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Characterization of retroendocytosis in rat liver parenchymal cells and sinusoidal endothelial cells

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After receptor-mediated endocytosis, internalized ligands may be recycled to the cell surface instead of being routed to lysosomes for degradation, a process termed retroendocytosis. We have investigated the kinetics and extent of retroendocytosis of neoglycoproteins after internalization via two carbohydrate-specific receptors in rat liver cells: galactose receptors in parenchymal cells (PC) and mannose receptors in sinusoidal endothelial cells (EC). Retroendocytosis in both cell types occurred with first-order kinetics, and the rate of recycling of internalized ligands was about 4 times higher in EC than in PC. As the length of the internalization pulse was increased, the extent of subsequent retroendocytosis decreased, indicating that retroendocytosis takes place from a relatively early stage in the endocytic pathway. Furthermore, as the degree of carbohydrate substitution of the neoglycoprotein ligands increased, the affinities of the receptors for the ligands and the extent of ligand retroendocytosis increased. In the EC, the relationship between degree of substitution and extent of retroendocytosis was not immediately apparent, as some of the neoglycoprotein ligands used may also bind to and be internalized by scavenger receptors on the EC, causing a decreased apparent retroendocytosis. However, when this interaction was inhibited, this relationship was restored. We conclude that retroendocytosis mainly occurs because of incomplete dissocation of ligands from receptors before receptor recycling to the cell surface and that the affinities of a receptor for its ligand at the cell surface and in the endosomal environment are major factors in determining the extent of retroendocytosis.

INTRODUCTION

Receptor-mediated endocytosis is a process by which eukaryotic cells selectively take up macromolecules from their environment (for reviews see Gruenberg & Howell, 1989; Rodman *et al.*, 1990). It is a major pathway for ingestion of nutrients or hormones, as well as for scavenging of harmful compounds. The process of receptor-mediated endocytosis may be divided into binding, internalization, sorting, intracellular transport and processing of the endocytosed compounds, and the kinetics and characteristics of each of these events may differ to a considerable extent between cell types and receptor systems.

After internalization, many endocytosed compounds dissociate from their receptors in the endosomal compartment, and subsequently receptors and ligands are physically segregated into separate vesicles of different destinations. While many endocytic receptors are recycled back to the plasma membrane for reutilization, most of the internalized ligands are delivered to the lysosomes for degradation. However, a fraction of the internalized ligands may be recycled to the cell surface in a process called retroendocytosis. The ultimate case of retroendocytosis is the itinerary of transferrin, which on internalization releases its bound iron in the endosomal compartment and is subsequently recycled to the cell surface as apotransferrin, together with its receptor (Octave *et al.*, 1983). Also, retroendocytosis has been observed for, e.g., asialotransferrin (Tolleshaug *et al.*, 1981) and other galactose-terminated ligands (Connolly *et al.*, 1982; Weigel & Oka, 1984) endocytosed via the galactose receptor (asialoglycoprotein receptor) of rat liver parenchymal cells (PC), low-density lipoprotein in bovine aortic smooth-muscle cells (Aulinskas *et al.*, 1981) and fibroblasts (Greenspan & St. Clair, 1984), mannose-terminated ligands endocytosed via the mannose receptor of rat alveolar macrophages (Tietze *et al.*, 1982) and liver endothelial cells (Magnusson & Berg, 1989), insulin in bovine aortic endothelial cells (Jialal *et al.*, 1984), growth hormone in rat adipocytes (Roupas & Herington, 1987), and ricin in bladder carcinoma cells (McIntosh *et al.*, 1990) and rat liver endothelial cells (Magnusson *et al.*, 1991). In some cases, the recycled ligands are apparently unaltered (Simmons & Schwartz, 1984; Marshall, 1985), whereas transferrin is recycled as apotransferrin, and recycled low-density lipoprotein (Greenspan & St. Clair, 1984; Aulinskas *et al.*, 1985), insulin (Levy & Olefsy, 1986; Dahl *et al.*, 1989) and parathyroid hormone (Diment *et al.*, 1989) appear to have been subjected to proteolytic cleavage or otherwise modified before recycling.

