Intracellular transport of endocytosed proteins in rat liver endothelial cells

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1. Receptor-mediated endocytosis of mannose-terminated glycoproteins in rat liver endothelial cells has been followed by means of subcellular fractionation and by immunocytochemical labelling of ultrathin cryosections after intravenous injection of ovalbumin. For subcellular-fractionation studies the ligand was labelled with ¹²⁵I-tyramine-cellobiose adduct, which leads to labelled degradation products being trapped intracellularly in the organelle where the degradation takes place. 2. Isopycnic centrifugation in sucrose gradients of a whole liver homogenate showed that the ligand is sequentially associated with three organelles with increasing buoyant densities. The ligand was, 1 min after injection, recovered in a light, slowly sedimenting vesicle and subsequently (6 min) in larger endosomes. After 24 min the ligand was recovered in dense organelles, where also acid-soluble degradation products accumulated. 3. Immunocytochemical labelling of ultrathin cryosections showed that the ligand appeared rapidly after internalization in coated vesicles and subsequently in two larger types of endosomes. In the 'early' endosomes (1 min after injection) the labelling was seen closely associated with the membrane of the vesicle; after 6 min the ligand was evenly distributed in the lumen. At 24 min after injection the ligand was found in the lysosomes. 4. A bimodal distribution of endothelial cell lysosomes with different buoyant densities was revealed by centrifugation in iso-osmotic Nycodenz gradients, suggesting that two types of lysosomes are involved in the degradation of mannose-terminated glycoproteins in liver endothelial cells. Two populations of lysosomes were also revealed by sucrose-density-gradient centrifugation after injection of large amounts of yeast invertase. 5. In conclusion, ovalbumin is transferred rapidly through three endosomal compartments before delivering to the lysosomes. The degradation seems to take place in two populations of lysosomes.

INTRODUCTION

Hepatic endocytosis of plasma proteins and other macromolecules is essential for blood homeostasis. By this process blood levels of several nutrients, peptide hormones, immune complexes, macromolecules that have leaked out into blood, and denatured proteins, are controlled. Hepatic endocytosis takes place in at least four types of cells, the parenchymal cells (PC), the Kupffer cells, the endothelial cells (EC), and the stellate cells. The various cells have different receptors that mediate uptake of various ligands; there is therefore a 'division of labour' between the cells. In addition, the cells may co-operate in endocytosis of some ligands (Berg *et al.*, 1986).

During recent years it has become clear that the hepatic EC have a particularly important function in removing denatured proteins (Blomhoff *et al.*, 1984b) and proteins and glycosaminoglycans that have leaked out into the circulation (Smedsrød *et al.*, 1990). It has furthermore been suggested that the EC bind and endocytose proteins that are modified in the EC and subsequently presented for the PC (Tavassoli *et al.*, 1986).

The receptors recognize lysosomal enzymes (Stahl *et al.*, 1978), mannose-, fucose- and *N*-acetylglucosamine-terminated glycoproteins (Hubbard *et al.*, 1979), denatured or chemically modified proteins (Blomhoff *et al.*, 1984b), caeruloplasmin (Kataoka & Tavassoli, 1984), transferrin (Soda & Tavassoli, 1984a), glycosaminoglycans (Smedsrød *et al.*, 1984), collagen (Smedsrød *et al.*, 1985), acetyl-LDL (Blomhoff *et al.*, 1984a), tissue plasminogen activator (Einarsson *et al.*, 1988) and insulin (Soda & Tavassoli, 1984a).

Although a variety of specific receptors have been identified in

the liver EC, relatively little is known about the intracellular transport and degradation of the endocytosed ligands in these cells. It has previously been shown that EC express scavenger and mannose receptors that mediate an extremely rapid internalization of ligands (Eskild *et al.*, 1989; Magnusson & Berg, 1989), and subcellular-fractionation studies have revealed that ligands recognized by the scavenger receptor are transported very rapidly from endosomal to lysosomal compartments (Kindberg *et al.*, 1987; Eskild *et al.*, 1989). Electron-microscopic studies have indicated that glycosaminoglycans (Smedsrød *et al.*, 1988) and modified low-density lipoprotein ('acetyl-LDL') are internalized into unusually large endosomes before transfer to secondary lysosomes (Mommaas-Kienhuis *et al.*, 1985).

