Use of glycyl-L-phenylalanine 2-naphthylamide, a lysosome-disrupting cathepsin C substrate, to distinguish between lysosomes and prelysosomal endocytic vacuoles

Trond Olav BERG,* Per Eivind STRØMHAUG,* Torunn LØVDAL,† Per Ottar SEGLEN* and Trond BERG†‡

*Department of Tissue Culture, Institute for Cancer Research, The Norwegian Radium Hospital, Montebello, 0310 Oslo, Norway, and †Division of Molecular Cell Biology, University of Oslo, P.O. Box 1050, Blindern, 0316 Oslo, Norway.

Lysosome-disrupting enzyme substrates have been used to distinguish between lysosomal and prelysosomal compartments along the endocytic pathway in isolated rat hepatocytes. The cells were incubated for various periods of time with ^{125}I-labelled tyramine cellobiose (^{125}I-TC) covalently coupled to asialo-orosomucoid (AOM) (^{125}I-TC–AOM); this molecule is internalized by receptor-mediated endocytosis and degraded in lysosomes, where the degradation products (acid-soluble, radiolabelled short peptides) accumulate, Glycyl-L-phenylalanine 2-naphthylamide (GPN) and methionine *O*-methyl ester (MOM), which are hydrolysed by lysosomal cathepsin C and a lysosomal esterase respectively, both diffused into hepatocytic lysosomes after electrodisruption of the cells. Intralysosomal accumulation of the hydrolysis products (amino acids) of these substrates caused osmotic lysis of more than 90% of the lysosomes, as measured by the release of acid-soluble radioactivity derived from ^{125}I-TC–AOM degradation. The acid-soluble radioactivity coincided in sucrose-density gradients with a major peak of the lysosomal marker enzyme acid phosphatase at 1.18 g/ml; in addition a minor, presumably endosomal, acid phosphatase peak was observed around 1.14 g/ml. The major peak of acid phosphatase was almost completely released by GPN (and by MOM), while the minor peak seemed unaffected by GPN. Acid-insoluble radioactivity, presumably in endosomes, banded (after 1 h of ^{125}I-TC–AOM uptake) as a major peak at 1.14 and a minor peak at 1.18 g/ml in sucrose gradients, and was not significantly released by GPN. GPN thus appears to be an excellent tool by which to distinguish between endosomes and lysosomes. MOM, on the other hand, released some radioactivity and acid phosphatase from endosomes as well as from lysosomes.

INTRODUCTION

Lysosomes are end points for both the endocytic and the autophagic pathways [1]. In addition, lysosomes receive hydrolytic enzymes and lysosomal glycoproteins from a biosynthetic pathway that enters the endocytic pathway at a prelysosomal level [2–5]. It has been notoriously difficult to determine whether molecules that are transported towards the lysosomes by the endocytic or the autophagic route are present in lysosomal or prelysosomal organelles. This is partly due to the fact that lysosomal components are introduced into the endocytic pathway before endosomes reach lysosomes [5]. Acid hydrolases or lysosomal glycoproteins are therefore not unique markers for lysosomes that could be used in immunocytochemical or cell fractionation studies. After cell fractionation by isopycnic centrifugation the density distribution of lysosomes and prelysosomal (endosomes, phagosomes) organelles may overlap to a considerable extent [6]. As an additional complication, a partial convergence between the endocytic and autophagic pathways at the prelysosomal level has been demonstrated, defining a common autophagic/endocytic vacuole called an amphisome [7,8]. Furthermore, the delivery of material to lysosomes by autophagy leads to a reduced buoyant density of the lysosomes, causing the density distribution of prelysosomal and lysosomal autophagic vacuoles to coincide, e.g. in metrizamide gradients [9].

It has been shown that methyl esters of amino acids [10], as well as certain tripeptides [11] that are lysosomal enzyme substrates, may selectively cause rupture of lysosomes. Such substrates diffuse across the lysosomal membrane in intact cells as well as under cell-free conditions, and are hydrolysed to free amino acids within the lysosome. The amino acid molecules do not readily diffuse back out of the lysosome due to their polarity, resulting in osmotic lysis. As lysosomal enzymes are present in prelysosomal endocytic organelles [12–14], it is conceivable that the lysosome-disruptive substrates may cause osmotic lysis even of endosomes and amphisomes.

The purpose of the present study was to obtain information about the step(s) in the endocytic pathway at which endosomes might become sensitive to lysosomal enzyme substrates. Two substrates that have been shown to cause osmotic lysis of lysosomes were tested: methionine *O*-methyl ester (MOM), which is hydrolysed by an esterase (possibly cathepsin G) [15], and glycyl-L-phenylalanine 2-naphthylamide (GPN), which is a substrate for cathepsin C [11]. As an endocytic marker we employed asialo-orosomucoid (AOM) which is endocytosed by the galactosyl receptor in rat liver parenchymal cells [16]. AOM was radiolabelled by conjugation with ^{125}I-tyramine cellobiose (^{125}I-TC) [17]. The labelled degradation products formed by proteolysis of this ligand are trapped in the degradative organelle and may therefore serve as markers for proteolytic compartments [18]. To expose the organelles to enzyme substrates the cells were either homogenized or electrodisrupted [7,19] and the cell-free preparation incubated with the selected substrates under the appropriate conditions. To relate the effect of the substrates to

Abbreviations used: β-AGA, *N*-acetyl-β-D-glucosaminidase; AOM, asialo-orosomucoid; GPN, glycyl-L-phenylalanine 2-naphthylamide; MOM, methionine *O*-methyl ester; ^{125}I-TC, ^{125}I-labelled tyramine cellobiose; N-fraction, nuclear fraction; ML-fraction, large-granule fraction; P-fraction, particulate fraction; MLP-fraction, high-speed centrifugation sediment.