For most ligands, retroendocytosis serves no known physiological function, transferrin being a well-known exception, but several theories have been put forward that involve retroendocytosis in physiological processes. Cast in the mould of the transferrin itinerary, a model has been proposed for the delivery of retinol (vitamin A) to cells by endocytosis of retinol in complex with retinol-binding protein followed by extraction of retinol from the complex and subsequent recycling of the apo-(retinol-binding protein) to the cell surface (Blomhoff *et al.*, 1991). Extraction of cholesterol from cells may also be achieved in a similar manner, by endocytosis of high-density lipoprotein and association of cellular cholesterol with the internalized high-density lipoprotein particles which are thereupon released from the cells (Schmitz *et al.*, 1985). Moreover, it has been suggested that internalization, processing and retroendocytosis may be necessary steps in the activation and physiological functions of

Abbreviations used: EC, liver sinusoidal endothelial cells; fBSA, formaldehyde-treated BSA; K_d, dissociation constant; PC, liver parenchymal cells; PITC, phenyl isothiocyanate. Neoglycoprotein nomenclature is as follows: PITC-glycoside derivatives of BSA are termed Gly_n-BSA and iminomethoxyethylthioglycoside derivatives of BSA are termed Gly_n-AI-BSA, where Gly = Gal or Man and n = number of glycosyl groups attached per protein molecule.

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some peptide hormones (Diment *et al.*, 1989). Finally, processing and presentation of exogenous antigens by internalization, limited proteolysis and recycling of antigen fragments to the cell surface in association with MHC class II molecules (reviewed by Braciale & Braciale, 1991) is a process highly reminiscent of retroendocytosis.

After internalization, ligands *en route* to lysosomes are thought to traverse sequentially different components of the endosomal compartment, i.e. early endosomes, intermediate transport vesicles and late endosomes (prelysosomal compartment) (Gruenberg & Howell, 1989). It is not clear which of these structures can give rise to recycling vesicles that carry receptors and ligands to the cell surface, nor have the exact mechanisms for direction of ligands to either degradation or recycling to the cell surface been elucidated. Early observations of retroendocytosis led to the proposal of a bifurcation model where receptor–ligand complexes were internalized into one of two functionally different pools: a rapidly dissociating pool that delivered ligand to the lysosomes for degradation and a non-dissociating or slowly dissociating pool which recycled intact to the cell surface (Tietze *et al.*, 1982; Weigel *et al.*, 1986). Prerequisites for such a model are either two pre-existing populations of endosomes that differ functionally, e.g. in acidification, or two receptor populations that differ in their binding characteristics or intracellular handling, as proposed for the galactose receptor of rat PC (Weigel *et al.*, 1986).

Alternatively, an incomplete dissociation model has been proposed, based on the assumption that neither dissociation nor segregation of most receptor–ligand complexes is likely to go to completion during a single pass through the endosomal compartment (Levy & Olefsky, 1986). As a consequence, a fraction of any internalized ligand will remain associated with its receptors at the time that the receptors are segregated into recycling vesicles committed for return to the cell surface and will thus undergo retroendocytosis. Moreover, the latter model predicts that efficient routing of endocytosed ligands into the degradative pathway may only be achieved through repeated cycling of receptors and ligands between the cell surface and endosomes (Dunn *et al.*, 1989).

If retroendocytosis is simply a manifestation of incomplete dissociation during a single endocytic itinerary, the extent of retroendocytosis should be inversely proportional to the efficiency of dissociation and segregation of ligands from receptors. Furthermore, it should be influenced by any attribute of the cells, receptors or ligands that can affect this efficiency, such as the affinity of the receptor for the ligand. On the other hand, routing of receptor–ligand complexes into either of two functionally different pools (bifurcation model) would not be expected to be directly dependent on the efficiency of dissociation, nor to be influenced by factors affecting this efficiency. Thus analysis of retroendocytosis of ligands with different binding characteristics under otherwise similar conditions may allow one to distinguish between the two models proposed.

