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Extremely rapid endocytosis mediated by the mannose receptor of sinusoidal endothelial rat liver cells

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Isolated sinusoidal endothelial rat liver cells (EC) in suspension bound and internalized ovalbumin, a mannose-terminated glycoprotein, in a saturable manner. The binding and uptake were Ca^{2+}-dependent and were effectively inhibited by α-methyl mannoside and yeast mannan, but not by galactose or asialoglycoproteins. This corresponds to the binding specificity described for the mannose receptor of macrophages and non-parenchymal liver cells. Binding studies indicated a surface pool of 20000–25000 mannose receptors per cell, with a dissociation constant of 6×10^{-8} M. Uptake and degradation of ovalbumin by isolated EC were inhibited by weak bases and ionophores which inhibit acidification of endocytic vesicles and dissociation of receptor–ligand complexes. Cycloheximide had no effect on uptake or degradation. Degradation, but not uptake, was inhibited by leupeptin. We conclude that ovalbumin dissociates from the mannose receptors in the endosomal compartment and the receptors are recycled to the cell surface, while the ovalbumin is directed to the lysosomes for degradation. A fraction of the internalized ovalbumin was recycled intact to the cell surface and escaped degradation (retroendocytosis). The rate of internalization of ovalbumin by isolated EC was very fast, with a K_e (endocytotic rate constant) of 4.12 min^{-1}, which corresponds to a half-life of 10 s for the surface pool of receptor–ligand complexes. To our knowledge, this is the highest K_e reported for a receptor-mediated endocytosis system.

INTRODUCTION

Cell-surface receptors which recognize and mediate the internalization of glycoproteins or glycoconjugates with terminal non-reducing mannose, L-fucose or *N*-acetylglucosamine residues (mannose receptors) have been extensively studied in macrophages of different mammalian tissues (Stahl *et al.*, 1984). In particular macrophages bind and internalize several lysosomal enzymes by mannose receptors (Stahl *et al.*, 1978; Shepherd *et al.*, 1981; Stahl & Gordon, 1982). Upon intravenous administration, however, lysosomal enzymes are mainly cleared from the rat circulation by the non-parenchymal cells of the liver (Schlesinger *et al.*, 1978; Hubbard *et al.*, 1979; Steer & Clarenburg, 1979). Of the non-parenchymal liver cells, the sinusoidal endothelial cells (EC) have been found to be the most active in binding and internalization of mannose-terminated ligands (Hubbard *et al.*, 1979; Summerfield *et al.*, 1982). More recently, mannose receptors have been demonstrated on rat alveolar macrophages as well as Kupffer cells and EC of rat liver by the same immunocytochemical methods, and the mannose receptors of those tissues are therefore now believed to be identical (Haltiwanger & Hill, 1986*a*,*b*).

Lysosomal enzymes that are secreted or leak out from damaged cells must be effectively removed from the extracellular space, in order to diminish tissue degradation (Stahl *et al.*, 1984). Furthermore, it has been claimed that mannose receptors participate in the binding and phagocytosis or killing of yeast cells (Warr, 1980; Schwocho & Moon, 1981; Karbassi *et al.*, 1987), parasitic micro-organisms (Chang, 1981) and certain tumour cell lines (Brunda *et al.*, 1983). These putative functions are compatible with the hypothesis that carbohydrate-specific receptors on phagocytic vertebrate cells participate in a non-immune cellular defence system (Weir & Ögmundsdóttir, 1977). Furthermore, the mannose receptor binds and mediates the internalization of the toxin ricin (Skilleter *et al.*, 1981; Simmons *et al.*, 1986), and may also have a potential function in cellular recognition or adhesion (Largent *et al.*, 1984).

The EC share many characteristics of the reticulo-endothelial system, and are now assumed to be a structural and functional part of it (Praaning-van Dalen *et al.*, 1981; Pulford & Souhami, 1981; Brouwer *et al.*, 1985). We are interested in investigating the possibility that mannose receptors on the EC may constitute a non-immune defence mechanism against blood-borne invading micro-organisms and elucidating the kinetic parameters of the handling of mannose-terminated ligands by these cells. In the present study, we have studied the binding, internalization and degradation of a mannose-terminated glycoprotein by isolated EC.