‡ To whom correspondence should be addressed.

the intracellular localization of the ligand, subcellular fractionation was employed in combination with measurement of the formation of degradation products. To get further information about the sensitivity of early endosomal compartments to the substrates we also incubated cells with a microtubule inhibitor (vinblastine). This treatment has been shown to block the transport of endocytosed material to the lysosomes, probably by interfering with microtubule-mediated transport of a carrier vesicle from early to late endosomes [20–23].

MATERIALS AND METHODS

Biochemicals

Vinblastine sulphate, collagenase, leupeptin, GPN, and MOM were obtained from Sigma Chemical Co. (St. Louis, MO, U.S.A.). Metrizamide was purchased from Nyegaard & Co, Oslo, Norway. $Na^{125}I$ was from the Radiochemical Centre (Amersham, Bucks., U.K.).

Cell preparation and incubation

Hepatocytes were prepared by collagenase perfusion from male Wistar rats (250–300 g) starved for 18 h. The cells were incubated as suspensions (0.4 ml aliquots shaken in centrifuge tubes at 37 °C, usually 50–75 mg wet wt/ml) in suspension buffer [24] fortified with pyruvate (20 mM) and Mg^{2+} (2 mM).

Cell disruption

After incubation, the hepatocytes were washed twice in 10% (w/v) sucrose solution. The cell pellet was then suspended in 0.5 ml of 10% (w/v) sucrose and subjected to electrodisruption by a single high-voltage pulse as described previously [25]. The resulting preparation will be referred to as 'cell disruptate'.

Separation of sedimentable cell components ('cell corpses') from cytosol

After electrodisruption 0.5 ml of phosphate-buffered sucrose (100 mM potassium phosphate, pH 7.5, 2 mM EDTA; adjusted to 300 mosM with sucrose) was added and the suspension of disrupted cells was layered on top of a 3 ml ice-cold layer of phosphate-buffered metrizamide/sucrose [8% (w/v) metrizamide/50 mM potassium phosphate, pH 7.5/1 mM dithiothreitol/1 mM EDTA; adjusted to 300 mosM with sucrose]. After centrifugation at 0 °C for 30 min at 7000 *g* the metrizamide/sucrose layer was aspirated and the pellet (referred to later as 'cell corpses') was suspended in 0.5 ml of 0.25 M sucrose with 10 mM Hepes, pH 7.3.

Enzyme assays

Lactate dehydrogenase activity was determined spectrophotometrically by measuring the oxidation of NADH with pyruvate as substrate at 340 nm in a Beckman spectrophotometer or in an autoanalyser [26]. Acid phosphatase activity (β-glycerophosphatase) was determined according to Ames [27].

Determinations of radioactivity

Radioactivities were measured in a Kontron γ-counter. Degradation of ^{125}I-TC–AOM was followed by measuring radioactivity soluble in 10% (w/v) trichloroacetic acid (acid-soluble radioactivity). BSA (0.5%) was added as a carrier.

Measurement of release of endocytosed ligand and/or enzymes from organelles

To assess GPN- or MOM-induced release or radioactivity from organelles 0.5 ml aliquots of the cell disruptates were mixed with equal volumes of 0.25 M sucrose solution containing MOM (5–10 mM) or GPN (0.25–0.5 mM). These substrate concentrations were found, in preliminary experiments, to yield maximal organelle lysis. Controls contained the solvent (dimethyl sulphoxide) in which the lytic substrates were added. The 0.25 M sucrose solution was buffered with 20 mM Hepes (pH 7.0). NaCl (5 mM) was added to samples incubated with or without GPN, as cathepsin C requires chloride ions for full activity [11]. The samples were incubated at the temperatures found to be optimal (0 °C and 37 °C for MOM and GPN respectively), and after incubation for the optimum period of time (5 min with GPN and 30 min with MOM) the cell disruptates were either centrifuged at 48000 *g* for 60 min or placed on layers of metrizamide (4 ml) and centrifuged for 30 min at 7000 *g*. Before centrifugation at 48000 *g* for 60 min the disruptates were diluted with 3 ml of 0.25 M sucrose/10 mM Hepes (pH 7.3). High-speed centrifugation was carried out in a Sorvall centrifuge with an SS24 rotor. Acid-soluble and acid-insoluble radioactivities, together with lysosomal enzymes, were measured in pellets and supernatants after high-speed centrifugation, or in the pellets (cell corpses) as well as in the cell disruptates after separation in metrizamide.

