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Endocytosed ricin and asialoorosomucoid follow different intracellular pathways in hepatocytes

Andreas Brech [a], Rune Kjeken [a], Marianne Synnes [a], Trond Berg [a,*], Norbert Roos [a], Kristian Prydz [b]

[a] *University of Oslo, Department of Biology, Division of Molecular Cell Biology, Oslo, Norway*
[b] *Institute of Biochemistry, Postbox 1050 Blindern, 0316 Oslo, Norway*

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Abstract

Earlier studies have suggested that fluid phase endocytosis in rat hepatocytes takes place via a clathrin-independent mechanism [1,2]. This observation suggests that a relatively large amount of plasma membrane outside coated pits may be involved in hepatic endocytosis. Ricin, which binds to galactose residues on glycoproteins and glycolipids, has, in this report, been used as a general marker for the plasma membrane of hepatocytes. The endocytosis of ricin was compared with that of asialoorosomucoid (AOM) which is taken up exclusively via clathrin-coated pits. Hypertonic medium has been shown to inhibit uptake via coated pits more effectively than clathrin-independent uptake [3–5]. It was found, in this study, that the addition of 100 mM sucrose to the incubation medium inhibited the uptake of ^{125}I-tyramine-cellobiose-asialoorosomucoid (^{125}I-TC-AOM) more extensively than that of ^{125}I-tyramine-cellobiose-ricin (^{125}I-TC-ricin), compatible with the notion that the two probes are internalised via different mechanisms. Subcellular fractionation experiments indicated that ^{125}I-TC-ricin entered a denser endocytic organelle than that receiving ^{125}I-TC-AOM. To determine whether the separation of the two probes was due to a different transport kinetics (i.e. that ^{125}I-TC-ricin is transported more rapidly to a later, denser compartment than ^{125}I-TC-AOM) the cells were incubated at 18°C to allow a slower internalisation/transport of the labelled probes. The results obtained showed, again, that the early endosomes containing ^{125}I-TC-ricin were significantly denser than those containing ^{125}I-TC-AOM. We also employed the horseradish peroxidase (HRP)-diaminobenzidine (DAB) density shift technique of Courtoy et al. [6] to determine whether ^{125}I-TC-ricin and ^{125}I-TC-AOM were in separate endosomes early after their uptake. The results showed that early endosomes containing ^{125}I-TC-AOM were density shifted whereas those containing ^{125}I-TC-ricin were unaffected by the density shift procedure. The use of probes labelled with ^{125}I-TC allowed us to identify compartments involved in the degradation of ^{125}I-TC-AOM and ^{125}I-TC-ricin, by measuring acid soluble radioactivities in the gradient fractions. It was found that ^{125}I-TC-ricin was degraded mainly in endosomes, whereas ^{125}I-TC-AOM, as expected, was degraded mainly in lysosomes.

Keywords: Asialoglycoprotein receptor; Clathrin; Receptor-mediated endocytosis; Liver; Fluid phase

1. Introduction

Endocytosis was earlier thought to take place exclusively in clathrin-coated pits at the plasma membrane. More recently, it has, however, become clear

Abbreviations: AOM, asialoorosomucoid; ^{125}I-TC-AOM, ^{125}I-tyramine-cellobiose-asialoorosomucoid; ^{125}I-TC-ricin, ^{125}I-tyramine-cellobiose-ricin; HRP, horseradish peroxidase; DAB, diaminobenzidine; β-AGA, *N*-acetyl-β-D-glucosaminidase

* Corresponding author. Fax: +47 (2) 285-4726.

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that clathrin-independent endocytosis may take place in several cell types [3–5,7]. In COS-cells, clathrin-dependent and clathrin-independent endocytosis lead to the formation of primary endosomes of slightly different sizes [8,9]. The two types of endosomes evidently both fuse with the same early endosomes. A special type of clathrin-independent endocytosis is macropinocytosis which leads to the formation of large endocytic structures that may not communicate with the clathrin-dependent endocytic pathway prelysosomally [10–12].

Hepatocytes contribute to blood homeostasis by endocytosing a number of proteins present in plasma. Ligands taken up by receptor-mediated endocytosis include lipoproteins [13] (high density lipoproteins, low density lipoproteins, chylomicron remnants, β-very low density lipoprotein), peptide hormones (insulin, glucagon, epidermal growth factor) [14–16], catecholamines, haptoglobin, α_2-macroglobulin [17], asialoglycoproteins [18]. Few studies have been focused on the mode of endocytic internalisation of ligands in hepatocytes, notwithstanding the important physiological role of these processes. In addition to uptake of ligands via receptor-mediated endocytosis, the hepatocytes also internalise (macro)-molecules in the fluid phase of the forming endocytic vacuole, the primary endosome. The extent of fluid phase endocytosis in hepatocytes is uncertain, but may amount to about 0.1 μl per million cells per hour [19]. This value has been obtained using [^{14}C]sucrose, [^{3}H]raffinose or [^{125}I]polyvinylpyrrolidone as fluid phase markers. The actual uptake of fluid may be much higher, maybe 1 μl/h/10^6 cells, since a large proportion of the internalised fluid is recycled back to the extracellular medium. The amount of fluid taken up may be subject to regulation by adjusting the balance between endocytosis and retroendocytosis. It may amount to 10 ml/day in the liver of a 200 g rat.

It has been suggested that clathrin-dependent endocytosis may be selectively arrested by incubating the cells with hypertonic medium, by acidifying the cytosol, or by potassium depletion [1,20–22]. These treatments do not affect clathrin-independent endocytosis to the same extent, and may therefore be used to determine whether a given endocytic process takes place via clathrin-coated pits or via alternative mechanisms. By employing such treatments, it has been found (using [^{14}C]sucrose or lucifer yellow as fluid phase markers) that fluid phase endocytosis may not take place via coated pits in rat hepatocytes [1,2]. This result is surprising since hepatocytes are well equipped with coated pits and since some fluid, out of necessity, must be internalised via coated pits. The finding that fluid phase endocytosis is nearly unaffected by inhibitors of clathrin-dependent endocytosis therefore suggests that a very large proportion of endocytic plasma membrane invagination takes place independent of clathrin-coated pits.

In the present study, we have compared the uptake and intracellular transport in rat hepatocytes of [^{125}I]tyramine-cellobiose-labelled asialoorosomucoid (^{125}I-TC-AOM) and [^{125}I]tyramine-cellobiose-labelled ricin (^{125}I-TC-ricin). Ricin binds to numerous glycolipids and glycoproteins with terminal galactose in the plasma membrane of hepatocytes [23,24]. These binding sites are distributed all over the plasma membrane, and ricin may therefore be a useful tool to follow plasma membrane traffic in general [25]. Asialoorosomucoid, on the other hand, is taken up exclusively via clathrin-coated pits [26].