EXPERIMENTAL

Experimental animals

Male Wistar rats, weighing 150–250 g, were used for all experiments. The animals were fed on ordinary lab chow *ad lib.*

Chemicals and equipment

BSA, chloroquine, collagenase (type I), cycloheximide, invertase, leupeptin, mannan and ovalbumin were

Abbreviations used: BSA, bovine serum albumin; EC, sinusoidal endothelial rat liver cells; In/Sur, ratio of internalized to surface-bound ligand; K_e, endocytotic rate constant.

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obtained from Sigma Chemical Co., St. Louis, MO, U.S.A. Monensin was obtained from Calbiochem-Behring, San Diego, CA, U.S.A., and $Na^{125}I$ was from The Radiochemical Centre, Amersham, Bucks., U.K. Nycodenz was obtained from Nycomed A/S, Oslo, Norway. All additional chemicals were of analytical grade.

Ovalbumin was labelled with ^{125}I, with sodium hypochlorite as the oxidizing agent (Redshaw & Lynch, 1974).

Separation and purification of liver cells by differential centrifugation and concentration of elutriated cell fractions were carried out in 50 ml centrifuge tubes in a Hettich Universal/K2S centrifuge. The distance from the fluid surface to the bottom of the tube was 8 cm. All centrifugal elutriation separations were carried out in a Beckman J2-21 centrifuge equipped with a Beckman JE-6B elutriation rotor with a standard separation chamber. Radioactivity was measured in a Kontron MR252 γ-radiation counter.

Preparation of isolated EC

Experimental animals were anaesthetized with pentobarbital, and total liver cell suspension was obtained by enzymic perfusion of the rat liver (Berry & Friend, 1969; Seglen, 1976), by a modified two-step procedure (Berg & Blomhoff, 1983). The liver cell suspension was filtered through nylon gauze, and the hepatocytes were sedimented by centrifugation for 2 min at 16 ***g***. The pellet was resuspended in incubation buffer (Tolleshaug *et al.*, 1977) and the centrifugation repeated. Non-parenchymal liver cells were sedimented from the supernatants by centrifugation for 4 min at 310 ***g***. The cells were resuspended in 50 ml of incubation buffer containing 1 % BSA, and centrifuged for 2 min at 16 ***g***, and the resulting supernatant was finally centrifuged for 4 min at 310 ***g***. The pellet consisted mainly of non-parenchymal cells.

Remaining hepatocytes were quantitatively removed from the non-parenchymal liver cells by centrifugal elutriation at 1500 rev./min. Non-parenchymal cells were collected in 100 ml at a flow rate of 25 ml/min and separated further by centrifugal elutriation at 2500 rev./min. The EC were collected in 100 ml at a flow rate of 22 ml/min.

For further purification, the EC were resuspended in 20 % (w/v) Nycodenz in incubation buffer (1.12 g/ml), carefully overlaid with incubation buffer containing 1 % BSA, and centrifuged for 15 min at 1450 ***g*** (3800 rev./min). The EC were collected from the interface, washed and resuspended in incubation buffer containing 1 % BSA. The yield of elutriated EC was typically 8×10^6–12×10^6 cells/g of liver (wet wt.).

Cell viability was assessed by the Trypan Blue exclusion test (Berg *et al.*, 1972). The Nycodenz purification step yielded routinely essentially 100 % viable cells. For cytochemical identification of different non-parenchymal liver cell types, cell preparations were stained for endogenous peroxidase activity (Knook & Sleyster, 1977). Contamination of EC with Kupffer cells was consistently found to be less than 1 %.

Experimental design

Suspensions of elutriated EC in incubation buffer containing 1 % BSA were incubated in sealed Erlenmeyer flasks in the presence of ^{125}I-labelled ovalbumin and various concentrations of unlabelled ovalbumin, with or without addition of possible inhibitors. Binding studies were carried out at 4 °C. Samples of the incubation mixtures were removed at various time points, the cells were washed three times with incubation buffer containing 1 % BSA, and cell-associated radioactivity was measured. Uptake and degradation studies were carried out at 37 °C. Samples of the incubation mixtures were layered on top of a mixture of dibutyl phthalate and dinonyl phthalate (3:1, v/v), centrifuged for 30 s at 2000 ***g***, and radioactivity associated with the cell pellets was measured. Samples of the supernatants were precipitated with an equal volume of 4 % (w/v) phosphotungstic acid, and both supernatant and pellet were assayed for radioactivity. Uptake was defined as the sum of cell-associated radioactivity and acid-soluble radioactivity in the supernatant.