Subcellular fractionation

Cell disruptates or cell corpses [in 10% (w/v) sucrose] were mixed with equal volumes of 0.25 M sucrose/10 mM Hepes/1 mM EDTA (pH 7.3) and homogenized by five strokes in a Dounce homogenizer (tight-fitting pestle). The homogenates, containing material from about 50 mg of cells (wet wt)/ml, were fractionated by differential centrifugation and/or by isopycnic centrifugation in Nycodenz or sucrose gradients. Differential centrifugation started 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 0.25 M sucrose/10 mM Hepes/1 mM EDTA, homogenized (five strikes) and centrifuged again at 2000 *g* for 2 min. A large-granule fraction (ML-fraction) was sedimented from the postnuclear fraction by centrifugation at 25000 *g* for 9 min, and a particulate fraction (P-fraction) was sedimented from the postmitochondrial fraction at 48000 *g* for 60 min. In some experiments an MLP-fraction (high-speed centrifugation sediment) was prepared by centrifuging the postnuclear fraction at 48000 *g* for 60 min. Differential centrifugation was carried out in a Sorvall RC-2B centrifuge using an SS24 rotor and 13 ml capacity centrifuge tubes. Homogenizations and centrifugations were carried out at 0–4 °C. In isopycnic centrifugation experiments 2–4 ml aliquots of an ML-fraction or an MLP-fraction were initially layered either at the bottom or at the top of the gradients. The centrifuge tubes (38 ml) were centrifuged at 85000 *g* in a Beckman SW 28 rotor at 4 °C for 2 h (Nycodenz gradients) or 4 h (sucrose gradients). After centrifugation the gradients were divided into 18 × 2 ml fractions by upward displacement using Maxidens as displacement fluid. The densities of the fractions were calculated from the refractive indices [18].

RESULTS

Recovery of markers in cell corpses prepared by electrodisruption and centrifugation through a layer of metrizamide

Lysosome disruption was measured as the release of intralysosomal endocytosed ligand or lysosomal enzymes from cell

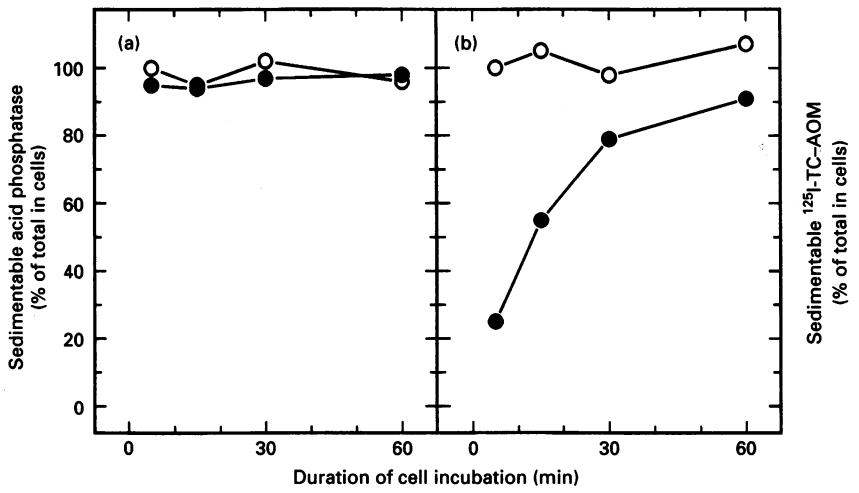

Figure 1 Recovery of acid phosphatase and endocytosed ^{125}I-TC–AOM in cell corpses

Hepatocytes were incubated for 1 h at 0 °C with ^{125}I-TC–AOM (10 nM). The cells were then washed and incubated at 37 °C to internalize the labelled ligand. Aliquots of cells were removed at the indicated times and cell disruptates and cell corpses were prepared as described in the Materials and methods section. Aliquots of the disruptates were centrifuged at 48000 ***g*** for 1 h to measure total sedimentable radioactivity. (**a**), Acid phosphatase activity; (**b**), radioactivity, in cell corpses (●) and in the high-speed sediment (○), as percent of the total activity in the disruptate.

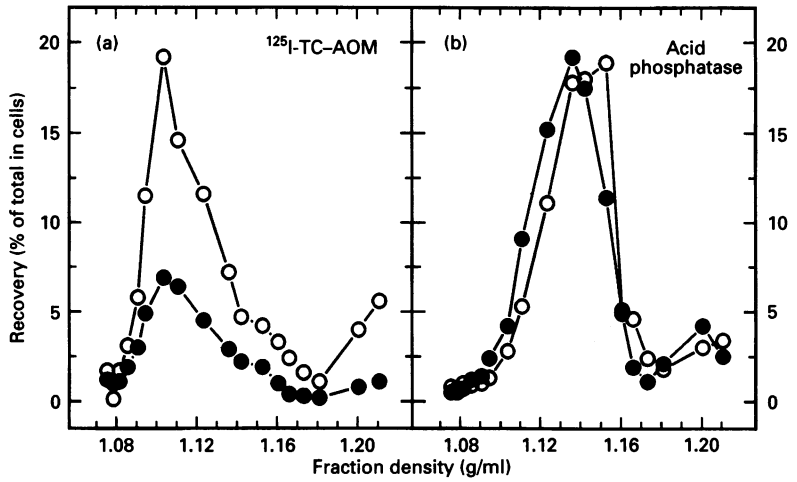

Figure 2 Fractionation of cell corpses and high-speed sediments early after initiation of endocytosis of ^{125}I-TC–AOM in hepatocytes