In this study, we have investigated the relationship between receptor–ligand affinity and the extent of retroendocytosis of ligands endocytosed via two carbohydrate-specific receptors in rat liver: the galactose receptors of PC and the mannose receptors of liver endothelial cells (EC). We have taken advantage of the finding that the affinity of carbohydrate-specific endocytic receptors for their ligands increases with increasing carbohydrate-substitution density of the ligands (Connolly *et al.*, 1983; Hoppe & Lee, 1983). We have previously shown that the kinetics of endocytosis and intracellular transport of internalized compounds are markedly different in the different cell types under study (Magnusson & Berg, 1989; Kindberg *et al.*, 1990). Furthermore, we have studied how retroendocytosis is affected when ligands are able to interact with more than one structure (receptor) at the cell surface and in the endosomal membrane.

EXPERIMENTAL

Animals and chemicals

Male Wistar rats, weighing 120–200 g, were used for all experiments. The animals were fed standard laboratory chow *ad libitum*. Nycodenz was obtained from Nycomed A/S, Oslo, Norway, and $Na^{125}I$ was from The Radiochemical Centre, Amersham, Bucks, U.K. All other chemicals were obtained from Sigma (St. Louis, MO, U.S.A.).

Preparation of ligands

To exclude complications caused by different interactions of dissimilar ligands with the cells, the ligands that we used were neoglycoproteins constructed from BSA, differing only in their carbohydrate moieties. Neoglycoproteins were prepared by allowing BSA to react with phenyl isothiocyanate (PITC) derivatives of mannose or galactose, to a varying degree of substitution (Man_{10}-BSA, Man_{21}-BSA, Man_{30}-BSA, Gal_8-BSA, Gal_{28}-BSA) (Monsigny *et al.*, 1984). The carbohydrate content of the products was quantified by the phenol/H_2SO_4 assay (Dubois *et al.*, 1956) and protein concentration was determined as described by Bradford (1976). Neoglycoproteins prepared by the reaction of BSA with iminomethoxyethylthioglycosides (Man_{45}-Al-BSA, Gal_{44}-Al-BSA) (Lee *et al.*, 1976) were generously provided by Dr. Y. C. Lee, Johns Hopkins University, Baltimore, MD, U.S.A. Formaldehyde-treated BSA (fBSA) was prepared as described by Mego & McQueen (1965).

Proteins were radioiodinated with $Na^{125}I$ by oxidation with sodium oxychloride (Redshaw & Lynch, 1974). The specific radioactivity was 0.7×10^8–2×10^8 c.p.m./μg of protein.

Preparation of cells

Animals were anaesthetized by intraperitoneal injection of pentobarbital (0.05 mg/g body wt.) and total liver cell suspensions were prepared by collagenase perfusion of the liver and dispersion of the liver cells in ice-cold incubation buffer (Berg & Blomhoff, 1983). Isolated PC and EC were prepared by centrifugal elutriation. PC were sedimented by centrifugation for 2 min at 16 *g*, resuspended in ice-cold incubation buffer containing 1 % BSA, and the remaining non-parenchymal cells were removed by centrifugal elutriation at 1200 rev./min in a Beckman JE 5.0 elutriation rotor, equipped with a standard separation chamber. Fractions eluted at flow rates of 25 ml/min or less were discarded, and essentially pure PC fractions were then collected at a flow rate of 60 ml/min. Centrifugal elutriation and Nycodenz purification of EC was carried out as described previously (Magnusson & Berg, 1989). The viability of PC and EC was routinely approx. 90 and 99 % respectively as determined by Trypan Blue exclusion.

Experimental design

All experiments were carried out with suspensions of freshly isolated cells in incubation buffer containing 1 % BSA kept in sealed Erlenmeyer flasks on a shaking water bath adjusted to the appropriate temperature. The cells were incubated at 37 °C for 30 min before the start of each experiment to stabilize their surface content of receptors (Weigel & Oka, 1983). Subsequently, the incubation temperature was shifted to 4 °C for binding studies or maintained at 37 °C for uptake and retroendocytosis studies respectively. Each experiment was initiated by addition of ^{125}I-labelled ligands to the incubation mixture. When used, additional effector compounds were added to the cells 15 min before addition of the labelled ligands.