To characterise the endocytic pathways followed by the two probes, we used subcellular fractionation. We, furthermore, induced density shifts in the pathway followed by ^{125}I-TC-AOM to see if this affected the density distribution of ^{125}I-TC-ricin. Finally, we measured the uptake of the two markers under hypertonic conditions (a treatment that inhibits clathrin-dependent endocytosis) to obtain further information about the mode of uptake of the ligand and the fluid phase marker.

2. Materials and methods

2.1. Biochemicals

Vinblastine sulphate, collagenase, leupeptin, and horseradish peroxidase were obtained from Sigma (St. Louis, MO). Na^{125}I was from the Radiochemical Centre (Amersham, UK). Tyramine-cellobiose was a gift from professor Helge Tolleshaug (Nycomed, P.O. Box 4220, Torshov, N-0401 Oslo, Norway).

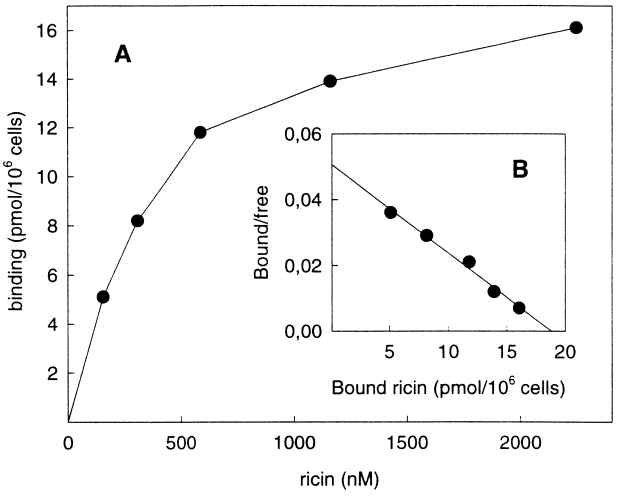

Fig. 1. Binding of ^{125}I-TC-ricin to galactose residues on rat hepatocytes at 0°C. Suspensions of rat hepatocytes (3×10^6 cells/ml) were incubated at 0°C with ^{125}I-TC-ricin at the indicated concentrations, and then washed thrice in the presence and absence of lactose (50 mM). The results represent the lactose-sensitive binding and are presented as pmol/10^6 cells in (A). In (B), the data are presented as a Scatchard plot. The results suggest that ^{125}I-TC-ricin binds to about 10^7 sites with an apparent K_d of 10^{-6} M. The figure shows results from a representative experiment.

2.2. Cell preparation and incubation

Hepatocytes were prepared from 18 h starved male Wistar rats (250–300 g) by collagenase perfusion [27]. The cells were incubated as suspensions (0.4 ml aliquots in shaking centrifuge tubes at 37°C, usually 50–75 mg wet wt/ml) in suspension buffer [27] fortified with pyruvate (20 mM) and Mg^{2+} (2 mM).

2.3. Enzyme assays

β-Acetylglucosaminidase was determined as described by Barrett [28].

2.4. Determinations of radioactivity

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

2.5. Subcellular fractionation

Cells were mixed with equal volumes of 0.25 M sucrose/10 mM HEPES/1 mM EDTA (pH 7.3) and homogenised by five strokes in a Dounce homogeniser (tight-fitting pestle). The homogenates, containing material from about 50 mg cells (wet weight)/ml, were fractionated by differential centrifugation and/or by isopycnic centrifugation in sucrose gradients. Differential centrifugation started by centrifuging a 5 ml sample of the homogenate at $2000\times g$ for 2 min, the resulting nuclear fraction (N-fraction) was resuspended in 5 ml 0.25 M sucrose/10 mM HEPES/1 mM EDTA, homogenised (five strokes) and centrifuged again at $2000\times g$ for 2 min. A large granule fraction (ML-fraction) was sedimented from the postnuclear fraction by centrifugation at $25\,000\times g$ for 9 min, and a particulate fraction

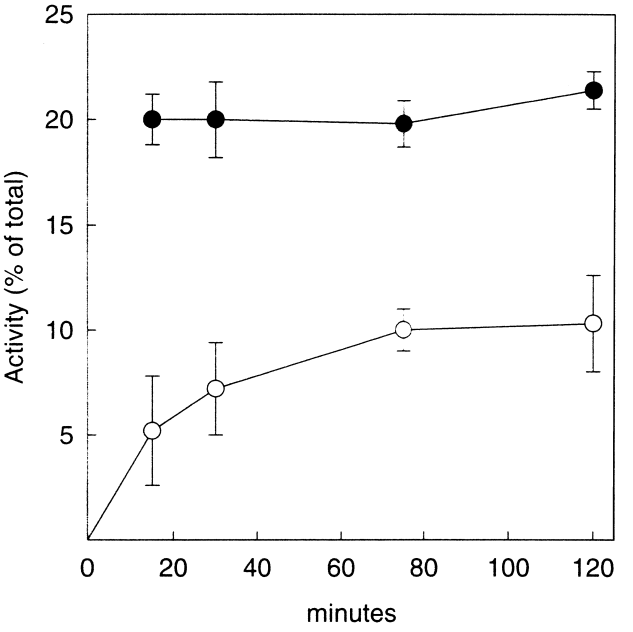

Fig. 2. Uptake of ^{125}I-TC-ricin in rat hepatocytes. Suspensions of rat hepatocytes (5×10^6 cells/ml) were incubated at 0°C in the presence of ^{125}I-TC-ricin (10 nM) for 20 min. The cells were then washed in ice-cold PBS and incubated at 37°C. Aliquots of cells were removed at the indicated times and washed thrice in PBS with or without 50 mM lactose. Cell-associated (closed symbols) and internalized (open symbols) radioactivities are presented as percent of total radioactivity added. Internalized radioactivity is calculated as the difference between total cell-associated radioactivity and radioactivity remaining in cells washed in the presence of 50 mM lactose. The figure shows mean values ± S.D. from at least two identical experiments.

