathepsin B and cathepsin C, were measured (results not shown).

Figure 3 Effects of wortmannin on subcellular distribution of [125 I]TC-ASOR and β -AGA

(A)–(C) Hepatocytes were pre-incubated in the presence or absence of wortmannin ($0.1 \mu\text{M}$) for 15 min. Subsequently [125 I]TC-ASOR (5 nM) was added. Samples of cells were fractionated by isopycnic centrifugation after 5, 45 and 180 min. Total (A) and (B) and acid-soluble (C) radioactivity was measured in the fractions. The results are percentages of total recovered activities in the gradient. The distribution of total radioactivity in the gradients is shown for control cells (A) and wortmannin-treated cells (B) after 5, 45 and 180 min. (C) shows the distribution of acid-soluble radioactivity after 45 and 180 min for controls and wortmannin-treated cells. (D) Density distribution of β -AGA for cells incubated in the presence of wortmannin for 5 min and 270 min. The recovery of radioactivity or enzyme activity in the gradients varied between 75 and 85 % of values for the original homogenate.

Wortmannin induces formation of swollen endosomes at the expense of dense lysosomes

To investigate the effect of wortmannin on the morphology of organelles constituting the endocytic pathway, and especially the fate of dense lysosomes, we employed ASOR-labelled gold particles (20 nm) to label late endosomes and lysosomes. Following a 270 min pulse with gold particles, cells were incubated in the presence or absence of wortmannin for increasing times. Photomicrographs from a 270 min time point is shown in Figure 4. Whereas in control cells we observed dense lysosomes (Figures 4A, 4B and 4D) and MVBs (Figures 4C and 4D) with normal morphology, the most prominent feature of the wortmannin-treated cells was the appearance of large, swollen vesicles (Figure 4E). These vesicles still contained some internal membrane structures, although the increased size of the swollen endosomes often made these structures less prominent. The morphology of the internal membrane structures also deviated from those of the untreated cells. In control cells, gold particles were found in numerous dense lysosomes and, to a lesser extent, in MVBs. Cells that were treated with wortmannin showed a dramatic 80 % decrease in the number of dense lysosomes (see Table 1 for quantification). Instead the endocytic marker was mostly found in the large swollen endocytic vesicles.

The wortmannin-induced density change of degradative organelles is reversible

To determine whether the wortmannin-induced change in the density distribution of degraded (acid-soluble) [125 I]TC-ASOR

Table 1 Number of dense lysosomes in control and wortmannin treated cells

Cells that were incubated with ASOR-coated 20 nm gold particles ($A_{520} = 5.0$) for 270 min were treated with wortmannin ($0.1 \mu\text{M}$) for increasing lengths of time and were then processed for electron microscopy. Endocytic vesicles containing internalized tracer were characterized according to their morphology. Only dense lysosomes were scored and expressed as the mean $\pm$ S.D. number of dense lysosomes per cell section. Two separate experiments were performed, and 30 cells were counted for each condition. Statistical significance was determined using Student's *t* test.

Time (min)	Number of dense lysosomes/cell section ($\pm$ S.D.)	
	Control	Wortmannin
0	1.31 ± 0.03	1.30 ± 0.03
120	1.27 ± 0.03	0.92 ± 0.09
270	1.28 ± 0.02	0.18 ± 0.12

was reversible, wortmannin-treated cells, in which a pulse of [125 I]TC-ASOR had been processed for 90 min (at which time most of the ligand is in 'hybrid organelles'), were washed and re-incubated in the absence of wortmannin. The results (Figure 5) showed that, upon removal of wortmannin, the compartments containing labelled ligand became denser and banded in the gradient at the same position as corresponding control organelles (presumably lysosomes). Conversely, the addition of wortmannin to control cells (that had been given a pulse of [125 I]TC-ASOR and were incubated in parallel with the wortmannin-treated cells) caused a shift of the [125 I]TC-ASOR to lower densities (Figure 5).