The aim of the present study was to obtain information about the intracellular transport and degradation of ligands endocytosed via the mannose receptor in rat liver EC. We have monitored the intracellular pathway of the mannose-terminated glycoprotein ovalbumin (OVA) by means of subcellular-fractionation studies in sucrose and Nycodenz density gradients and by immunocytochemical labelling of OVA on ultrathin cryosections of rat liver.

The intracellular distribution of OVA was determined in pieces of liver removed at various time points after intravenous injection of the labelled ligand; it was thereafter important to verify that the ligand was taken up exclusively in the EC. We have in the companion paper (Kindberg *et al.*, 1990) shown that negligible amounts of intravenously injected OVA are taken up by the Kupffer cells and that a small proportion is taken up via the galactose receptors in the parenchymal cells (PC). The uptake in PC is due to the presence of terminal galactose residues in some of the OVA molecules. To prevent uptake of OVA in the PC, a

Abbreviations used: AOM, asialo-orosomucoid; EC, endothelial cell; FSA, formaldehyde-treated serum albumin; OVA, ovalbumin; PC, parenchymal cell; TC, tyramine-cellobiose adduct.

huge excess of asialo-orosomucoid (AOM) was injected together with the OVA. AOM will block uptake via the galactose receptors in the PC. OVA was labelled with ^{125}I -tyramine-cellobiose adduct (^{125}I -TC-OVA) or ^{131}I -tyramine-cellobiose (^{131}I -TC-OVA) for use in the subcellular-fractionation studies; radioactive degradation products formed from this ligand are trapped in the organelle in which degradation takes place and may therefore serve as markers for these organelles. As OVA is taken up exclusively in the EC, the labelled degradation products will be markers for the lysosomes of the EC. The present study indicates that EC lysosomes are denser in sucrose gradients than the 'average' liver lysosomes. Hepatic lysosomal enzymes such as β -acetylglucosaminidase would therefore be unreliable markers for EC lysosomes.

The present study indicates that the ligand is transferred through at least three endosomal compartments before delivery to lysosomes. Moreover, degradation seems to take place sequentially in two groups of lysosomes that differ in buoyant density in iso-osmotic Nycodenz gradients.

MATERIALS AND METHODS

Chemicals

Collagenase (type I), BSA (fraction V), OVA, invertase and orosomucoid were purchased from Sigma Chemical Co, St. Louis, MO, U.S.A. Orosomucoid was desialylated as described previously (Tolleshaug *et al.*, 1979). Human serum albumin was from Kabi and was treated with formaldehyde as previously described (Eskild & Berg, 1984). Nycodenz and Maxidens were obtained from Nycomed, Oslo, Norway. Na^{125}I and Na^{131}I were from The Radiochemical Centre, Amersham, Bucks., U.K. OVA and formaldehyde-treated serum albumin (FSA) were labelled by covalent attachment of a TC adduct as described by Pittman *et al.* (1983). The TC adduct was kindly provided by Dr. H. Tolleshaug (Nycomed). Antibodies used were rabbit anti-(hen's egg albumin) (Bio Science Products A.G., Emmenbrucke, Switzerland) and pig anti-rabbit IgG (Nordic Immunology, Tilburg, The Netherlands). The Protein A-gold complexes (5 and 9 nm) were kindly provided by Dr. J. Slot, University of Utrecht, Utrecht, The Netherlands. Protein A was from Pharmacia A.B. All other reagents were of analytical grade.

Animals

Male Wistar rats, weighing 150–250 g, were given food and water *ad libitum*. They were anaesthetized by intraperitoneal injection of Pentobarbital (0.05 mg/g body wt.).