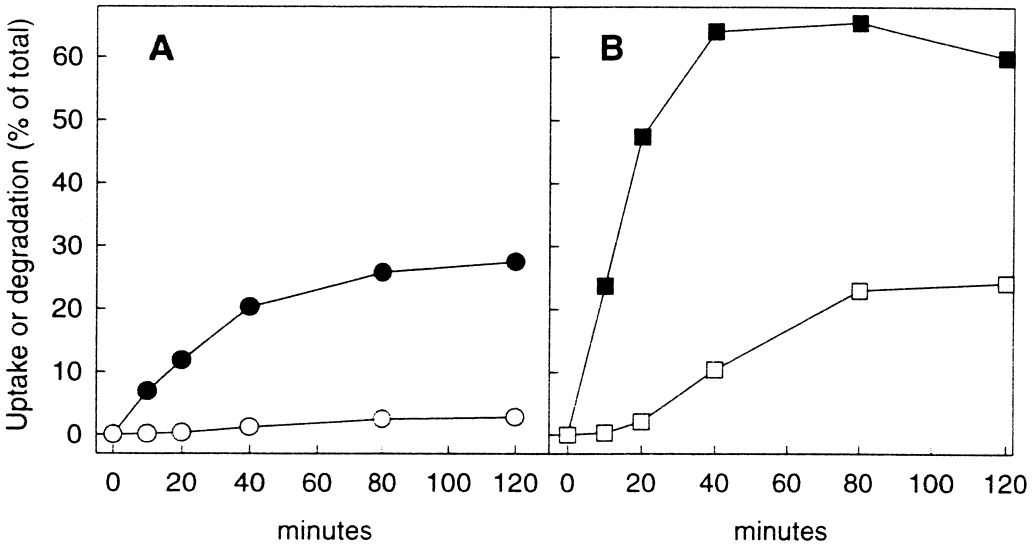

Fig. 3. Endocytosis of ^{125}I-TC-ricin and ^{125}I-TC-AOM in rat hepatocytes. Suspensions of rat hepatocytes (5×10^6 cells/ml) were incubated at 37°C in the presence of either ^{125}I-TC-ricin (A) or ^{125}I-TC-AOM (B). Aliquots (0.25 ml) of cells were removed at the indicated times, washed thrice in either 10% sucrose (^{125}I-TC-AOM) or in PBS containing 50 mM lactose (^{125}I-TC-ricin) and then treated with 10% (w/v) trichloroacetic acid. Acid soluble (open symbols) and acid precipitable (closed symbols) radioactivities were measured and the results obtained are presented as % of total radioactivity (minus acid soluble radioactivity present initially) in the cell suspension. The figure shows results from a representative experiment.

(P-fraction) was sedimented from the postmitochondrial fraction at $48\,000\times g$ for 60 min. In some experiments a MLP-fraction (high speed sediment) was prepared by centrifuging the postnuclear fraction at $48\,000\times g$ for 60 min. Differential centrifugation was carried out in a Sorvall RC-2B centrifuge using a SS24 rotor and 13 ml centrifuge tubes. Homogenisations and centrifugations were carried out at 0–4°C. In isopycnic centrifugation experiments 2–4 ml aliquots of a ML-fraction or a MLP-fraction were initially layered at the top of the gradients. The centrifuge tubes (38 ml) were centrifuged at $85\,000\times g$ in a Beckman SW 28 rotor at 4°C for 4 h. Following 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 [29].

2.6. DAB-induced density shift of endosomes

The DAB-induced density modification [6,30] was based on the endocytic uptake of AOM-HRP followed by a cytochemical reaction on isolated subcellular fractions (mostly the postnuclear fraction). Hepatocytes (2 million cells/ml) were incubated with AOM-HRP (500 nM) at 37°C for the chosen time interval. Five ml portions of cells were washed in ice-cold 10% sucrose. Postnuclear fractions (5 ml) were subsequently prepared and incubated in the presence of DAB (3.2 ml) at room temperature in the dark for 15 min. Aliquots of the PNF were then incubated in the presence and absence of H_2O_2 (36.8 μl 6% H_2O_2 added to 3.5 ml PNF) and the incubation was continued for another 30 min in the dark. The fractions were finally layered on top of linear sucrose gradients and centrifuged at $85\,000\times g$ for 4 h.

3. Results

3.1. Binding of ^{125}I-TC-ricin to isolated hepatocytes

To analyse binding of ^{125}I-TC-ricin to hepatocytes, the cells were incubated at 0°C with increasing concentrations of the labelled probes, and subsequently washed thrice in PBS in the presence and absence of 50 mM lactose. Surface bound ^{125}I-TC-ricin or ^{125}I-ricin was calculated as the difference between cell-

associated radioactivity with and without lactose in the washing medium. The results (Fig. 1) suggest that ^{125}I-TC-ricin binds to about 10^7 sites with an apparent K_d of 5×10^{-6} M. These data are comparable with the numbers found by Decastel et al. [24].

3.2. Uptake and degradation of ^{125}I-TC-ricin and ^{125}I-TC-AOM in isolated hepatocytes

To differentiate between internalised and surface-bound ^{125}I-TC-ricin, the cells were washed thrice in PBS with or without 50 mM lactose. It was found that this treatment released a maximum amount of the toxin from the cells. Incubation of cells for extended periods of time at 0°C with increasing concentrations of lactose did not alter the amount of surface-bound ^{125}I-TC-ricin further. Fig. 2 shows cell-associated and the time-dependent increase in internalised ^{125}I-TC-ricin in hepatocytes that were incubated at 37°C after having been given a pulse of ^{125}I-TC-ricin (20 nM) at 0°C. The results indicate that at steady state, about 50% of the cell-associated ^{125}I-TC-ricin were bound to galactose residues at the cell surface.

To measure uptake and degradation of ^{125}I-TC-ricin and ^{125}I-TC-AOM hepatocytes were incubated with the labelled probes (10 nM) at 37°C. At increasing time intervals, aliquots of the cell suspensions were washed in presence of lactose (ricin) or 10% sucrose (AOM). Washing in 10% sucrose effectively removes surface-bound ^{125}I-TC-AOM, the addition of EGTA to the washing solution does not remove additional surface-bound ligand. The amounts of internalised and degraded (acid soluble) ligands were determined. Fig. 3 shows that both probes were internalised effectively; after 60 min about 65 and 25% of, respectively, ^{125}I-TC-AOM and ^{125}I-TC-ricin were internalised. ^{125}I-TC-AOM was degraded much more effectively than ^{125}I-TC-ricin. After 60 min, 20% of the labelled AOM and only 1.5% of the ^{125}I-TC-ricin were degraded.