For determination of surface-bound radioactivity during uptake studies, samples of the incubation mixture were removed at various time points; the cells were washed and resuspended in ice-cold incubation buffer containing 5 mM-EGTA, and radioactivity released to the medium was measured. For retroendocytosis studies, the EC were allowed to take up ^{125}I-ovalbumin for 5 min, washed three times with ice-cold incubation buffer containing 1 % BSA and 5 mM-EGTA, incubated further at 37 °C in the absence or presence of 5 mM-EGTA, and the medium was assayed for acid-precipitable radioactivity at various time points.

RESULTS

Binding of ovalbumin to isolated EC

The binding of ovalbumin to isolated EC was greatly decreased by EGTA, yeast mannan and α-methyl mannoside (Table 1). The binding measured in the presence of these substances was defined as non-specific. Galactose had no effect on the binding (Table 1).

The time course of the specific binding showed that it reached equilibrium after 2 h (Fig. 1). The rate constant for the binding was $5.1\times10^5\ M^{-1}\cdot min^{-1}$. Pre-bound ovalbumin dissociated rapidly from the EC in the presence of EGTA, whereas in the absence of EGTA dissociation was negligible (results not shown).

The specific binding was concentration-dependent and saturable (Fig. 2*a*). The Scatchard plot (Fig. 2*b*) yielded a value of 23000 receptors per cell, with a dissociation constant of 5.9×10^{-8} M.

Table 1. Effects of possible inhibitors on binding of ovalbumin to isolated EC

For this experiment 7.1×10^6 cells/ml were incubated for 3 h at 4 °C in the presence of 1.0 nM-^{125}I-ovalbumin, and in the absence (control) or presence of the following compounds. Binding was measured in the presence of each compound and expressed as % of the control value. Each value represents the mean ± S.E.M. of triplicate assays.

Compound added	Binding (%)
None (control)	100.0 ± 9.3
EGTA (4 mM)	5.2 ± 1.0
Yeast mannan (2 mg/ml)	1.9 ± 4.1
α-Methyl mannoside (50 mM)	4.8 ± 1.9
Galactose (50 mM)	94.7 ± 4.9

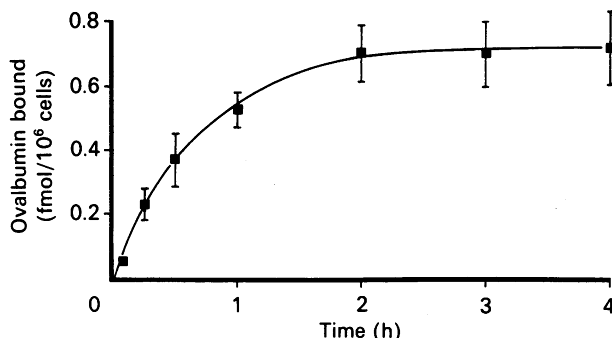

Fig. 1. Binding of ovalbumin to isolated EC as a function of time

For this experiment 8.1×10^{6} cells/ml were incubated at 4 °C in the presence of 1.0 nM-^{125}I-ovalbumin, samples were removed at the time points shown and binding was assayed as described in the Experimental section. Each point represents the mean ± S.E.M. of triplicate assays. Non-specific binding, assayed in the presence of 5 mM-EGTA, has been subtracted.

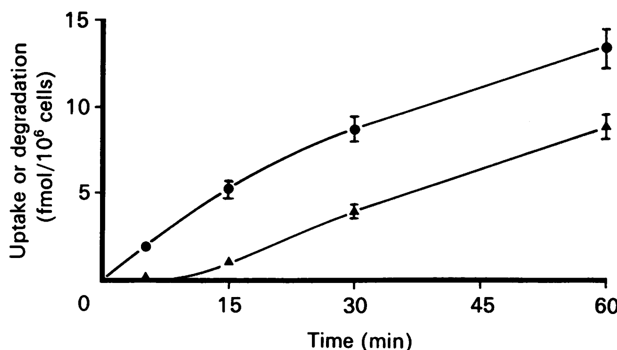

Fig. 3. Uptake and degradation of ovalbumin by isolated EC as a function of time

For this experiment, 4.2×10^{6} cells/ml were incubated at 37 °C in the presence of 1.0 nM-^{125}I-ovalbumin, samples were removed at the time points shown, and uptake (●) and degradation (▲) were assayed as described in the Experimental section. Each point represents the mean ± S.E.M. of triplicate assays. Non-specific uptake and degradation, assayed in the presence of 5 mM-EGTA, have been subtracted.