Design of experiments

^{125}I -TC-OVA (or ^{131}I -TC-OVA) (0.8 nmol) added together with 25 nmol of AOM, or 0.8 nmol of FSA, or alone was injected into the femoral vein. At different time points afterwards, liver lobules were tied off and cooled immediately in 0.25 M-sucrose solution containing 1 mM-Hepes, pH 7.2, and 1 mM-EDTA. Isolated rat liver cells were prepared essentially by the Seglen (1976) technique described previously (Berg & Blomhoff, 1983). The EC were also isolated as described previously (Magnusson & Berg, 1989).

Subcellular fractionation

The liver was homogenized in iso-osmotic sucrose using a Dounce homogenizer (Kindberg *et al.*, 1987). Isolated EC were homogenized in a LOX-press (Eskild *et al.*, 1989). A postnuclear fraction was prepared by centrifuging the homogenate at 2000 g for 2 min at 4 °C. The nuclear fraction was rehomogenized once. A 4 ml sample of the postnuclear fraction was layered on to linear gradients made either of Nycodenz (density ranging from

1.05 to 1.18 g/ml) or sucrose [densities ranging from 1.10 to 1.24 (or 1.32) g/ml]. The volume of the gradient itself was 34 ml. The tubes were centrifuged at 85000 g in a Beckman SW 28 rotor for 45 min (Nycodenz gradients) or 4 h (sucrose gradients) at 4 °C. After centrifugation the gradients were divided into 18 × 2 ml fractions by upward displacement using Maxidens as displacement fluid.

Enzyme and chemical assays

The densities of the fractions were calculated from the refractive indices (Rickwood, 1983). Degradation of the labelled ligand was monitored by measuring radioactivity soluble in 10 % (w/v) trichloroacetic acid. Albumin was added to a final concentration of 0.5 % (w/v) and served as a carrier during the acid precipitation.

The plasma marker enzyme 5'-nucleotidase was assayed as described by El-Aaser & Reid (1969). β -Acetylglucosaminidase and acid β -galactosidase were assayed fluorometrically as previously described in detail (Andersen *et al.*, 1983). Cathepsin B was assayed at pH 6.5 using benzyloxycarbonylarginylarginine naphthylamide methyl ester as substrate (Andersen & Dobrota, 1986). Dipeptidyl peptidase II was assayed at pH 5.5 on lysylalanine 2-naphthylamide (Andersen & McDonald, 1989).

Immunocytochemical labelling experiments

The rats were fasted overnight before injection of 23 nmol of OVA and 75 nmol of AOM. At different time points afterwards the liver was perfusion-fixed for 10 min using 2.0 % formaldehyde/0.5 % glutaraldehyde (Geuze *et al.*, 1987) in 0.2 M-Pipes

Fig. 1. Intracellular distribution of ^{125}I -TC-OVA in the liver

Postnuclear fractions prepared from total liver of rats which had received ^{125}I -TC-OVA together with AOM 1 (■, □), 6 (▲, △) and 24 (●, ○) min before liver removal were loaded on to linear sucrose gradients and centrifuged for 4 h at 85000 g. Undegraded (closed symbols) and degraded (open symbols) ligand were determined in each fraction and presented as a percentage of total recovered radioactivity from the gradient as function of the density of the fraction, except for the three upper fractions, which contain material not entering the gradient itself.

with 2 mM- CaCl_2 , pH 7.4. After perfusion the liver was sliced and immersed for 1 h in the same fixative. Small blocks of liver were further processed for freezing in liquid N_2 , cryosectioned (nominal section thickness 100 nm) and immunolabelled as described by Griffiths *et al.* (1983). Double labelling was performed as described by Slot & Geuze (1984). The pig anti-rabbit antibody was used to increase the sensitivity of the method (Geuze *et al.*, 1987).