To test whether the endocytosis of ^{125}I-TC-AOM was affected by ricin, cells were incubated with and without ricin (1 μM) for 1 h prior to the addition of ^{125}I-TC-AOM (10 nM) (ricin and AOM could not be added simultaneously since ricin binds AOM). Uptake and degradation of ^{125}I-TC-AOM were measured in aliquots of cells removed after time intervals

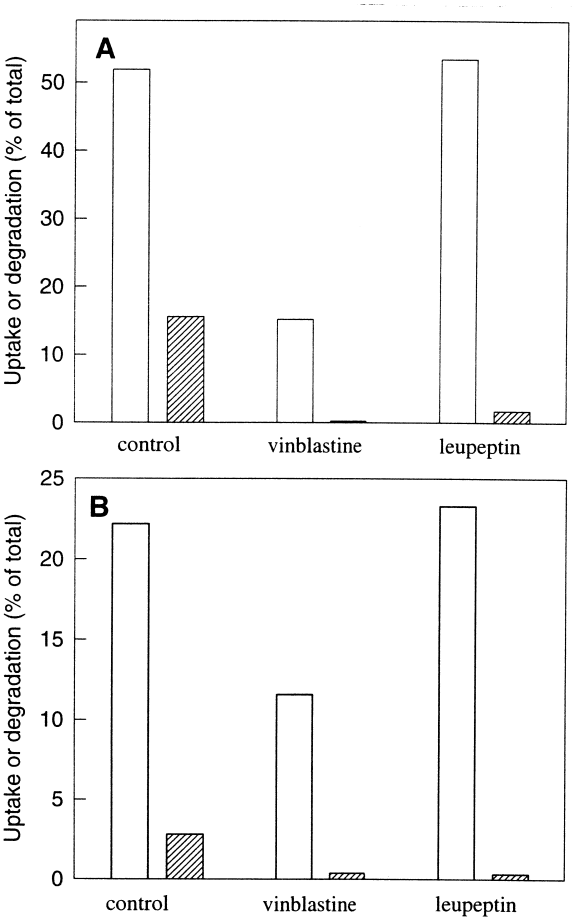

Fig. 4. The effects of vinblastine (25 μM) and leupeptin (100 μM) on the endocytosis of ^{125}I-TC-AOM and ^{125}I-TC-ricin in hepatocytes. The cells were incubated with the inhibitors for 15 min at 37°C prior to the addition of ^{125}I-TC-AOM (100 nM, A) and ^{125}I-TC-ricin (10 nM, B). Acid soluble (hatched bars) and acid precipitable (open bars) radioactivities were measured in control cells and inhibitor-treated cells after 60 min of incubation at 37°C. The figure shows results from a representative experiment.

up to 3 h after the addition of the labelled ligand. The lectin did not significantly affect uptake or degradation of ^{125}I-TC-AOM under these conditions (results not shown).

3.3. The effects of vinblastine and leupeptin on the uptake and the degradation of ^{125}I-TC-AOM and ^{125}I-TC-ricin

Fig. 4 shows the results of a typical experiment in which the cells were preincubated for 15 min at 37°C with vinblastine (25 μM) or leupeptin (100 μM). La-

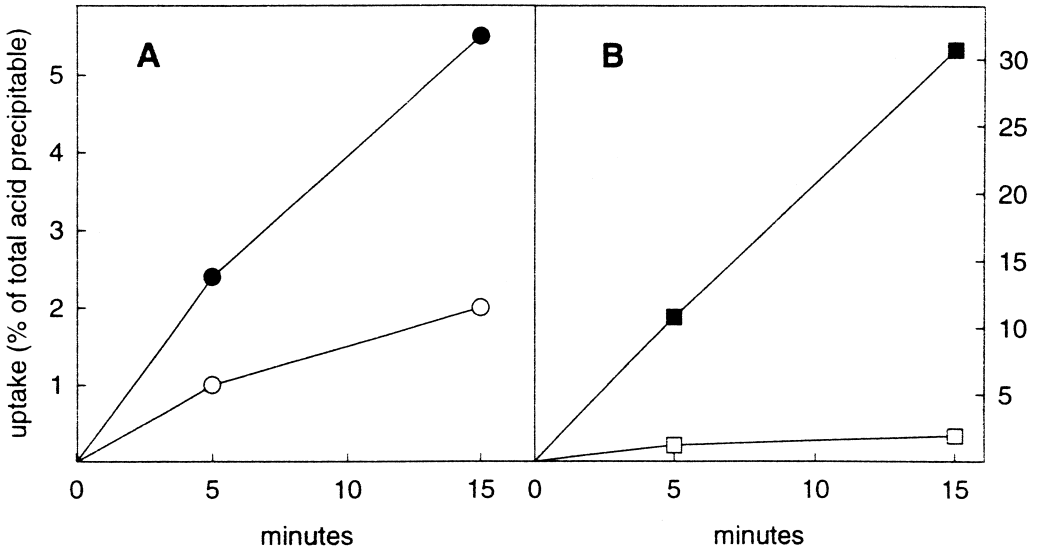

Fig. 5. Effects of hypertonic medium on the uptake and degradation of ^{125}I-TC-ricin and ^{125}I-TC-AOM in rat hepatocytes. Rat hepatocytes were suspended in incubation medium without additions (filled symbols) or in incubation medium containing 100 mM sucrose (open symbols) and incubated at 37°C for 15 min. ^{125}I-TC-ricin (10 nM) (A) or ^{125}I-TC-AOM (100 nM) (B) were then added and the incubation at 37°C was continued. Aliquots (0.25 ml) of cells were removed at the indicated times and washed thrice in PBS containing 50 mM lactose (A) or 10% sucrose (B). Uptake is presented as percent of total initial acid precipitable radioactivity in the suspensions. The figure shows results from a representative experiment.

belled AOM (Fig. 4A) and ricin (Fig. 4B) were then added, and cell-associated and degraded ligands were determined at increasing time intervals. The results presented in Fig. 4 show acid precipitable and acid soluble radioactivities after 60 min of incubation with the labelled probes. It is evident that leupeptin inhibits degradation of either probe, whereas uptake is unaffected. Vinblastine strongly inhibited uptake of both ^{125}I-TC-ricin and ^{125}I-TC-AOM.

3.4. *Effects of hypertonic medium on the uptake and degradation of ^{125}I-TC-AOM and ^{125}I-TC-ricin*

Numerous studies have shown that hypertonic medium, potassium depletion or acidification of cytosol inhibit clathrin-dependent endocytosis selectively, while clathrin-independent endocytosis is less affected by these treatments [4]. To study the effect of hypertonic medium, we incubated hepatocytes with or without 100 mM sucrose (the total osmolality was 400 mM). It was found consistently that the hypertonic medium reduced uptake of both ^{125}I-TC-ricin and ^{125}I-TC-AOM; the uptake of ^{125}I-TC-ricin was, however, reduced significantly less than that of ^{125}I-TC-AOM. The results of a typical experiment are shown in Fig. 5. It can be seen that the uptake of ^{125}I-TC-AOM (Fig. 5B) after 15 min was 90% reduced, whereas the uptake of ^{125}I-TC-ricin (Fig. 5A) was about 65% reduced.

3.5. *Subcellular distribution of ^{125}I-TC-AOM and ^{125}I-TC-ricin*

In order to determine whether ^{125}I-TC-ricin and ^{125}I-TC-AOM are endocytosed by different pathways, we studied the subcellular distribution of both probes in a series of experiments employing differential and isopycnic centrifugation.