Cell corpses (7000 ***g***; 30 min) or high-speed sediments (48000 ***g***; 60 min) were prepared from cell disruptates (by centrifugation through metrizamide) 5 min after the addition of ^{125}I-TC–AOM to the cells at 37 °C. The sediments were homogenized and placed at the bottom of linear Nycodenz gradients. The gradients were centrifuged at 85000 ***g*** for 4 h. The recoveries of ^{125}I-TC–AOM as percentages of that measured in the cell disruptates were 83.7% and 33.0% in gradients with material from high-speed sediments and cell corpses respectively. (**a**), Density distribution of ^{125}I-radioactivity; (**b**) acid phosphatase in high-speed sediments (○) and cell corpses (●).

corpses or cell disruptates, whereas endosome disruption was measured as the release of endocytosed ligand present in endosomes (in cell corpses or disruptates). It was therefore important to verify that the recovery of these lysosomal/endosomal markers in the separated corpses was satisfactory.

Figure 1(a) shows that the lysosomal marker enzyme, acid phosphatase, was virtually quantitatively recovered (90–100%) in the cell corpses. In contrast, the recovery of endocytosed ^{125}I-TC–AOM in cell corpses prepared from cells that had internalized surface-bound ^{125}I-TC–AOM at 37 °C for various periods of time was initially low but increased with time, approaching a complete recovery after about 1 h (Figure 1b). The low recovery of ligand at early time points after the start of the uptake could be due to rupture of early endosomes during electropermeabilization and/or to loss of early endosomes from the corpses. If early endosomes were lost from the corpses one would expect them to be sedimentable at higher speed. This turned out to be the case: both acid phosphatase (Figure 1a) and ^{125}I-TC–AOM (Figure 1b) were quantitatively recovered in a sediment from high-speed centrifugation (48000 ***g***; 60 min) at any time after the start of the endocytosis. It thus seems clear that early endosomes (in contrast with late endosomes and lysosomes) do not sediment quantitatively at the centrifugation force routinely used to isolate cell corpses. Figure 2 shows the results of an experiment in which resuspended postnuclear particles, prepared from high-speed sediments (MLP-fractions) or cell corpses 5 min after the addition of ^{125}I-TC–AOM, were fractionated in Nycodenz gradients. Much fewer early endosomes (peak at 1.10–1.11 g/ml) were

recovered in the cell corpses than in the high-speed sediments at this early time point (Figure 2a). The recovery of lysosomes (peak at 1.13–1.14 g/ml) was, on the other hand, high, as indicated by the activity of acid phosphatase in the gradients (Figure 2b). These results suggest that it is, particularly at early time points, preferable to assess the release of endocytosed ligand from cell disruptates rather than from cell corpses, as the corpses may have lost most of the early endosomes.

GPN and MOM released mainly degraded ^{125}I-TC–AOM from cell disruptates

To determine when or whether endocytosed ^{125}I-TC–AOM entered a GPN- or MOM-sensitive compartment, cell disruptates were prepared from cells that had been allowed to internalize a pulse of surface-bound ligand at 37 °C for various times. The cell disruptates were incubated with GPN for 5 min at 37 °C or with MOM for 30 min at 0 °C and were subsequently centrifuged at 48000 *g* for 60 min. Figure 3 shows total, acid-soluble and acid-insoluble radioactivity released (rendered unsedimentable) by GPN (left-hand panels) or MOM (right-hand panels) in cell disruptates prepared at various times after the start of ^{125}I-TC–AOM internalization. Figures 3(c) and 3(d) also shows total acid-soluble radioactivity formed in the cells during the incubation at 37 °C. The results obtained show that both GPN and MOM induced release of radioactivity above control values. The proportion of radioactivity released increased with time of cell incubation. Cell disruptates prepared 15 min after the start of internalization of ^{125}I-TC–AOM were not affected by GPN, whereas a slight release above control values could be seen with MOM. Figures 3(c) and 3(d) show that the radioactivity released by either substrate was mainly acid-soluble, suggesting its origin in a lysosomal compartment. In fact, most of the acid-soluble radioactivity formed in the cells was released by the lytic substrates. MOM consistently released a small amount of acid-insoluble radioactivity, which explains the effect of MOM on unsedimentable total radioactivity after 15 min (Figures 3e and 3f). Acid phosphatase was also measured in the sedimentable and unsedimentable fractions prepared from the cell disruptates incubated in the presence and absence of GPN and MOM. Both substrates released about 70% of the enzyme at all time points (results not shown).