Binding studies. For determination of binding parameters, cells were incubated with increasing concentrations of ^{125}I-labelled neoglycoproteins for 90 min at 4 °C, which was found to be sufficient for the binding of neoglycoproteins to the cells to reach equilibrium. Triplicate samples of the incubation mixture were removed, the cells were washed three times with ice-cold incubation buffer containing 1 % BSA and cell-associated radioactivity was measured.

Internalization studies. For studies of internalization, EC were incubated with ^{125}I-labelled proteins at 37 °C, samples of the incubation mixture were removed at various time-points and the cells were centrifuged through a mixture of dibutyl phthalate and dinonyl phthalate (3:1, v/v). After centrifugation, samples of the supernatant (medium) were precipitated by addition of an equal volume of 20 % (w/v) trichloroacetic acid. Radioactivity associated with the cell pellets and acid-soluble radioactivity in the supernatant (degradation products) was measured, and total internalization was defined as the sum of cell-associated radioactivity and acid-soluble radioactivity in the medium.

Retroendocytosis studies. Since recycled ligands may be internalized anew on reaching the cell surface, the true extent of retroendocytosis of an internalized 'pulse' of ligand must be determined in conditions that prohibit association of the ligand with its receptor at the cell surface during the 'chase' period. When binding and internalization is blocked, recycled ligand will be released from the cells to the medium. As the galactose- and mannose-specific receptors studied here are Ca^{2+}-dependent, addition of EGTA to the medium abolishes their binding activity and thus allows quantification of retroendocytosis.

For studies of retroendocytosis, cells were incubated with ^{125}I-labelled neoglycoproteins for various lengths of time at 37 °C (pulse), washed three times with ice-cold dissociation buffer (incubation buffer containing 5 mM-EGTA) and incubated further in incubation buffer or dissociation buffer at 37 °C (chase). Samples of the incubation mixture were removed at various time-points, the cells were centrifuged through dibutyl phthalate (PC) or a mixture of dibutyl phthalate and dinonyl phthalate (3:1, v/v) (EC), and portions of the supernatant (medium) were precipitated by addition of equal volumes of 20 % trichloroacetic acid. Acid-precipitable radioactivity in the medium was measured (taken to represent retroendocytosed ligand) and expressed as percentage of total cell-associated radioactivity at the start of the chase period. Samples of the medium at the latest chase time points were analysed by SDS/PAGE (Laemmli, 1971) and autoradiography.

RESULTS AND DISCUSSION

Kinetics of retroendocytosis

The kinetics of release of endocytosed Gal_{28}-BSA from PC and Man_{21}-BSA from EC are shown in Fig. 1. Internalized neoglycoproteins were released rapidly to the incubation medium during the first minutes of the chase, but after about 30 and 10 min for the PC and EC respectively the release levelled off (Figs. 1*a* and 1*b*). SDS/PAGE showed that the M_r of the released neoglycoproteins was unchanged (not shown). Transforming the data according to the equation $y = \ln[1-(\text{obs.}/\text{limit})]$ (obs. is the measured release at each time point, and limit is the maximal release) showed that retroendocytosis from both cell types occurred with first-order kinetics (Fig. 1*c*). For both cell types, the rate of release was the same for all ligands tested (not shown), indicating that it is an attribute of the cells rather than the ligands. This rate presumably reflects the rate of receptor recycling from the endosomal compartment to the cell surface, independent of which of the two proposed models best describes the mechanisms leading to retroendocytosis. The rate of release was about four times higher in the EC than in the PC (rate constants of 0.34 min^{-1} and 0.083 min^{-1} respectively). We have shown previously that the rate of internalization in the EC is very high (Eskild *et al.*, 1989; Magnusson & Berg, 1989) and that transport from endosomes to lysosomes is much faster in the EC than in the PC (Kindberg *et al.*, 1990). In addition, the present data show that recycling of ligands (and presumably receptors) from the endosomes to the cell surface is considerably faster in the EC than in the PC.