3.5.1. *Subcellular distribution of ^{125}I-TC-AOM and ^{125}I-TC-ricin determined by means of differential centrifugation*

We also followed the time-dependent appearance of the probes in fractions obtained by classical differential centrifugation (Fig. 6). The results obtained show that ^{125}I-TC-ricin, initially found mainly in the P-fraction, accumulated in the L- and the M-fractions over time. ^{125}I-TC-AOM, on the other hand, showed a temporary accumulation in the L-fraction after 10–20 min. At later times, however,

the amount of ^{125}I-TC-AOM in the M-fraction increased at the expense of the L- and P-fractions.

3.5.2. *Subcellular distribution of ^{125}I-TC-AOM and ^{125}I-TC-ricin determined by means of isopynic centrifugation in sucrose gradients*

In initial experiments, MLP-fractions were subjected to isopycnic centrifugation in linear sucrose gradients. Fig. 7 shows the density distribution of undegraded (acid precipitable) and degraded (acid soluble) ^{125}I-TC-AOM and ^{125}I-TC-ricin in sucrose gradients 30 and 120 min after the addition of the labelled probes to the cells. The results indicated, in accordance with earlier data [31,32], that ^{125}I-TC-AOM was early after uptake in endosomes banding at relatively low density in the gradients; peak activity was at about 1.11–1.13 g/ml (Fig. 7). The ligand was subsequently found in denser endosomes (at 1.14–1.16 g/ml, not shown) and eventually in organelles coinciding with the lysosomal enzyme β-acetylglucosaminidase (density about 1.18–1.20 g/ml). Acid soluble degradation products from ^{125}I-TC-AOM were already present after 30 min in this region of the gradient. ^{125}I-TC-ricin seemed to enter endosomes of higher density than the early ^{125}I-TC-AOM-containing endosomes. The density distribution of ^{125}I-TC-ricin 120 min after initiation of uptake indicated that some of the toxin had entered vesicles coinciding with lysosomes. ^{125}I-TC-ricin was degraded at a much slower rate than ^{125}I-TC-AOM, as noted above (Fig. 3). The density distribution of degradation products formed from ^{125}I-TC-ricin showed after 2 h of incubation two peaks, one coinciding with the endosomes at 1.14–1.15 g/ml and one at the lysosomal position in the gradient.

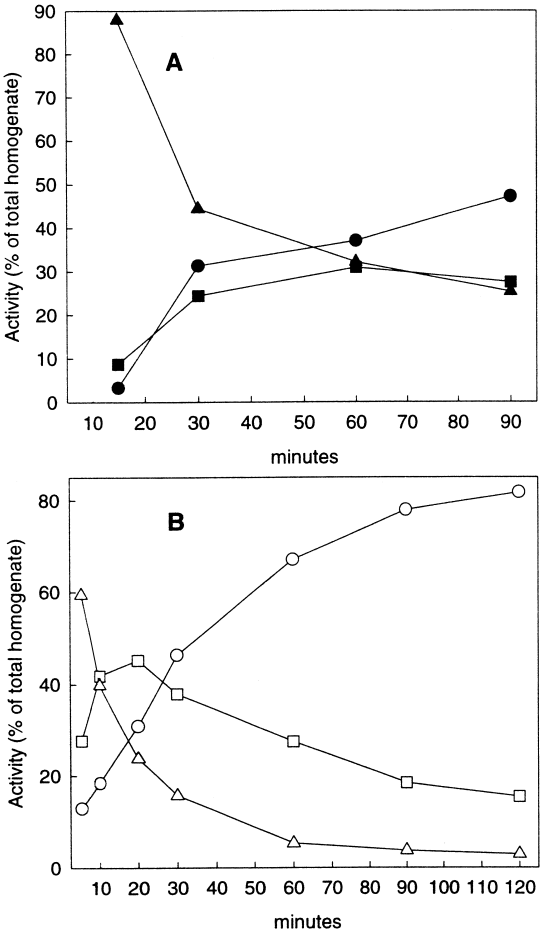

Fig. 6. Subcellular distribution of ^{125}I-TC-ricin and ^{125}I-TC-AOM following differential centrifugation of homogenates from rat hepatocytes. The cells (5×10^6 cells/ml) were incubated with 10 nM ^{125}I-TC-ricin (A) or 10 nM ^{125}I-TC-AOM (B). Aliquots of cells (5 ml) were removed at the indicated times, homogenized, and fractionated into M- (circles), L- (squares), and P-fractions (triangles), as described in Section 2. The amounts of radioactivities in the fractions are presented as percent of the radioactivity in the homogenate from which the fractions were prepared. Each fraction is derived from a homogenate prepared from 25×10^6 cells. The figure shows results from a representative experiment.

3.6. *Intracellular degradation of ^{125}I-TC-ricin and ^{125}I-TC-AOM studied by means of subcellular fractionation*

Labelled degradation products formed from ^{125}I-TC-labelled probes do not readily pass biological membranes and will therefore be trapped temporarily at the site of formation [29,33]. They may, therefore, serve as markers for the degradative compartments that are involved in their formation. To identify endocytic compartments involved in the degradation of ^{125}I-TC-ricin, we fractionated further both P-fractions and ML-fractions by means of isopycnic centrifugation in sucrose gradients. Parallel experiments were performed on cells that had endocytosed ^{125}I-TC-AOM. Fig. 8 shows the results of a typical experiment in which cells incubated with ^{125}I-TC-ricin (Fig. 8A) or ^{125}I-TC-AOM (Fig. 8B) were fractionated after 30 and 90 min. Undegraded (acid precipitable) and degraded (acid soluble) probes were measured in the fractions. Only degraded ligands