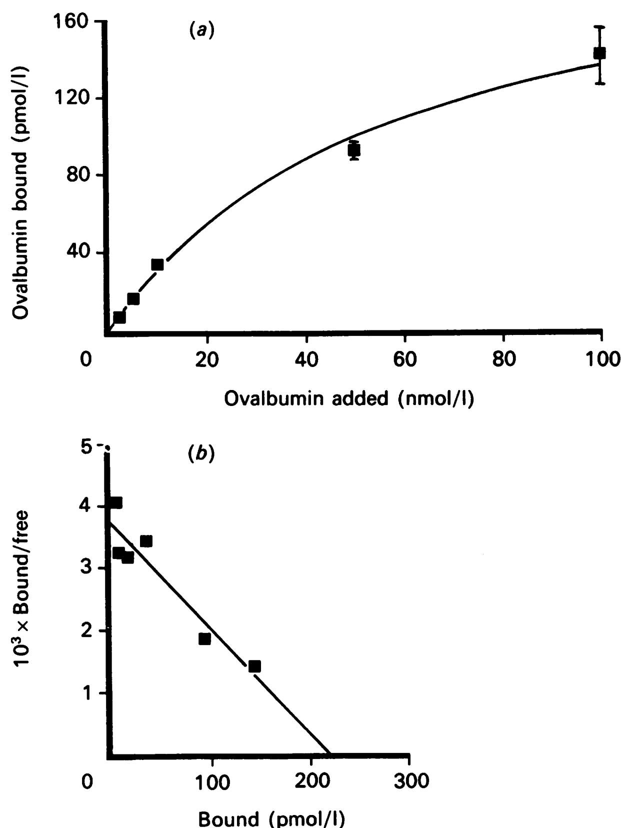

Fig. 2. Binding of ovalbumin to isolated EC as a function of concentration

For this experiment 5.7×10^{6} cells/ml were incubated for 180 min at 4 °C, in the presence of 1.0 nM-^{125}I-ovalbumin and increasing concentrations of unlabelled ovalbumin, and binding was assayed as described in the Experimental section. Each point represents the mean ± S.E.M. of triplicate assays. Non-specific binding, assayed in the presence of 5 mM-EGTA, has been subtracted. In (*a*), the amount of ovalbumin bound is expressed as a function of the ovalbumin concentration, whereas in (*b*) the same data are plotted according to Scatchard (1949).

Uptake and degradation of ovalbumin by isolated EC

Specific uptake and degradation of ovalbumin by isolated EC was approximately linear for at least 60 min (Fig. 3). Degradation products could be detected in the medium after a lag time of approx. 10 min, after which time degradation proceeded at the same rate as the uptake.

The specific uptake was concentration-dependent and saturable (Fig. 4). From a double-reciprocal plot (not shown), the maximal uptake rate was determined to be 43.5 fmol/min per 10^{6} cells, and the K_{uptake} was determined to be 190 nmol/l. Degradation was found to proceed at the same rate as the uptake up to the micromolar concentration range (results not shown), and is therefore not likely to be a limiting factor in the overall uptake and degradation process.

The effects of possible inhibitors on the uptake and degradation of ovalbumin by isolated EC are summarized in Table 2. EGTA, yeast mannan, α-methyl mannoside and *N*-acetylglucosamine were effective inhibitors of uptake, whereas mannose 6-phosphate and galactose showed only slight inhibition. The effects of all these compounds on the degradation of ovalbumin were probably merely due to the decreased uptake, rather than to any direct interaction with the degradation mechanism. Furthermore, yeast invertase, a mannose-terminated glycoprotein, was strongly inhibitory, whereas asialo-orosomucoid, a galactose-terminated glycoprotein, had no significant effect. These results confirm the carbohydrate specificity of the binding of ovalbumin to isolated EC (Table 1), and extend it to include *N*-acetylglucosamine, but exclude mannose 6-phosphate. The carbohydrate specificity and Ca^{2+}-dependence indicate that the uptake of ovalbumin in isolated EC was mediated by a receptor similar to or identical with the macrophage mannose receptor.