Effect of pulse length on extent of retroendocytosis

In both cell types, the extent of retroendocytosis was significantly decreased by increasing the pulse length (Table 1). As the pulse is prolonged, a larger proportion of the internalized ligand therefore appears to become unavailable for retroendocytosis. This result is easily accommodated with either of the proposed models for retroendocytosis, assuming that retroendocytosis takes place from a relatively early stage in the endocytic pathway (presumably early endosomes) and that any internalized ligand that is transported beyond this stage to later compartments (late endosomes or lysosomes) is denied access to the recycling pathway and becomes irreversibly destined for the degradative pathway.

Under similar conditions (pulse length, affinity for ligand etc.) the extent of retroendocytosis was consistently higher from PC than from EC (e.g. Fig. 1*a* and *b*). This agrees well with our earlier finding that intracellular transport is much faster in the EC than in the PC (Kindberg *et al.*, 1990). As a consequence of the difference in transport kinetics, less of the internalized ligand has been transported beyond the stage competent of recycling at the end of the pulse in the PC than in the EC and correspondingly more is available for recycling.

Effect of affinity on the extent of retroendocytosis

According to the incomplete-dissociation model, the affinity of a receptor for its ligand in the endosomal environment is likely to be an important factor in determining the extent of retroendocytosis. Even in conditions that favour dissociation of ligands from receptors, such as low endosomal pH, high-affinity ligands presumably remain bound to their receptors to a larger extent than low-affinity ligands and should therefore also be recycled to a larger extent. On the other hand, the bifurcation model predicts that similar ligands, albeit with subtle differences in affinity, would partition identically into the pre-existing functionally different intracellular pools, and it cannot easily be envisaged how differences in affinity would affect this partitioning.

In agreement with earlier data (Connolly *et al.*, 1983; Hoppe & Lee, 1983), the affinities of the two carbohydrate-specific receptors for the neoglycoproteins used in this study increased with increasing carbohydrate-substitution density of the neoglycoproteins, when measured at neutral pH (Table 2). Supposedly, the higher affinities observed for the higher-substituted neoglycoproteins are due to interactions of more carbohydrate determinants on each neoglycoprotein molecule with multiple receptors, or multiple binding sites on single receptors. Although each interaction is weaker at the lower pH found in endosomes, the number of possible interactions for each neoglycoprotein is likely to be unchanged, and accordingly, although dissociation constants for the neoglycoproteins were not measured at endosomal pH, it may be assumed that the relationship between the individual affinities is similar at lower pH to that found at neutral pH. It should be noted that dissociation constants for binding of Man_{10}-BSA and Man_{30}-BSA to mannose receptors on EC were determined in the presence of excess unlabelled fBSA to

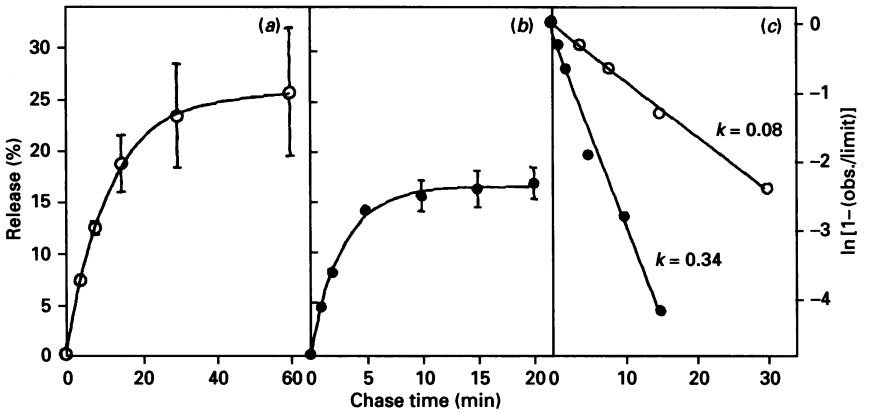

Fig. 1. Kinetics of retroendocytosis of neoglycoproteins from rat liver cells

Cells were pulsed for 10 min at 37 °C with 1.5 nM-^{125}I-labelled neoglycoproteins, washed with cold dissociating buffer and incubated further at 37 °C in dissociating buffer (containing 5 mM-EGTA). At the time-points indicated, release of endocytosed neoglycoprotein was measured as described in the Experimental section. (*a*) Release of Gal_{28}-BSA from PC; (*b*) release of Man_{21}-BSA from EC; (*c*) transformation of data in (*a*) (○) and (*b*) (●) according to the equation $y = \ln[1-(\text{obs.}/\text{limit})]$; k = rate constants for the release. The Figure shows mean values ± S.D. from at least two identical experiments.