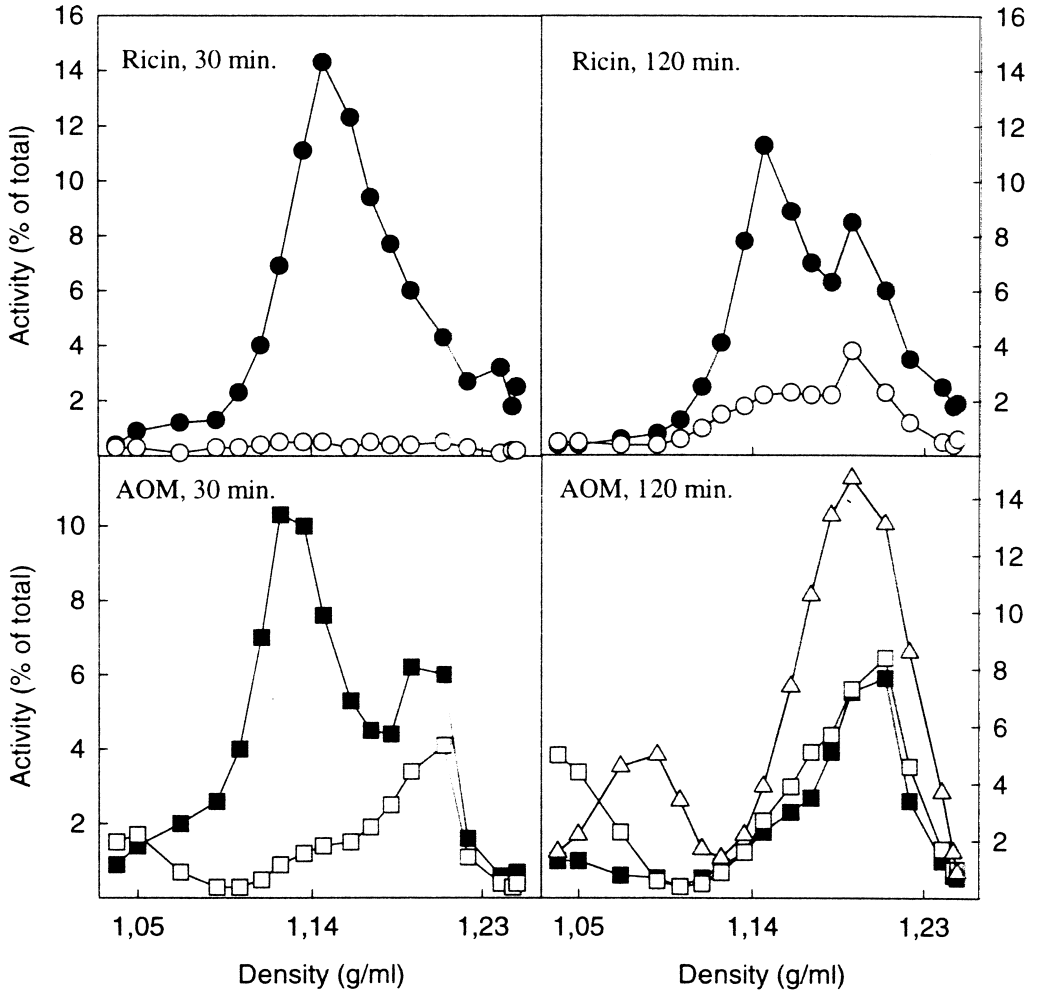

Fig. 7. Density distribution of ^{125}I-TC-ricin or ^{125}I-TC-AOM following isopycnic centrifugation of MLP-fractions prepared from cells that had endocytosed ^{125}I-TC-ricin (upper panels) or ^{125}I-TC-AOM (lower panels). The cells were incubated with 10 nM ^{125}I-TC-ricin or 10 nM ^{125}I-TC-AOM. Aliquots of cells (5 ml) were removed after 30 min (left panels) and 120 min (right panels), homogenized, and MLP-fractions were prepared as described in Section 2. The amounts of acid soluble (open symbols) and acid precipitable (closed symbols) radioactivities in the fractions are presented as percent of total recovered radioactivity in the gradients. The distribution of β-acetylglucosaminidase (triangles) is shown in the lower right panel. The figure shows results from a representative experiment.

(as percent of total recovered radioactivity in the gradients) are shown. The main degradation of ^{125}I-TC-AOM is taking place in organelles with densities around 1.18 g/ml (Fig. 8B). The degradation products coincide in the gradient with β-acetylglucosamindase (not shown) and are most likely present in lysosomes. The density distribution of labelled degradation products from ^{125}I-TC-ricin was distinctly different from that of ^{125}I-TC-AOM: Acid-soluble products formed from ^{125}I-TC-ricin seemed to be present both in endosomes and lysosomes. In fact, the time-dependent distribution of degradation products in endosomes and lysosomes suggested that degradation is initiated in endosomes and proceeds further in lysosomes. To check whether the acid soluble degradation products from ^{125}I-TC-ricin were in lysosomes of low density, we also measured β-acetylglucosaminidase following fractionation of P-fractions in sucrose gradients. The trace of β-acetylglucosaminidase in the P-fraction was found to band at about 1.18 g/ml (results not shown).

The prelysosomal degradation of ^{125}I-TC-ricin seemed to take place in the endosomes present in the P-fraction. This is consonant with the observation that degradation of ^{125}I-TC-ricin commences very early after initiation of endocytosis. The degra-

dation of ^{125}I-TC-AOM, on the other hand, starts after a lag of about 12 min.

3.7. ^{125}I-TC-AOM and ^{125}I-TC-ricin are internalised into early endosomes that may be separated by isopycnic centrifugation

The differential centrifugation experiments had shown that initially most of the labelled probes sedimented in the P-fraction (Fig. 6). Early endosomes, containing ^{125}I-TC-AOM or ^{125}I-TC-ricin should, therefore, be present in this fraction. Fig. 9A shows the density distribution of ^{125}I-TC-AOM and ^{125}I-TC-ricin following isopycnic centrifugation of P-fractions prepared from cells that had internalised the probes for 10 min at 37°C. The results show that the early endosomes containing ^{125}I-TC-AOM and ^{125}I-TC-ricin can be clearly separated in sucrose gradients, the ^{125}I-TC-AOM-containing endosomes equilibrate at 1.12 g/ml whereas those containing ^{125}I-TC-ricin are banding at 1.16 g/ml.

A possible explanation of the different distribution of ^{125}I-TC-AOM and ^{125}I-TC-ricin (early after initiation of uptake) could conceivably be that ^{125}I-TC-ricin very rapidly passes the early ^{125}I-TC-AOM-containing endosomes. To slow down the transport, we therefore allowed the cells to internalise the labelled probes at 18°C. Fig. 9B shows the density distribution of endosomes containing ^{125}I-TC-AOM and ^{125}I-TC-ricin after 10 min of incubation at 18°C. Since the internalisation (particularly of ^{125}I-TC-ricin) was reduced at this low temperature, the cells were treated at 0°C for 60 min with pronase (0.3%) prior to homogenisation to remove remnants of surface-bound ligands. The gradient profiles show that ^{125}I-TC-AOM bands at about 1.08 g/ml, whereas ^{125}I-TC-ricin is found at 1.13 g/ml. These data support the notion that ^{125}I-TC-AOM and ^{125}I-TC-ricin are internalised into different populations of early endosomes.