Number of experiments

All experiments were performed at least four times with similar results. In all Figures, results from one typical experiment are shown.

RESULTS

The intracellular steps in endocytosis of OVA in liver EC was studied by subcellular fractionation of the whole rat liver homogenate in sucrose gradients. ^{125}I -TC-OVA was injected intravenously together with an excess of AOM to ensure uptake of OVA exclusively in EC. Fig. 1 shows the distributions of acid-precipitable and acid-soluble radioactivity in sucrose gradients 1, 6 and 24 min after injection of the ligand. The distribution curves show three intracellular steps with which the ligand was sequentially associated in EC. In the 1 min after injection, OVA was in endocytic vesicles, banding at about 1.13 g/ml, but was rapidly transferred to denser endosomes banding at 1.17 g/ml.

Fig. 2. Intracellular distribution of ^{125}I -TC-OVA and ^{125}I -TC-FSA in the liver

Postnuclear fractions were prepared from total liver of rats which had received ^{125}I -TC-OVA together with AOM (a) or ^{125}I -TC-FSA (b) 1 (■), 6 (▲) and 24 (●) min before liver removal. The homogenate was loaded on to sucrose gradients and centrifuged for 4 h at 85000 g. Undegraded ligand was determined in each fraction and presented as a percentage of total recovered radioactivity in the gradient as a function of the density of the fraction, except for the three upper fractions, which contain material not entering the gradient itself.

At 24 min after injection and later, the ligand was recovered in organelles banding at 1.21 g/ml. These organelles were evidently lysosomes, as acid-soluble radioactivity formed from ^{125}I -TC-OVA also accumulated in this region of the gradient.

To compare the intracellular pathway of ligands taken up by the scavenger receptor with those taken up by the mannose receptor in liver EC, ^{125}I -TC-FSA (taken up by the scavenger receptor) was also injected. Liver lobules were tied off after various time points and fractionated in sucrose gradients. Fig. 2 shows the distribution of undegraded ligand in the gradients 1, 6 and 24 min after injection of the ligand. The curves show that the rapid transfer through two endosomal steps to the lysosomes is identical for ligands taken up by the scavenger receptor and the mannose receptor in liver EC.

Because EC account for only a small fraction of the total liver homogenate, a lysosomal marker enzyme for the whole liver may not be representative for the EC lysosomes. In order to verify that acid-soluble radioactivity formed from OVA really is a marker for EC lysosomes, liver cells were prepared 20 h after intravenous injection of the labelled ligand. The EC were then isolated and fractionated in sucrose gradients. Fig. 3(a) shows that the density distribution in sucrose gradients of undegraded and degraded OVA was identical for isolated EC and for whole liver. After fractionating isolated EC it was also found (Fig. 3b) that the lysosomal marker enzyme β -acetylglucosaminidase coincided with acid-soluble radioactivity. Therefore the labelled degradation products are reliable markers for the EC lysosomes.

Fig. 3. Intracellular distribution of ^{125}I -TC-OVA in isolated liver endothelial cells

Postnuclear fractions were prepared from isolated EC of a rat which had received ^{125}I -TC-OVA together with AOM 20 h before liver perfusion. The EC were isolated and fractionated in sucrose gradients. The distribution of undegraded (●) and degraded (○) ligand (a), 5'-nucleotidase (■) and β -acetylglucosaminidase (▲) (b) were determined and presented as a percentage of total recovered activity in the gradient, except for the three upper fractions, which contain material not entering the gradient itself.

Fig. 4. Immunocytochemical labelling of OVA

At 1 min after injection (parts a-c) OVA is localized in coated vesicles (CV) (localization of OVA is shown by arrows in parts a and b) and along the membrane of tubulovesicular or cisternal compartments (part c). After 6 min (part d) the ligand is concentrated in the lumen of large electron-lucent endosomes. At 24 min after injection (part e) the ligand is localized in electron-dense lysosomes (L). Abbreviations: E, endosome; N, nucleus; MV, microvilli; S, sinusoid, sD, space of Disse. The bar represents 0.1 μm .