Table 1. Effect of pulse length on the extent of retroendocytosis of neoglycoproteins from rat liver cells

Cells were pulsed for 5 or 10 min at 37 °C with 1.0 nM-^{125}I-labelled Gal_{28}-BSA (PC) or Man_{21}-BSA (EC), washed with cold dissociating buffer and incubated further at 37 °C in dissociating buffer (containing 5 mM-EGTA). Released of endocytosed neoglycoprotein was measured after 60 min (PC) or 15 min (EC), as described in the Experimental section. The values shown are means ± S.D. from at least two identical experiments.

		Release (% of cell-associated radioactivity after pulse)	
Cell type	Neoglycoprotein	5 min pulse	10 min pulse
PC	Gal_{28}-BSA	28.6 ± 1.7	19.5 ± 2.5
EC	Man_{21}-BSA	19.4 ± 2.1	11.5 ± 1.7

Table 2. Affinities of carbohydrate-specific receptors on rat liver cells for differently substituted neoglycoproteins

Rat liver cells were incubated with various concentrations of ^{125}I-labelled and unlabelled neoglycoproteins for 90 min at 4 °C, washed three times with cold incubation buffer and cell-associated radioactivity was measured. In experiments with EC, all buffers were supplemented with 150 nM-fBSA. Dissociation constants (K_d) for the binding were determined by Scatchard analysis. The values shown are means ± S.D. from at least two identical experiments; n.d., not determined.

	K_d (nM)	
Neoglycoprotein	Mannose receptor (EC)	Galactose receptor (PC)
Man_{10}-BSA	11.4 ± 3.0	n.d.
Man_{30}-BSA	2.2 ± 1.7	n.d.
Gal_8-BSA	n.d.	42.3 ± 1.6
Gal_{44}-AI-BSA	n.d.	0.63 ± 0.36

avoid interference from possible binding to scavenger receptors (see below).

After a short pulse of internalization, all the ligands tested were released from the cells to some degree (Table 3), even when binding and re-internalization of recycled ligand were allowed to take place during the chase period (i.e. in the absence of EGTA). In these conditions, lower-substituted ligands were released to a larger extent than higher-substituted ones. Indeed, as lower-substituted ligands show lower affinities (Table 2), they would be expected to dissociate more readily from active receptors than would ligands with higher affinities, to attain a higher free concentration at equilibrium. Incubation under dissociating conditions during the chase period (i.e. in the presence of EGTA) increased the release of all ligands substantially. As dissociation occurs spontaneously on exposure to EGTA, all of the recycled ligand will dissociate before the receptors are internalized anew, even in the EC, where internalization is extremely fast. The release in the presence of EGTA was therefore initially assumed to represent the total amount of ligand recycled to the cell surface.

Table 3. Effect of carbohydrate-substitution density on extent of retroendocytosis of neoglycoproteins from rat liver cells

Cells were pulsed for 2 min (EC) or 15 min (PC) at 37 °C with 1.5 nM-^{125}I-labelled neoglycoproteins as indicated, washed with cold dissociating buffer and incubated further at 37 °C in incubation buffer (control) or dissociating buffer (+EGTA). Release of endocytosed neoglycoprotein was measured after 20 min (EC) or 60 min (PC) as described in the Experimental section. The values shown are means ± S.D. from at least two identical experiments.