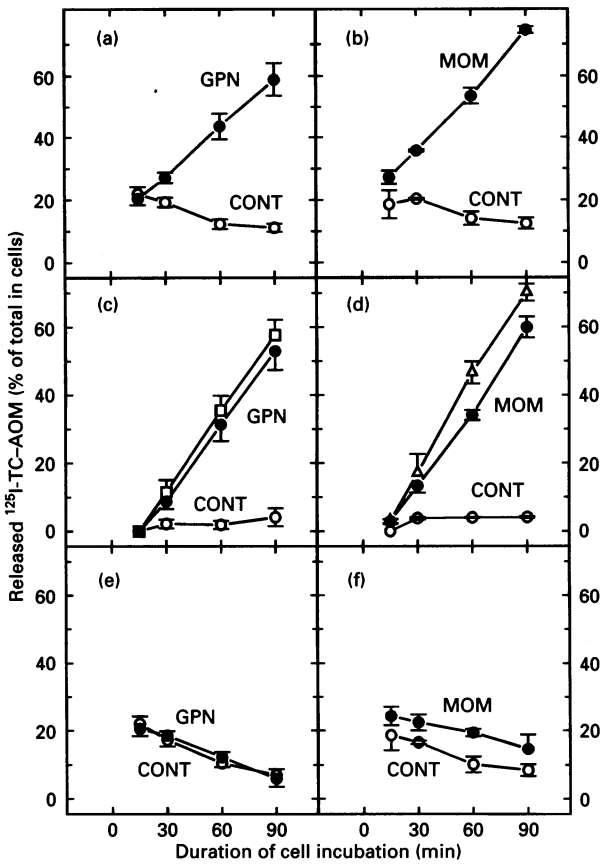

Figure 3 Effects of GPN and MOM on release of ^{125}I-TC–AOM radioactivity and acid phosphatase from endosomes and lysosomes

Rat hepatocytes were first incubated with 50 nM ^{125}I-TC–AOM at 4 °C for 60 min. The cells were then washed and re-incubated at 37 °C to internalize surface-bound ligand for the length of time indicated. Cell disruptates were prepared and incubated in the presence or absence (CONT) of 0.5 mM GPN (**a**, **c** and **e**) or 10 mM MOM (**b**, **d** and **f**). The cell disruptates were then diluted with 0.25 M sucrose/10 mM Hepes, pH 7.3, and centrifuged for 60 min at 48000 *g*. Acid-soluble and acid-insoluble radioactivities were measured in the supernatants and in the pellets. The curves show total released radioactivity (**a** and **b**), released acid-soluble radioactivity (**c** and **d**), and released acid-insoluble radioactivity (**e** and **f**). Open circles show control values where closed circles show release in the presence of the lytic substrates. Total acid-soluble radioactivity formed in the cells is shown in (**c**) and (**d**) (□ and △). All values are expressed as percentages of total radioactivity in the cells. Each value is the mean ± S.E.M. of five experiments.

Vinblastine prevents GPN and MOM from releasing endocytosed ligand from the disruptates

To get more information about the identity of the compartment that had been reached by ^{125}I-TC–AOM at various times, cell samples were incubated in the presence of 50 μM vinblastine. This treatment has been shown to prevent transport of ligand from endosomes to lysosomes [21,23]. Therefore, disruptive effects seen in vinblastine-treated cells should be due to the presence of the relevant enzymes in endosomes.

Figure 4 shows uptake and degradation of ^{125}I-TC–AOM in hepatocytes incubated in the presence and absence of vinblastine (50 μM) at 37 °C. The drug decreased uptake of ^{125}I-TC–AOM considerably; after 15 min control cells had accumulated about 60% of the labelled ligand (initial concentration 10 nM) whereas the corresponding value for the vinblastine-treated cells was about 25%. Vinblastine nearly abolished degradation of the ligand. These results confirm earlier data [20–23]. Cell disruptates prepared from cells and vinblastine-treated cells various times after the addition of ligand were incubated in the presence and absence of GPN or MOM. Table 1 shows release of radioactivity in disruptates prepared from cells that had been incubated with ^{125}I-TC–AOM for 1 h at 37 °C. Disruptates from control cells released, as expected, a substantial amount (40–50%) of radioactivity in the presence of the lytic substrates. Most of the released radioactivity (> 80%) was acid-soluble (results not shown). Vinblastine prevented the GPN-induced release of radioactivity from the disruptates, whereas MOM induced a small release in disruptates from vinblastine-treated cells. The vacuolar compartment accumulating ^{125}I-TC–AOM in the presence of vinblastine would thus seem to be completely GPN-resistant, but moderately sensitive towards MOM.

Subcellular fractionation experiments indicate that GPN releases ^{125}I-TC–AOM selectively from terminal lysosomes

The kinetics of the effects of GPN or MOM on the sedimentability of radioactivity, and the fact that mainly acid-soluble radio-

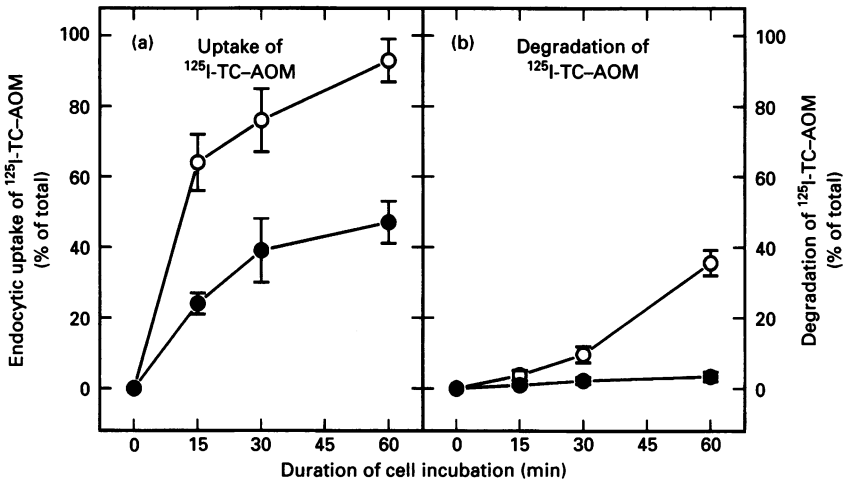

Figure 4 Effect of vinblastine on uptake and degradation of ^{125}I-TC–AOM in hepatocytes

The cells (1×10^7 cells/ml) were preincubated for 30 min with (●) and without (○) 50 μM vinblastine at 37 °C. ^{125}I-TC–AOM (10 nM) was then added and cell aliquots were removed at the indicated times. The cells were washed twice in 10% (w/v) sucrose solution, and total and acid-soluble radioactivities in the cells were measured. The results are presented as percentages of the total radioactivity added to the cells initially. Each value is the mean ± S.E.M. of four experiments.