Formaldehyde-treated human serum albumin, a good ligand for the scavenger receptor of EC (Blomhoff *et al.*, 1984), had no effect on uptake or degradation of ovalbumin by isolated EC.

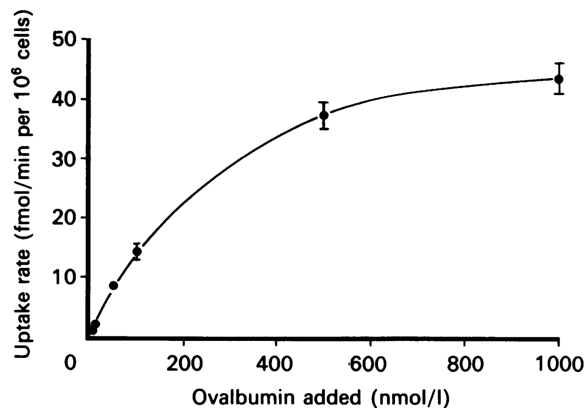

Fig. 4. Uptake of ovalbumin by isolated EC as a function of concentration

For this experiment, 4.1×10^6 cells/ml were incubated for 60 min at 37 °C, in the presence of 1.0 nM-^{125}I-ovalbumin and increasing concentrations of unlabelled ovalbumin, and uptake was assayed as described in the Experimental section. Each point represents the mean ± S.E.M. of triplicate assays. Non-specific uptake, assayed in the presence of 5 mM-EGTA, has been subtracted.

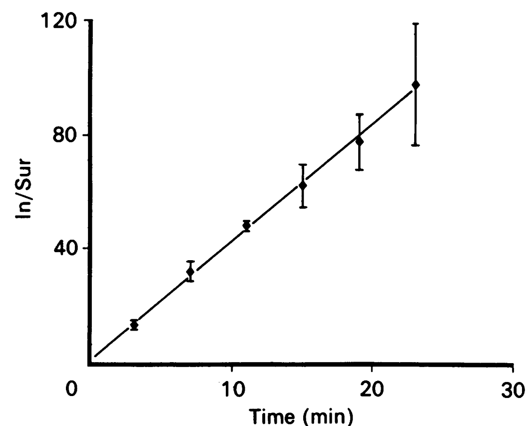

Fig. 5. In/Sur ratio for internalization of ovalbumin by isolated EC as a function of time

For this experiment, 7.1×10^6 cells/ml were incubated at 37 °C in the presence of 1.0 nM-^{125}I-ovalbumin, and samples were removed at the time points shown, internalized and surface-bound ovalbumin were assayed and the In/Sur ratio and the endocytotic rate constant (K_e) calculated as described in the Experimental Section. Each point represents the mean ± S.E.M. of triplicate assays.

Table 2. Effects of possible inhibitors on the uptake and degradation of ovalbumin by isolated EC

For this experiment 4.2×10^6 cells were incubated for 1 h at 37 °C in the presence of 1.0 nM-^{125}I-ovalbumin and in the absence (control) or presence of the following compounds. Uptake and degradation were measured in the presence of each compound and expressed as % of the control values. Each value represents the mean ± S.E.M. of triplicate assays.

Compound added	Uptake (%)	Degradation (%)
None (control)	100.0 ± 16.0	100.0 ± 16.0
EGTA (5 mM)	12.0 ± 1.9	12.4 ± 2.0
Yeast mannan (50 mM)	10.6 ± 1.7	10.7 ± 1.7
α-Methyl mannoside (50 mM)	11.1 ± 1.8	11.9 ± 1.9
N-Acetylglucosamine (50 mM)	26.4 ± 4.2	27.2 ± 4.4
Mannose 6-phosphate (50 mM)	80.5 ± 12.9	77.5 ± 12.4
Galactose (50 mM)	73.9 ± 11.8	71.1 ± 11.4
Invertase (100 nM)	21.0 ± 3.3	19.7 ± 3.2
Asialo-orosomucoid (100 nM)	99.8 ± 16.0	86.8 ± 13.9
Formaldehyde-treated human serum albumin (100 nM)	113.5 ± 18.1	98.5 ± 15.8
NH_4Cl (10 mM)	8.5 ± 1.4	4.1 ± 0.7
Chloroquine (100 μM)	14.9 ± 2.4	4.5 ± 0.7
Monensin (5 μM)	5.6 ± 0.9	6.1 ± 1.0
Leupeptin (50 μg/ml)	82.5 ± 13.2	52.1 ± 8.3
Cycloheximide (10 μg/ml)	93.1 ± 14.9	87.7 ± 14.3