		Release (% of cell-associated radioactivity after pulse)	
Cell type	Neoglycoprotein	Control	+EGTA
PC	Gal_8-BSA	6.5 ± 2.1	9.5 ± 2.4
	Gal_{44}-BSA	2.0 ± 0.7	16.0 ± 2.6
EC	Man_{10}-BSA	7.7 ± 4.0	19.0 ± 4.2
	Man_{30}-BSA	1.3 ± 0.7	7.8 ± 1.8
	Man_{45}-AI-BSA	1.7 ± 0.8	34.4 ± 1.9

In the presence of EGTA, the extent of retroendocytosis from the PC increased with increasing substitution density, as predicted by the incomplete dissociation model (Table 3). On the other hand, retroendocytosis from the EC deviated from this pattern, as Man_{30}-BSA was released to a lesser extent than Man_{10}-BSA,

Table 4. Involvement of scavenger receptors in endocytosis of differently mannosylated BSA in EC

EC were incubated at 37 °C with 1.5 nM-^{125}I-labelled neoglycoproteins or fBSA as indicated and without (control) or with 5 mM-EGTA, 300 nM-fBSA or both compounds added. After 20 min, samples of the suspension were assayed for cell-associated radioactivity and acid-soluble radioactivity in the medium (degradation products), which combined to yield a measure of the total uptake. Results are expressed as percentage of control values. The values shown are means ± S.D. from at least two identical experiments.

	Release (% of cell-associated radioactivity after pulse)			
Ligand	Control	+EGTA	+fBSA	+EGTA and fBSA
Man_{45}-AI-BSA	100	2.6±2.0	97.6±1.1	2.6±0.1
Man_{10}-BSA	100	51.9±9.2	74.2±7.3	17.7±9.9
Man_{30}-BSA	100	83.2±3.6	49.2±2.1	25.3±4.7
fBSA	100	107.8±10.0	4.6±1.1	4.6±1.0

indicating that factors other than the affinity of the mannose receptors for these ligands were involved in determining the extent of their retroendocytosis from the EC.

Involvement of scavenger receptors in endocytosis of neoglycoproteins

Apart from their affinities for the receptor under study, any additional interactions of ligands with other cell surface structures may influence the characteristics of both their endocytosis and retroendocytosis. Recently, Jansen *et al.* (1991) showed that glycosylation of proteins with PITC derivatives of carbohydrates, but not with thioglycosides, increases the net negative charge of proteins, and renders them susceptible for uptake via scavenger receptors on EC. In studies of endocytosis and retroendocytosis of mannosylated BSA by the EC, this must therefore be taken into account.

The scavenger receptor is not Ca^{2+}-dependent, but can be blocked by adding an excess of fBSA, a good ligand for this receptor. The data in Table 4 show that, whereas uptake of Man_{45}-AI-BSA is completely inhibited by EGTA and not with fBSA, increasing PITC mannosylation (Man_{10}-BSA, Man_{30}-BSA) leads to less inhibition with EGTA and stronger inhibition with fBSA. This confirms that, whereas endocytosis of Man_{45}-AI-BSA takes place exclusively via mannose receptors, scavenger receptors become increasingly involved in endocytosis of PITC-mannosylated BSA in the EC as the degree of PITC substitution increases. As expected, the uptake of fBSA takes place exclusively via scavenger receptors.

Effect of scavenger receptor uptake on retroendocytosis of neoglycoproteins

As a consequence of the participation of scavenger receptors in endocytosis of PITC-mannosylated ligands, the extent of their retroendocytosis may be underestimated under the usual conditions (in the presence of EGTA). Whereas mannose receptors lose their binding activity in the presence of EGTA, the scavenger receptors are not Ca^{2+}-dependent, and will, even in these conditions, be available for binding and internalization of retroendocytosed ligand, resulting in a decreased net release of ligand from the cells. This possibility was investigated by including excess fBSA in the dissociating buffer to block surface scavenger receptors during the chase period.

Addition of fBSA, but not EGTA, during the chase period did not induce retroendocytosis of any of the ligands tested (results not shown). However, as shown in Table 5, fBSA and EGTA

Table 5. Effect of blocking scavenger receptors on the extent of retroendocytosis of differently mannosylated BSA from EC

EC were pulsed for 2 min at 37 °C with 1.5 nM ^{125}I-labelled neoglycoproteins as indicated, washed with cold dissociating buffer and incubated further at 37 °C in dissociating buffer (+EGTA) or dissociating buffer supplemented with 300 nM-fBSA (+EGTA and fBSA). Release of endocytosed neoglycoprotein was measured after 20 min as described in the Experimental section. The values shown are means ± S.D. from at least two identical experiments.