Table 1 Vinblastine inhibits GPN- and MOM-induced release of endocytosed ^{125}I-TC–AOM from cell disruptates

Hepatocytes (1×10^7 cell/ml) were incubated for 30 min at 37 °C in the presence or absence of vinblastine (50 μM). ^{125}I-TC–AOM (10 nM) was then added and the cells were incubated further at 37 °C for 1 h. Disruptates were prepared from the cells and incubated in the presence or absence (controls) of GPN (0.50 mM) or MOM (5 mM). The total amount of radioactivity released, i.e. rendered unsedimentable, in GPN-, MOM- or untreated cell disruptates is given as the percentage of total radioactivity. Each value is the mean ± S.E.M. of the number of experiments given in parentheses.

	Release of radioactivity from cell disruptates (% of total radioactivity in cells)		
	Control	+ GPN	+ MOM
Control cells	15.5 ± 3.2 (4)	39.0; 45.0 (2)	47.6; 53.6 (2)
Vinblastine-treated cells	12.7 ± 1.3 (4)	10.9; 13.1 (2)	14.8; 18.8 (2)

activity was released, suggested that the lytic substrates acted selectively on lysosomes. However, as lysosomal enzymes are present in endosomes [5] it cannot be ruled out that the substrates also acted on prelysosomal compartments. Moreover, it has been shown that degradation of various ligands may take place in endosomes [12,13,18]. To characterize further the organelles that are sensitive to GPN/MOM treatment we fractionated cell disruptates that had been incubated with or without GPN or MOM by means of differential centrifugation and isopycnic centrifugation in sucrose-density gradients, and the disruptive effects of the substrates were then related to the subcellular distribution of the ligand at various times after the start of its uptake in the cells.

The cell disruptates were fractionated by differential centrifugation into a nuclear fraction, a combined mitochondrial fraction (ML-fraction), a microsomal fraction (P-fraction) and a final supernatant, as described in the Materials and methods section. It was found, consonant with earlier reports [16], that the ligand sedimented mainly in the P-fraction until 15 min after the start of the internalization (Figure 5a). It may therefore be assumed that early endosomes (as defined kinetically) are mainly present in the P-fraction. Isopycnic centrifugation of the P-fraction furthermore demonstrated that these early endosomes band at a low density in sucrose gradients (1.11–1.12 g/ml) (results not shown). The P-fraction, on the other hand, does not seem to contain later endocytic structures; these, therefore, sediment in denser and/or larger organelles and are also associated with cell corpses during centrifugation through a layer of metrizamide.

GPN had, as expected, no effect on the contents of ^{125}I-TC–AOM in the P-fraction, but induced a selective release of radioactivity from the ML-fraction at later time points (Figure 5a). Acid phosphatase was released (from the lysosomes) to a similar extent at all times (Figure 5b). Similar results were obtained with MOM (not shown).

To try to separate late(r) endosomes and lysosomes the ML-fraction (prepared from cell corpses) was fractionated further by sucrose-density-gradient centrifugation. Figure 6 shows the density distribution of acid-soluble and acid-insoluble radioactivities after fractionation of control corpses and corpses treated with GPN and MOM after 1 h of ^{125}I-TC–AOM uptake. At this time point the labelled ligand was associated with organelles (late endosomes/lysosomes) that were nearly quantitatively recovered in the cell corpses (following centrifugation through a layer of metrizamide). The distribution of a marker enzyme for lysosomes (acid phosphatase) is also included in the figure. Fractionation of control ML-fractions in sucrose-density gradients revealed two peaks of radioactivity, at 1.14 g/ml and 1.18 g/ml. Figures 6(a) and 6(b) indicate that most of the acid-insoluble radioactivity peaked at 1.14 g/ml, but a significant amount of material was also present around 1.18 g/ml, evident as a shoulder in Figure 6(a). None of the peaks of acid-insoluble radioactivity was significantly affected by GPN (Figure 6a). On the other hand, the acid-soluble radioactivity, which peaked at 1.18 g/ml, was nearly eliminated after GPN treatment (Figure 6b). The large amount of acid-soluble radioactivity in this peak, presumably representing the lysosomes, supports the notion that the rate-limiting