The plasma-membrane marker enzyme 5'-nucleotidase in EC was distributed with an activity peak at about 1.13 g/ml in sucrose gradients.

Immunocytochemical labelling of OVA on ultrathin cryosections of rat liver showed that the ligand was found in coated vesicles 1 min after injection (Figs. 4, parts a and b) and closely associated with the membrane of tubulovesicular or cisternal compartments (Fig. 4, part c). After 6 min the labelling was in large electron-lucent vesicles and was concentrated in the lumen of the vesicles (Fig. 4, part d). At 24 min after injection, the labelling was found in electron-dense organelles that ultrastructurally resemble lysosomes (Fig. 4, part e).

Lysosomes, and maybe endosomes, are endowed with an osmotic space impermeable to sucrose. The density distributions of such organelles will differ in the hyperosmotic sucrose gradients and in iso-osmotic gradients formed with, e.g., Nycodenz or Percoll. Conceivably, organelles that do not separate in the

hypo-osmotic sucrose gradients may do so in iso-osmotic gradients (and vice versa).

Fig. 5 shows the results obtained by fractionating liver homogenates in Nycodenz gradients after intravenous injection of ^{125}I -TC-OVA. Whereas fractionation in sucrose gradients (Fig. 3a) revealed only one peak of acid-soluble radioactivity banding at 1.21 g/ml, the data obtained using Nycodenz gradients suggested that degradation of ^{125}I -TC-OVA took place in two lysosomes with different buoyant densities. Early after injection of ^{125}I -TC-OVA the degradation products were banding at about 1.11 g/ml. Later (60 min and onwards) there was clearly a dual distribution of degradation products; in addition to the peak at 1.11 g/ml, another peak appeared about 1.14–1.15 g/ml. The latter peak increased in size with time after injection. However, even after 24 h a significant proportion of the degradation products was still seen at lower densities.

The distribution of acid-soluble degradation products after

Fig. 5. Distribution of degradation products in Nycodenz gradients

Postnuclear fractions were prepared from total liver of rats which had received ^{125}I -TC-OVA together with AOM (a) or ^{125}I -TC-FSA alone (b) 24 min (●), 60 min (△), 4 h (○) and 20 h (▲) before liver removal. The livers were homogenized, then fractionated in Nycodenz gradients. Degraded ligand was determined in each fraction and presented as a percentage of total acid-soluble radioactivity in the gradient as function of the density of each fraction.

Fig. 6. Subcellular fractionation of whole liver after injection of invertase

^{125}I -TC-OVA, together with AOM, were injected into rats. After 30 min, invertase (0.9 mg/100 g body wt.) was injected. Cytoplasmic extracts were prepared, then fractionated in sucrose gradients 2 h

Fig. 7. Subcellular distribution of newly degraded ligand in a rat that had received invertase 20 h in advance

^{125}I -TC-OVA, together with AOM, was injected into a rat 30 min before invertase (0.9 mg/100 g body wt.). ^{131}I -TC-OVA, together with AOM, was injected 20 h after invertase, and 24 min before a cytoplasmic extract was prepared and fractionated in a sucrose gradient. Acid-soluble radioactivity formed from ^{125}I -TC-OVA (○) and ^{131}I -TC-OVA (△) was determined in each fraction and presented as percentage of total activity in the gradient as a function of the density of each fraction.

injection of ^{125}I -TC-FSA (Fig. 5b) was identical with that observed for ^{125}I -TC-OVA.

Yeast invertase is taken up efficiently by the mannose receptor in rat liver EC. It is not readily degradable and accumulates in the EC lysosomes and renders these organelles considerably denser in sucrose gradients (Jadot *et al.*, 1985). Therefore, if the intracellular degradation in EC is a two- or multi-step process, one should expect that injection of invertase might separate 'early' and 'late' lysosomes in sucrose gradients.