	Release (% of cell-associated radioactivity after pulse)	
Neoglycoprotein	+EGTA	+EGTA and fBSA
Man_{10}-BSA	11.3±4.4	10.4±2.2
Man_{30}-BSA	6.6±2.9	14.4±2.9
Man_{45}-AI-BSA	32.7±3.0	30.8±3.3

have an additive effect on the observed extent of retroendocytosis of Man_{30}-BSA, the ligand that was endocytosed via scavenger receptors to the largest extent (Table 4). Addition of fBSA did not increase retroendocytosis of Man_{10}-BSA, which is a poor ligand for the scavenger receptor (Table 4), or Man_{45}-AI-BSA, which is not endocytosed via scavenger receptors at all. In the EC, release of PITC-mannosylated ligand in the presence of both EGTA and fBSA therefore appears to be a better estimate of the total amount of ligand recycled to the cell surface than the release in the presence of EGTA alone. When this was taken into account, the extent of retroendocytosis from the EC, as well as the PC, was found to increase with increasing substitution density (Table 5).

As fBSA was not included in the incubation buffer during the pulse period, a considerable proportion of the Man_{30}-BSA pulse was internalized, and probably also recycled, via scavenger receptors. In contrast with the mannose receptors, the ligand-binding activity of which is abolished during the chase period by addition of EGTA, the scavenger receptors remained in an active binding state throughout the experiment. Release of recycled Man_{30}-BSA from scavenger receptors by exchange with excess extracellular fBSA on reappearance at the cell surface is therefore a competitive process and is highly dependent on the rate of dissociation of Man_{30}-BSA. Although we did not measure this dissociation rate, it is likely to be much lower than the rate of the spontaneous EGTA-mediated dissociation from the mannose receptors and the high rate of internalization reported for the scavenger receptors. Some of the Man_{30}-BSA bound to and recycled by scavenger receptors may therefore yet be internalized anew before it dissociates and, accordingly, the total release of this ligand may still be underestimated.

The different cell types that make up the rat liver exhibit considerable diversity in many of their basic cellular functions. A case in point is the difference in the kinetics of several steps of membrane transport found to exist between the PC and the EC. We have previously shown that both internalization (Magnusson & Berg, 1989) and intracellular transport (Kindberg *et al.*, 1990) are much faster in the EC than in the PC, and our present data show that this is also the case for retroendocytosis. Although this process has been studied previously in the PC (Weigel *et al.*, 1986), the present report is the first attempt at characterization of the process in the EC. Our data strongly indicate a correlation between the carbohydrate density of glycoconjugate ligands, the affinity of carbohydrate-specific receptors for these ligands and the extent of their retroendocytosis. In our view, these findings are easier to reconcile with the incomplete dissociation model

than with the bifurcation model. In retrospect, we also find that many of the data previously interpreted to be in support of the bifurcation model may alternatively be interpreted to be in support of the incomplete-dissociation model. Finally, we believe that the affinities of endocytic receptors for their ligands both at cell surfaces and in the endosomal compartment are major factors in determining the extent of retroendocytosis, although any interactions of the ligand with other cell surface components, which may or may not be genuine receptors in their own right, are likely to influence the process as well.

The model outlined here is compatible with the observed behaviour of several receptors and ligands that recycle from the endosomal compartment to the plasma membrane, ranging from transferrin, which does not dissociate in endosomes and therefore recycles quantitatively, to other ligands that dissociate readily and therefore undergo little retroendocytosis. On the other hand, other receptors may carry signals that dictate their routing along different intracellular pathways and therefore do not behave according to the described model. Good examples of this are the receptor for epidermal growth factor, which on internalization is largely directed to lysosomes and degraded (Haigler *et al.*, 1979), and the polymeric immunoglobulin receptor, which carries a signal in its cytoplasmic tail that destines it for transcytosis in polarized cells, e.g. rat PC, whereupon its ligands are released by proteolytic cleavage at the opposite plasma-membrane domain (Casanova *et al.*, 1990). A particular consequence of this different routing is that, although polymeric immunoglobulins are covalently bound to their receptors and accordingly do not dissociate from those in endosomes, they are not recycled back to the same cell surface domain, like transferrin and the neoglycoproteins studied here.

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