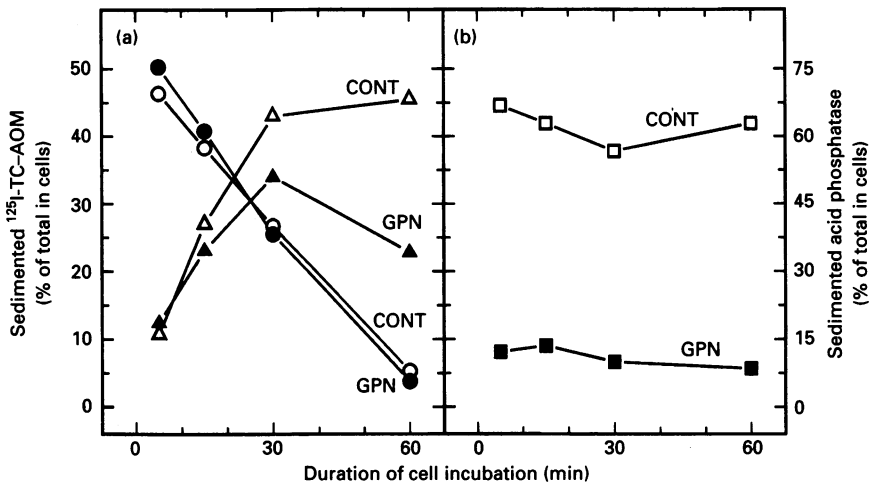

Figure 5 Effect of GPN on the distribution of (a) radioactivity and (b) acid phosphatase in subcellular fractions

Hepatocytes (1×10^7 cells/ml) were incubated with 10 nM ^{125}I-TC–AOM at 37 °C. At the indicated times after initiation of endocytosis of ^{125}I-TC–AOM aliquots of cells were removed and cell disruptates were prepared and incubated in the presence (filled symbols) or absence (open symbols) of 0.50 mM GPN for 5 min at 37 °C. The disruptates were subsequently fractionated by differential centrifugation into a nuclear fraction, an ML-fraction, a P-fraction and a soluble fraction as described in the Materials and methods section. (**a**) Radioactivity in the P-fraction (circles) and in the ML-fraction (triangles) in control (open symbols) or GPN-treated (filled symbols) disruptates. (**b**) Acid phosphatase activity in ML-fractions prepared from control and GPN-treated disruptates. All values are expressed as percentage of the total activities in the disruptates from which the fractions were prepared.

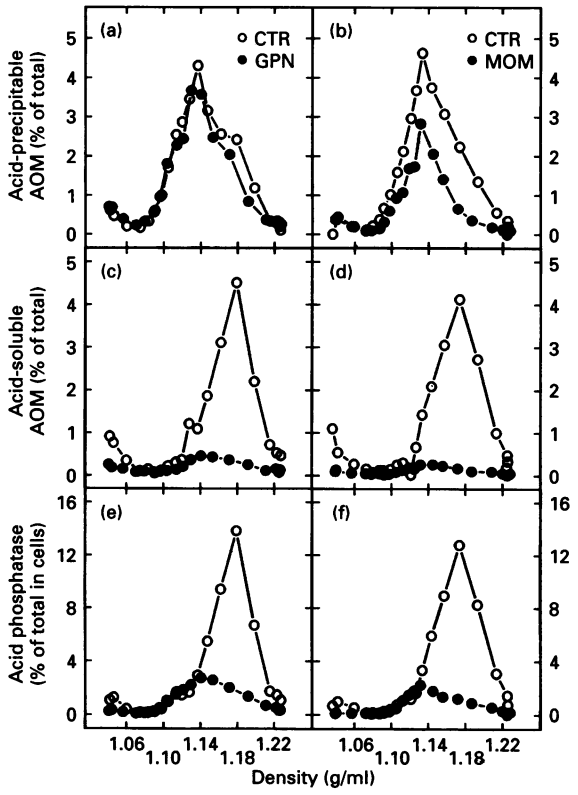

Figure 6 Effects of GPN and MOM on the density distribution of acid-soluble radioactivity, acid-insoluble radioactivity, and acid phosphatase in sucrose-density gradients

Cell disruptates, prepared from cells that had been incubated with ^{125}I-TC–AOM (10 nM) at 37 °C for 1 h, were incubated in the presence or absence of GPN (0.5 mM) (**a**, **c** and **e**) or MOM (10 mM) (**b**, **d** and **f**). ML-fractions were prepared from the cell corpses and fractionated further in sucrose gradients. The values are presented as percentages of the amounts present in the total disruptates from which the corpses were derived. Filled symbols represent disruptates treated with the substrates. Open symbols are controls.

step in intracellular degradation of ^{125}I-TC–AOM is not in lysosomes but at an earlier step in the process [16].

GPN reduced the major peak of acid phosphatase at 1.18 g/ml effectively (Figures 6e and 6f). However, a minor peak at 1.14 g/ml was unmasked by the GPN treatment. This peak is likely to represent acid phosphatase in endosomes (cf. Runquist and Havel [14] who found a considerable fraction of the acid phosphatase activity in purified endosomal fractions). Vesicles made leaky by GPN or MOM treatment obviously have an equilibrium density that is different from that of the corresponding intact vesicles. It is therefore conceivable that the density distribution of membrane-bound acid phosphatase may deviate from that of lysosomes.

The effect of MOM on the subcellular distribution of acid-soluble ^{125}I-TC–AOM (Figure 6d) and acid phosphatase (Figure 6f) was similar to that of GPN. However, MOM also released some acid-insoluble radioactivity (Figure 6b), suggesting that it might have a lytic effect on endosomes as well as on lysosomes.