We first determined the effect of invertase injection on the density distribution of EC lysosomes; labelled degradation products formed from ^{125}I -TC-OVA served as markers for the EC lysosomes. The labelled ligand was injected in advance and invertase was injected at a time when the EC lysosomes were labelled with acid-soluble radioactivity.

The results obtained (Fig. 6) indicated clearly that invertase changed the density distribution of acid-soluble radioactivity. To our surprise the injection of invertase led to a dual distribution of the lysosomal marker: two peaks of radioactivity were seen, at about 1.21 g/ml and 1.26 g/ml. Invertase gradually lead to accumulation of radioactivity in the denser lysosome; however, a steady state was reached after 6 h. Subsequently the relative size of the two peaks remained constant.

We then injected ^{125}I -TC-OVA into animals that had received

(●), 6 h (▲), 12 h (■) and 20 h (▼) after injection of invertase and 20 h after injection in a control rat (○). The distribution of degradation products was determined and plotted against the density of each fraction.

Fig. 8. Distribution of lysosomal marker enzymes in isolated EC

Invertase was injected 20 h before isolation, homogenization and fractionation of EC in sucrose gradients. All fractions were assayed for β -acetylglucosaminidase (▼), cathepsin B (▽), acid β -galactosidase (○) and dipeptidyl peptidase II (●) and presented as a percentage of the total activity in the gradient as a function of the density of each fraction.

invertase 24 h in advance and monitored the subcellular distribution of acid soluble radioactivity by sucrose-gradient centrifugation. The results clearly indicated that degradation products first appeared in the buoyant, and then in the dense, lysosome. Fig. 7 shows that, 24 min after injection, most of the degradation products are in the more buoyant organelle banding at 1.21 g/ml, whereas more acid-soluble radioactivity is associated with the denser peak after 24 h.

The subcellular distribution of radioactive degradation products seen after fractionation by means of gradient centrifugation clearly indicated that two types of lysosomes are involved in the degradation of ^{125}I -TC-OVA and ^{125}I -TC-FSA in rat liver EC.

To obtain further information about possible functional differences between the two lysosomal populations all fractions were assayed for lysosomal marker enzymes that typically have been reported to express lysosomal heterogeneity (Andersen & Dobrota, 1986; Andersen & McDonald, 1989). Invertase was injected 20 h before EC isolation, homogenization and fractionation in sucrose gradient. The results obtained using this model (Fig. 8) gave the same distribution pattern for the lysosomal enzymes measured and the acid-soluble radioactivity.

DISCUSSION

Subcellular fractionation, using density gradients, and immunocytochemistry in combination with electron microscopy, have in the present study demonstrated that OVA, after binding to the mannose receptor in EC, passes several endosomal and lysosomal steps in EC.

Electron microscopy revealed that the ligand was internalized into coated vesicles and then appeared in relatively large, electron-lucent endosomes. The ligand was closely associated with the limiting membrane of the endosomes, indicating its association with the mannose receptor at this stage. Subcellular fractionation done 1 min after injection of ligand revealed that these 'early' endosomes banded at about 1.14 g/ml in sucrose gradients.

After 6 min the ligand appeared in a vesicle banding at 1.17 g/ml in sucrose gradients. Electron-microscopic studies at this time point showed that this organelle was an electron-lucent large endosome. The labelling was concentrated in the lumen of the vesicle, indicating that the ligand had dissociated from the receptor. A similar structure has been described previously (Mommaas-Kienhuis *et al.*, 1985) and resembles CURL ('Compartment of Uncoupling of Receptor and Ligand') described in liver PC (Geuze *et al.*, 1984).