3.8. Density distribution of ^{125}I-TC-AOM and ^{125}I-TC-ricin after DAB cytochemistry

In order to determine whether AOM and ricin were present in the same endocytic organelle, we induced density shift in the AOM-containing compartment by means of HRP-induced cross-linking of

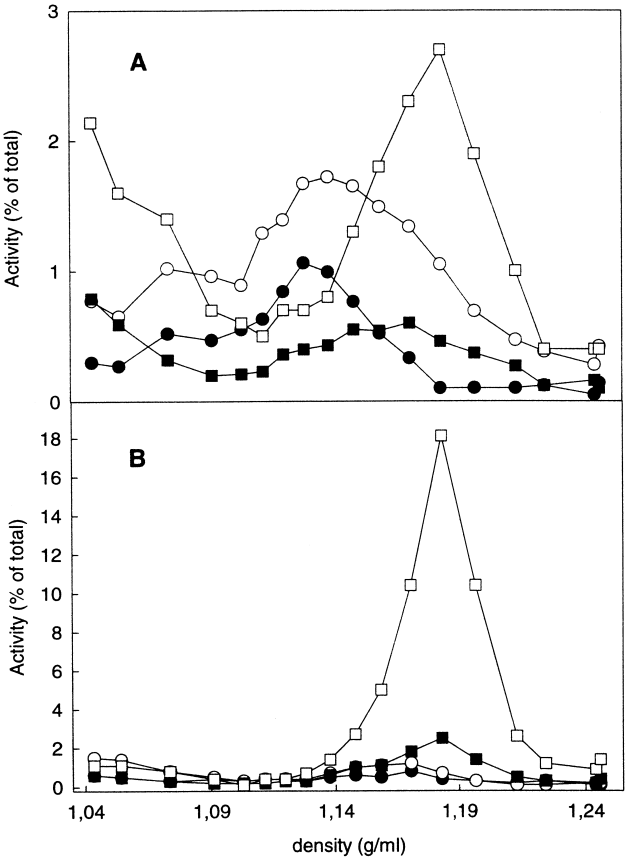

Fig. 8. Density distribution of degraded (acid soluble) ^{125}I-TC-ricin or ^{125}I-TC-AOM following isopycnic centrifugation. ML-fractions (squares) and P-fractions (circles) were prepared from cells that had endocytosed ^{125}I-TC-ricin (A) or ^{125}I-TC-AOM (B) for 30 min (closed symbols) or 90 min (open symbols). The fractions were dissolved in homogenization buffer, placed on top of linear sucrose gradients, and centrifuged at $85\,000 \times g$ for 7 h. Acid soluble radioactivities were measured in the fractions and are presented as % of total recovered radioactivities in the gradients. The figure shows results from a representative experiment.

DAB in the presence of H_2O_2 [6,30]. Hepatocytes were incubated with saturating concentrations of AOM conjugated to HRP for 30 min, in order to charge the endocytic pathway with the ligand. The cells were then washed in cold incubation medium and re-incubated at 37°C in the presence of either ^{125}I-TC-AOM or ^{125}I-TC-ricin. At various time intervals during the re-incubation, aliquots of cells were removed from the incubator, and MLP-fractions were prepared and incubated with DAB in the presence and absence of H_2O_2. Results obtained with cells incubated for 15 min are shown in Fig. 10.

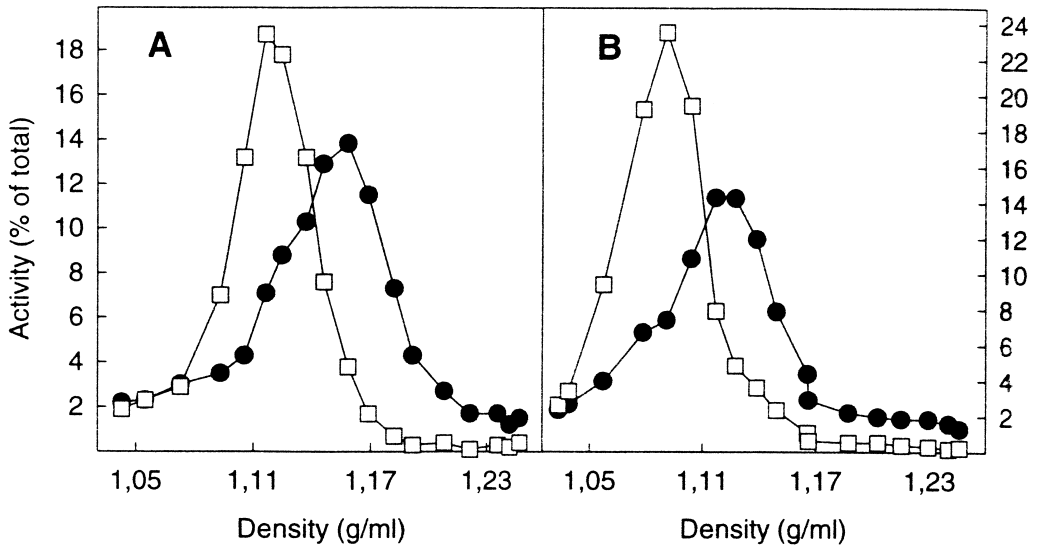

Fig. 9. The effect of reduced temperature on the density distribution of ^{125}I-TC-ricin and ^{125}I-TC-AOM following isopycnic centrifugation of P-fractions in sucrose gradients. Density distribution of ^{125}I-TC-ricin or ^{125}I-TC-AOM following isopycnic centrifugation of P-fractions prepared from cells that had endocytosed ^{125}I-TC-ricin or ^{125}I-TC-AOM. The cells were incubated with 10 nM ^{125}I-TC-ricin (circles) or 10 nM ^{125}I-TC-AOM (squares) at 37°C (A) or at 18°C (B). Aliquots of cells were removed after 10 min, homogenized, and P-fractions were prepared as described in Section 2. The fractions were dissolved in homogenization buffer, placed on top of linear sucrose gradients, and centrifuged at 85 000×g for 7 h. Radioactivities in the fractions are presented as % of total recovered activities in the gradients. The figure shows results from a representative experiment.

The data show that a clear density shift is obtained for the ^{125}I-TC-AOM after 15 min. The density distribution of ^{125}I-TC-ricin, on the other hand, was not affected after 15 min of incubation. Both probes were density shifted after 30 min or longer incubation times (not shown). These results are therefore compatible with the notion that the two probes, ^{125}I-TC-AOM or ^{125}I-TC-ricin enter the cells via different endocytic pathways, but that the two pathways meet.

4. Discussion

Ricin, injected intravenously into rats, is cleared rapidly from the blood by the liver [34–36]. The mannose receptor of the liver endothelial cells is responsible for the bulk of the hepatic uptake [34]. In the parenchymal liver cells, the binding and uptake are mediated by numerous ($\geq 10^7$ per cell) glycoproteins and glycolipids with terminal galactose. These binding sites are distributed all over the plasma membrane, and ricin may therefore be useful as a tool to follow plasma membrane traffic in general.