Figure 4 Effect of wortmannin treatment on endosome morphology

Hepatocytes were given a 270 min pulse with 20 nm ASOR-coated gold particles in order to label dense lysosomes, which were then re-incubated in ASOR-coated-gold-free medium in the presence or absence of wortmannin (0.1 μ M) for up to 270 min. (A)–(D) are photomicrographs of a control cell at the 270 min time point. Gold particles (arrowheads) were mainly found in numerous lysosomes (arrows) and in MVBs (stars). In wortmannin-treated cells (E), the gold particles were found in endocytic structures that appeared dilated, but which still contained some membrane structures in the vesicle lumen. Small arrows indicate sites of contact between vesicles. Bars represent 500 nm.

Inhibition of autophagy does not reduce the density of lysosomes

To determine whether the changes seen in the density distribution of [125 I]TC-ASOR and β -AGA following wortmannin treatment were a consequence of inhibited autophagy, we studied the density distribution of these components in cells that were treated with a

mixture of amino acids known to inhibit autophagy [26,27]. The results (not shown) indicated that the distributions of [125 I]TC-ASOR and β -AGA were not affected by the amino acid mixture. Moreover, the distribution pattern of [125 I]TC-ASOR in cells that were treated with both the amino acid mixture and wortmannin was similar to that in cells treated with wortmannin alone.

Figure 5 Effect of wortmannin on density distribution of acid-soluble radioactivity is reversible

Hepatocytes were pre-incubated in the absence (A) or presence (B) of wortmannin (0.1 μ M) for 15 min. Subsequently, [125 I]TC-ASOR (5 nM) was added and incubation at 37 $^{\circ}$ C continued for 70 min. The cells were then washed and re-incubated in the absence or presence of wortmannin (wort) for 70 min. Samples of cells were fractionated by isopycnic centrifugation after the 70 min pulse and after the additional 70 min chase. Subcellular distribution of acid-soluble radioactivity was measured in the fractions. For both (A) and (B), results for one typical experiment (from three independent experiments) are shown as percentages of the total recovered activities in the gradient compared with the original homogenate, varying between 75 and 85 %, and three independent experiments were performed.

These data show that wortmannin does induce a change in the distribution of [125 I]TC-ASOR independently of its effect on autophagy.

DISCUSSION

The main observations in this study were as follows: (i) low concentrations of wortmannin did not affect the internalization of [125 I]TC-ASOR, and did not interfere with the rate of transport of the ligand to a degradative compartment; (ii) wortmannin or LY294002 induced an increase in the rate of degradation of endocytosed [125 I]TC-ASOR; (iii) endocytosed [125 I]TC-ASOR was found in endosomes and lysosomes of lower density in wortmannin- or LY294002-treated cells than in control cells. In particular, labelled degradation products from [125 I]TC-ASOR accumulated in an organelle of lower buoyant density than that of normal lysosomes; (iv) with increasing length of incubation, the density distribution of lysosomal marker enzymes approached that of the labelled degradation products; and (v) electron microscopic investigations showed that the number of swollen endocytic organelles increased at the expense of dense lysosomes in cells treated with wortmannin or LY294002.

The lack of effects of wortmannin/LY294002 on the early parts of the endocytic pathways in hepatocytes is unexpected, as it has been shown that PtdIns3P binds FYVE-domain-containing proteins, such as EEA1 and Rabenosyn5 [13,35–37]. The finding that PI3K may be dispensable for early endocytic membrane traffic in hepatocytes is at variance with results obtained using liver endothelial cells. In the latter cell type, both internalization and transport of ligands (recognized by the mannose receptor or the scavenger receptor A1) to the lysosomes were strongly inhibited by the drug [19]. The present results are, on the other hand, compatible with data indicating that inhibition of PI3K activity by wortmannin or LY294002 does not block lysosomal targeting and degradation of epidermal growth factor receptor (EGFR) [38–40].