NH_4Cl, chloroquine and monensin, compounds that are known to interfere with acidification of endosomes and lysosomes, dissociation of receptor–ligand complexes and receptor recycling, inhibited uptake and degradation effectively. This indicates that endocytosis of ovalbumin via the EC mannose receptor follows a classical endocytic pathway. Leupeptin, which inhibits lysosomal cysteine proteinases (Umezawa, 1977), did not inhibit the uptake significantly, but decreased the degradation to about half of the control values. Cycloheximide had little effect on uptake or degradation, suggesting that internalized receptors are recycled to the plasma membrane and reutilized.

Endocytotic rate constant

An In/Sur plot (Wiley & Cunningham, 1982) was constructed to determine the endocytotic rate constant (K_e) for internalization of ovalbumin by isolated EC (Fig. 5). From the In/Sur plot, the K_e was found to be 4.12 min^{-1}, yielding a half-time of 10.1 s for internalization of the surface pool of receptor–ligand complexes.

Retroendocytosis of ovalbumin

Fig. 6 shows the release of acid-precipitable ovalbumin from pre-loaded and washed isolated EC in the presence of EGTA. About 8 % of the internalized ovalbumin was released from the cells in acid-precipitable form, judged to represent intact ovalbumin, upon incubation in the presence of EGTA. In the absence of EGTA, acid-precipitable ovalbumin was released at a much lower, constant, rate. After 10–15 min, no more acid-precipitable ovalbumin was released.

DISCUSSION

The number of mannose receptors on the EC surface is about one-quarter of the number of mannose receptors on the rabbit alveolar macrophage surface (Stahl *et al.*, 1980). The lower cell-surface density of mannose receptors on EC than on macrophages may be due to a generally different distribution of receptors on the surfaces of the two cell types. Although at least some receptors are randomly distributed over the whole macrophage surface (Kolb-Bachofen *et al.*, 1982), receptors on the EC surface tend to be found exclusively in coated pits (Kolb-Bachofen *et al.*, 1986; Eskild, 1986),

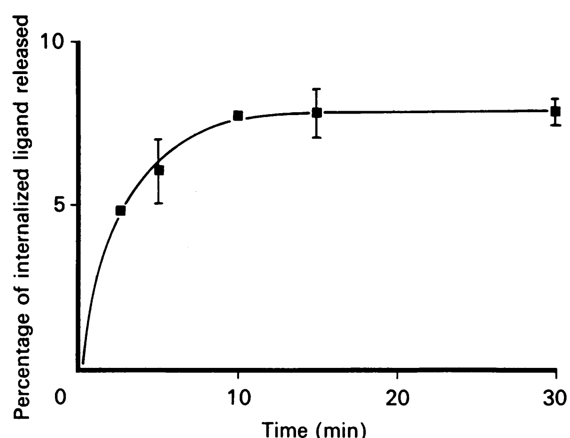

Fig. 6. EGTA-mediated release of intact internalized ovalbumin from pre-loaded EC as a function of time

For this experiment 8.9×10^6 cells/ml were incubated for 5 min at 37 °C in the presence of 1.0 nM-^{125}I-ovalbumin, washed with ice-cold incubation buffer containing 1% albumin and incubated further in the absence or presence of 5 mM-EGTA. Samples were removed at the time points shown, and acid-precipitable ovalbumin released by EGTA was assayed as described in the Experimental section and expressed as a percentage of the amount of cell-associated ovalbumin at the beginning of the latter incubation period. Each point represents the mean ± S.E.M. of triplicate assays.

which occupy only about 2% of the total cell surface in most cell types. The mannose receptor appears to be expressed to an extent comparable with that for the scavenger receptor on the EC surface (Eskild, 1986).

In most receptor systems studied, only a fraction of the total receptor population is found at the cell surface at any time, and the remainder is found on intracellular membranes. Thus 30% of the total mannose-receptor population of rat alveolar macrophages (Stahl *et al.*, 1980) and 30–40% of the total scavenger-receptor population of the EC (Eskild, 1986) are found at the cell surface. Since a large intracellular receptor pool is a prerequisite for rapid replacement of internalized receptors, it is likely that the total mannose-receptor population of the EC is significantly larger than the estimated surface pool of 23000 receptors per cell.