DISCUSSION

It was possible by cell fractionation to obtain three compartments that received the endocytosed ligand, ^{125}I-TC–AOM, sequentially. Up to 15 min after internalization started the ligand was in an organelle that sedimented in the P-fraction and banded at 1.11–1.12 g/ml in sucrose gradients. Later, the ligand entered endosomal compartments that sedimented in the ML-fraction and banded at about 1.14 g/ml and 1.18 g/ml in sucrose gradients. These later endosomes also sedimented with the cell corpses after centrifugation through a metrizamide layer. Finally, after 15–30 min, the labelled asialoglycoprotein entered a lysosomal compartment that sedimented in the ML-fraction and was found at about 1.18 g/ml in the sucrose gradient. The buoyant density of lysosomes, 1.18 g/ml, is slightly lower than values (about 1.20 g/ml) obtained for rat liver lysosomes by others [28,29]. The lower value in the present study is probably due to the high autophagic activity in suspended rat hepatocytes [30]. The

lysosomal localization was also revealed by the presence of ^{125}I-labelled degradation products in this region of the gradient. The labelled degradation products, ^{125}I-TC attached to short peptides, are trapped in the degradative organelles as acid-soluble radioactivity and may therefore serve as markers for these structures [18]. Following treatment of the cell disruptates with GPN it was found that radioactivity was released selectively from the lysosomes banding at 1.18 g/ml, negligible release of radioactivity occurring from the early endosomes sedimenting in the P-fraction or those found at 1.14 g/ml in the sucrose gradient. The release from the lysosomes was nearly complete (> 90 %). The fact that GPN had virtually no effect on acid-insoluble radioactivity would seem to allow a fairly clear distinction between lysosomes (containing acid-soluble radioactivity only) and dense endosomes (containing acid-insoluble radioactivity only) co-sedimenting around 1.18 g/ml. The data suggest, furthermore, that cathepsin C is active only in the terminal lysosomes, and not in prelysosomal compartments in the endocytic pathway. The present data suggest that lysosomal degradation of endocytosed ligand is a result of fusion with endosomes rather than a maturation of endosomes. The rate-limiting step in degradation is evidently the fusion step, once inside the lysosomes the ligand is degraded rapidly. The present data are in agreement with a model in which uptake of endocytosed material in lysosomes is the result of fusion between endosomes and lysosomes [31]. We have in a separate study also examined the effects of GPN and MOM on the autophagic pathway. The cytosolic enzyme lactate dehydrogenase was used as a marker for autophagosomes, and autophagosomes and lysosomes were separated in sucrose-density gradients. GPN was again found to act selectively on lysosomes in the autophagic pathway, leaving the autophagosomes intact (T. O. Berg, P. E. Strömhaug, T. Berg & P. O. Seglen, unpublished work). Cathepsin C is probably introduced into the endocytic pathway at a prelysosomal stage along with other lysosomal hydrolases [3–5] but, as indicated by the present results, the enzyme is probably not sufficiently active (or activated) to induce rupture of these prelysosomal organelles. GPN has previously been used by Wattiaux and co-workers [11,28] to differentiate between endosomal and lysosomal localization of ligands (e.g. invertase) that are selectively taken up in rat liver endothelial cells by the mannose receptor [32]. In those studies lysosomes were defined as organelles that were disrupted by GPN. Until recently, lysosomes were thought of as end-stage organelles where complete degradation occurred and endosomes were viewed as sites of sorting and segregation. However, an increasing number of studies have reported the presence of active hydrolases in endosomes of several cell types [12,13,18]. In macrophages cathepsin B and cathepsin D are active in early endosomes [12]. Casciola-Rosen and Hubbard [13] studied degradation in the endocytic pathway by assessing hydrolysis of various lysosomal enzyme substrates that were taken up by the perfused liver by endocytosis. It was demonstrated that some substrates (e.g. that of aryl sulphatase) were degraded in early endosomes, whereas other substrates such as that of *N*-acetyl-β-D-glucosaminidase (β-AGA) were transported to lysosomes before hydrolysis was initiated. Active cathepsin C would seem to be distributed similarly to β-AGA.

Like GPN, MOM induced a virtually complete release of ^{125}I-TC–AOM and enzymes from the lysosomes, but, unlike GPN, it also released a significant fraction of the acid-insoluble radioactivity from the endosomes. While this release might, at least in part, represent unspecific membrane damage, the possibility should also be considered that the MOM-hydrolysing esterase, cathepsin G [15], may (in contrast with cathepsin C) be partially active in prelysosomal (endosomal) organelles. The origin of this esterase is not known. It could be delivered from the Golgi or it could be introduced from the autophagic pathway. Freshly prepared hepatocytes in suspension do have a high autophagic activity [30]. The active esterase could, therefore, conceivably be derived from autophagically sequestered endoplasmic reticulum, which is rich in esterases [33]. We have found, in a parallel study, that MOM, in contrast with GPN, also partially ruptures prelysosomal autophagic vacuoles, some of which may able to fuse with endosomes [8].

The purpose of the present study was to determine the exact stage in the endocytic pathway at which an endocytosed ligand entered a GPN- or MOM-sensitive compartment. The data indicate that whereas MOM has effects both on endosomes and lysosomes, GPN evidently acts selectively on (terminal) lysosomes and not on prelysosomal compartments. This property makes GPN a valuable tool to distinguish between lysosomal and prelysosomal localization of components that are known to, or suspected of, being present in the autophagic or endocytic pathway.

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