A striking effect of wortmannin was the gradual formation of swollen late endosomes and the disappearance of dense lysosomes. It is known that the presence of PtdIns3P on the membrane of MVBs is required for the production of PtdIns(3,5) P_2 , which then recruits or activates effectors that regulate the maturation of MVBs [16]. Yeast Fab1p is a FYVE-containing protein with PtdIns3P 5-kinase activity that converts PtdIns3P into PtdIns(3,5) P_2 [41]. Fab1 mutant cells exhibit enlarged vacuoles that are reminiscent of those observed in wortmannin-treated cells, suggesting that Fab1p is involved in the sorting events during maturation of MVBs [16,17]. PtdIns(3,5) P_2 is present in fibroblasts [42], and enzymes with PtdIns3P 5-kinase activity have also been identified in mammalian cells [43,44]. Although these observations indicate that the formation of MVBs may be inhibited by wortmannin, our data do not indicate that this is the case in rat hepatocytes. The swollen large endosomes formed in wortmannin-treated hepatocytes, labelled with horseradish-peroxidase-reaction products or ASOR-coated gold particles, evidently contained small vesicles, as did control cells. The present data are in agreement with results published by Bright et al. [45] who showed that wortmannin in normal rat kidney ('NRK') cells did not interfere with the formation of intraluminal vesicles in the late swollen endosomes.

The formation of degradative compartments of lower density than lysosomes in wortmannin-treated cells is consistent with a model of direct fusion between late endosomes and lysosomes to form a pre-lysosomal compartment, a 'hybrid organelle', where degradation of endocytosed materials takes place [46,47]. In this model, the lysosomes function mainly as storage vesicles [48]. Since the endosomal fusion partner is of low density in wortmannin-treated cells (Figure 3B), the hybrid organelle will also be of lower density than lysosomes. In normal cells, the hybrid organelle may be a transient structure, because dense lysosomes will be regenerated. In the presence of wortmannin, the events that normally lead to the regeneration of dense lysosomes do not occur, and hybrid organelles (of low density) will therefore accumulate. This observation is consonant with an earlier report by Bright et al. [49]. This model predicts that conditions that restore the maturation process would lead to the disappearance of the accumulated hybrid organelles and reformation of dense lysosomes. Consistent with this prediction, we also found that [125 I]TC-ASOR resumed normal distribution following the removal of the inhibitor. The model also predicts that depletion of lysosomes, as they are used to form hybrid organelles in wortmannin-treated cells, would lead to a distribution pattern of lysosomal hydrolases that would eventually approach that of the [125 I]TC-ASOR. This was, in fact, what was found (Figure 3D). Although our results are compatible with the 'hybrid organelle model', we admit that these data alone cannot distinguish between this model and the 'kiss and run model' [50], because both models are based on fusion and fission events.

In agreement with an earlier study demonstrating enhanced lysosomal degradation of EGFR [51], we found increased degradation of [¹²⁵I]TC-ASOR in cells that were treated with wortmannin or LY294002. The reason for this effect is not known. It could be due to reduced pH or increased proteolytic activity in late endosomes. We found that [¹²⁵I]TC-ASOR reached degradative compartments simultaneously in wortmannin-treated and control cells; the increased rate of degradation is therefore not likely to be due to faster transport of the ligand to a degradative compartment. The 'paradoxical' effect could possibly be due to inhibition of autophagy [15], since an amino acid mixture known to inhibit autophagy [26,27] had the same effect as wortmannin or LY294002 and was not additive to these inhibitors. The density change in endocytic organelles in cells that were treated with wortmannin or LY294002 was independent of the effect on autophagy because the amino acid mixture did not affect the density distribution of endocytosed [¹²⁵I]TC-ASOR or its degradation products. Autophagy in itself may actually reduce the buoyant density of lysosomes [33].

Although the present study did not determine if PI3K activity was altered by wortmannin treatment, the fact that two unrelated inhibitors of PI3K gave similar biochemical and morphological results suggests that inhibition of PI3K activity is responsible for the observed changes. Considering that our experiments have been limited to the effects of wortmannin on unstimulated cells, it is unlikely that the observed biochemical and morphological changes are due to effects on signal-activated PI3Ks, class I, although an effect on basal activity of these enzymes cannot be ruled out. The most plausible target of wortmannin seems to be the PI3K hVPS34, which produces PtdIns3P through phosphorylation of PtdIns [4].

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