The EC mannose receptor exhibits similar binding characteristics to the macrophage mannose receptor

The dissociation constant for the binding of ovalbumin to the EC mannose receptor is comparable with dissociation constants reported for binding of various ligands to the macrophage mannose receptor (membrane-bound or isolated) (Stahl *et al.*, 1980; Wileman *et al.*, 1984, 1986; Lennartz *et al.*, 1987). The observed Ca^{2+}-dependency and carbohydrate specificity of the binding and uptake of ovalbumin *in vitro* by the EC also agrees well with the reported specificity of the macrophage mannose receptor (Stahl *et al.*, 1978, 1980; Shepherd *et al.*, 1981). Taken together, these results support the notion that the EC mannose receptor is identical with the macrophage mannose receptor.

Ovalbumin is internalized extremely rapidly by the EC

The rate constant for internalization of ovalbumin by EC that we report here is, to our knowledge, the highest endocytic rate constant reported. The high internalization rate may be due to either cell-specific characteristics of the endocytic apparatus or some intrinsic properties of the receptor molecules. Both the scavenger receptor of EC (Eskild, 1986) and the mannose receptor of macrophages (Hoppe & Lee, 1983) are internalized rapidly, and it is therefore difficult to predict whether the high rate of internalization of ovalbumin via the EC mannose receptor is a property of the cells or the receptors.

The physiological importance of a high internalization rate of the EC mannose receptor is not clear. In comparison with the galactose receptor of rat hepatocytes, the total surface population of mannose receptors in the liver is much smaller than the total surface population of galactose receptors (Kolset *et al.*, 1979), but the corresponding difference in endocytic capacity is partly compensated for by the high rate of internalization. The choice of a high internalization rate rather than a large surface population may offer some direct advantages, such as decreased expenditure of energy for receptor biosynthesis, but it may also render the system more sensitive to rapid and effective regulation, which may be of crucial importance in both homoeostatic regulation of extracellular enzyme concentrations and non-immune defence functions, which are among the putative functions of the mannose receptor.

Ovalbumin is degraded in the EC lysosomes

The degradation of internalized ovalbumin in the EC probably takes place in the lysosomes, as shown by the inhibitory effect of leupeptin. However, it cannot be excluded that the degradation may be initiated in the endosomal compartment, as found in some receptor-mediated endocytosis systems (Tolleshaug *et al.*, 1983; Diment & Stahl, 1985; Schaudies *et al.*, 1987). Further studies are required to clarify whether endosomal proteolysis also occurs in the EC.

Leupeptin has a less pronounced inhibitory effect on the degradation of ovalbumin in the EC than was reported for the degradation of asialoglycoproteins in rat hepatocytes (Berg *et al.*, 1981; Tolleshaug & Berg, 1981). This is in agreement with studies of the degradation of other endocytosed glycoproteins by these cells (Eskild *et al.*, 1986), and may be due to differences in the array of lysosomal enzymes found in these two cell types. Although cysteine proteinases probably are responsible for most of the lysosomal proteolysis in the parenchymal liver cells, this function may be carried out to a larger extent by other proteinases in the EC. Cathepsin D, which is an aspartic proteinase, and therefore not inhibited by leupeptin, has been reported to be present in 3 times as high concentrations in EC as in parenchymal liver cells (Ansorge *et al.*, 1984), and may be the main proteinase in the EC lysosomes. This may explain why lysosomal proteolysis appears to be less inhibitable by leupeptin in the EC than in hepatocytes.

Retroendocytosis occurs in the EC

Retroendocytosis has been shown to occur in alveolar macrophages (Tietze *et al.*, 1982), and parenchymal liver cells (Weigel & Oka, 1984), but it has not before been shown to occur in the EC. Like the endocytotic rate constant, the occurrence and extent of retroendocytosis may be either receptor-specific or cell-specific features. It is not clear whether retroendocytosis is the mechanism for, or a necessary part of, any physiological functions,

or whether it is merely a manifestation of inefficient sorting in the endosomal compartment. It is conceivable that regulation of the extent of retroendocytosis may, under certain circumstances, be of importance in the overall regulation of the endocytic pathway.

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