Electron-microscopic studies of the 24 min specimen revealed that the Protein A-gold-labelled antibodies bound to electron-dense lysosome-like structures. These structures have been identified as lysosomes by using antibodies against lgp 120 (Stang *et al.*, 1989) and cathepsin D (Stang *et al.*, 1990). A lysosomal localization of the ligand after 24 min was supported by the subcellular-fractionation experiments. Both degraded and undegraded ligand banded at 1.21 g/ml in sucrose gradients. Also, subcellular fractionation of isolated EC in sucrose gradients gave distributions of lysosomal marker enzymes such as β -acetylglucosaminidase, co-sedimenting with the labelled degradation products formed from ^{125}I -TC-OVA, which is consistent with the lysosomal nature of this compartment.

Although fractionation in sucrose gradients showed only one peak for the lysosomal markers, fractionation by means of iso-osmotic Nycodenz gradients revealed two populations of lysosomes identified by means of labelled degradation products. Two populations of EC lysosomes were also revealed by sucrose-density-gradient centrifugation after injection of large amounts of yeast invertase. This glycoprotein binds to the mannose receptor in EC. Provided large amounts are injected, invertase accumulates in lysosomes and renders them denser in sucrose gradients. When ^{125}I -TC-OVA was injected into rats that were given invertase 24 h in advance, it was found that the labelled degradation products banded at two densities in the sucrose gradient, at 1.21 and 1.26 g/ml. The distribution of acid-soluble radioactivity in Nycodenz gradients various times after injection of OVA indicated that degradation started in the buoyant lysosome and continued in the denser one. A similar degradation sequence has recently been demonstrated for formylated FSA in EC by Misquith *et al.* (1988) using iso-osmotic Percoll gradients and formaldehyde-treated FSA labelled with ^{125}I -TC. They found that two groups of lysosomes were sequentially involved in degradation of FSA, and they named these organelles 'transfer lysosomes' and 'accumulation lysosomes'. Our data are in full agreement with theirs.

Although one lysosome may act as a 'transfer lysosome' and the other as an 'accumulation lysosome', the two organelles are not simply connected in series: the degradation products were never completely transferred to the denser lysosome at the expense of the buoyant lysosome. Instead, a 'steady state' was reached in which the relative amounts in the two organelles were stable. This observation is compatible with a model in which material is recycled from the 'accumulation lysosome' to the 'transfer lysosome'. Such a recycling could be a reflection of recycling of membrane between the two types of lysosomes, and the content marker of the 'accumulation lysosome' would follow vesicles budding off from this organelle. Obviously membrane brought into the secondary lysosome by the endocytic pathway must somehow be removed, by for instance, a budding-off process.

The present results indicate, in agreement with data from Wattiaux' group (Misquith *et al.*, 1988), that lysosomal degradation in liver EC is a two-step process; degradation may be initiated in a 'transfer lysosome' and completed in an 'accumulation lysosome'. Such an intracellular digestive system would be more flexible than a process in which endocytosed

material were transported directly to one secondary lysosome. A two- or multi-step system would prevent constipation by undegradable material in the 'transfer lysosome'. Less digestible material (such as invertase) would be transferred to the 'accumulation lysosome' and, at the same time, the 'transfer lysosomes' would be available for new substrate. Possibly the two-step degradation would favour the extremely rapid uptake of endocytosed material in the EC.

Electron microscopy has so far not demonstrated more than one type of lysosome in the EC; it would be of interest in further studies to identify components (enzymes, receptors) that were unique for one of these putative organelles.

Several earlier studies demonstrated that degradation of endocytosed ligand may take place prelysosomally, and we have shown previously that degradation of asialoglycoproteins in rat hepatocytes may be initiated in endosomes (Berg *et al.*, 1985). A similar process has recently been described by Wattiaux *et al.* (1989). Stahl and co-workers have shown that degradation of mannosylated albumin may be initiated by cathepsin D in early endosomes in alveolar macrophages (Diment *et al.*, 1988). We did not demonstrate prelysosomal degradation of OVA in the EC. However, larger acid-insoluble degradation products might have appeared, and it has recently been shown that FSA is indeed partly degraded in the endosomal compartment of rat liver EC (Misquith *et al.* 1989).

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