Receptor-mediated endocytosis of a number of ligands has been shown to take place via coated pits and may therefore be termed clathrin-mediated endocytosis. The receptors, along with their ligands, are concentrated (10–20-fold) in coated pits, in part through direct interactions between defined internalisation signals on their cytoplasmic domains and the adaptor proteins of the coat [7]. Most of the glycolipids and glycoproteins that ricin binds to probably do not contain internalisation signals in their cytoplasmic domains. Such membrane constituents may, therefore, either be internalised via coated pits at a much slower rate than those containing the internalisation signal, or, alternatively, they may be internalised by a clathrin-independent mechanism. Evidence of clathrin-independent uptake of ricin has been obtained by exposing cells to conditions that selectively inhibit endocytosis via coated pits: i.e. hypertonic medium, K^+-depletion, acidification of cytosol [4]. Both biochemical and morphological data suggest that such conditions affect ricin uptake less than uptake known to take place exclusively via clathrin-coated pits [4,20,37,38]. In the present study, we found that hypertonic medium reduced ricin uptake about 50%. Uptake of AOM which takes place ex-

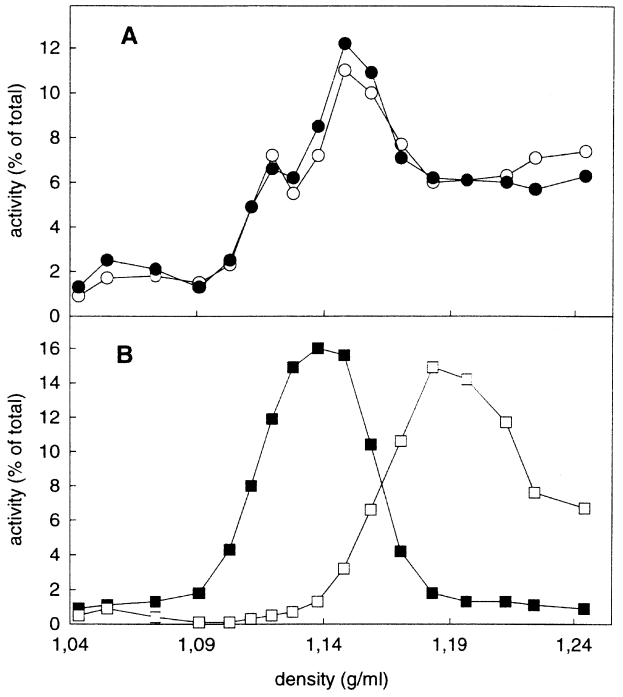

Fig. 10. Density distribution of ^{125}I-TC-ricin and ^{125}I-TC-AOM after DAB cytochemistry. Hepatocytes were first incubated at 37°C in the presence of AOM-HRP (200 nM) for 30 min. The cells were subsequently washed to remove extracellular AOM-HRP and then incubated at 37°C for 15 min in the presence of either 10 nM ^{125}I-TC-ricin (A) or 10 nM ^{125}I-TC-AOM (B). The cells were washed in 10% sucrose solution, homogenized in 0.25 M buffered sucrose solution (3 mM imidasol, pH 7.3) and centrifuged at $4000\times g$ for 2 min to remove nuclei. The nuclear fraction was resuspended in buffered sucrose and re-centrifuged. The combined postnuclear fractions were incubated with DAB in the presence (open symbols) or absence (closed symbols) of H_2O_2 as described in Section 2. The postnuclear fractions were placed on top of linear sucrose gradients and centrifuged at $85\,000\times g$ for 7 h. Radioactivities in the fractions are presented as % of total recovered radioactivity in the gradient. The figure shows results from a representative experiment.

clusively via coated pits was, however, nearly blocked by this treatment, compatible with clathrin-independent uptake of ricin.

Clathrin-dependent and -independent endocytosis may relate differently to the cytoskeleton. It has been shown, for instance, that cytochalasin D and colchicine inhibit uptake of ricin in Vero cells without reducing transferrin uptake (to the same extent) [20]. We found that cytochalasin (not shown) and vinblastine inhibited uptake of ricin and AOM to the same extent. In vinblastine-treated isolated hepatocytes gold-AOM conjugates are found in early endosomes [41], consonant with an effect of the drug on the microtubule-dependent conveyance of endosomes [39–41]. The comparable effect of vinblastine on the uptake of ricin and AOM suggests that the intracellular transport of ricin-containing endosomes is also mediated by microtubules. The inhibited transport to later compartments will also reduce the net uptake, since the recycling of the toxin continues.

Subcellular fractionation by isopycnic centrifugation in sucrose gradients showed that ricin was consistently found in denser endosomes than those containing AOM. Could the density distribution of the two probes differ if they were, after all, internalised via the same pathway? Two possibilities were considered. First, ricin could be transferred very rapidly through the early endosome containing AOM; second, surface-bound ricin could mask its true intracellular distribution. To check these possibilities we incubated the cells at 0°C with pronase to remove remnants of surface-bound ricin, and we also allowed the cells to internalise the probes at 16°C to retard the transport of the ligand to later endosomes. None of these treatments led to coinciding density distribution of ricin and AOM and we therefore believe that ricin and AOM are internalised into different endocytic pathways. These data are in agreement with results obtained in studies of fluid phase endocytosis in rat hepatocytes [1,2,42,43]: Weigel and coworkers showed that potassium depletion and hypertonic medium had only a modest effect on pinocytosis of [^{14}C]sucrose or Lucifer yellow in isolated rat hepatocytes, whereas uptake of AOM was nearly blocked [1,2,42]. Since fluid phase endocytosis is dependent on membrane internalisation, it is reasonable to assume that the bulk of membrane internalised in hepatocytes takes place outside coated pits.

AOM was transferred from the light endosomes (mainly present in the P-fraction) to denser endosomes (sedimenting in the ML-fraction) from which the ligand was transferred to lysosomes. These observations are in perfect agreement with earlier data [31,32,41]. The denser endosomes with which AOM was associated could conceivably also be receivers of ricin. To determine whether ricin and AOM colocalised in later endocytic compartments, we used the DAB cytochemistry, followed by gradient centrifugation in sucrose gradients. The results obtained indi-

cated that a rendezvous of ricin and AOM took place after 15–30 min. It is, therefore, likely that ricin and AOM enter the cells via different gates, but that they meet in a late(r) endocytic compartment.

Other studies of the relation between clathrin-dependent and clathrin-independent endocytosis have given divergent data. Tran et al. showed that α_2-macroglobulin-gold internalised via a clathrin-dependent pathway and cholera toxin-gold internalised via a clathrin-independent pathway meet in early endosomes [44]. Similar results were obtained by Hansen et al. [9] studying endocytosis of ConA-gold and transferrin in HEp2 cells. Other studies have indicated that a rendezvous may not take place: in ruffling A431 cells, clathrin-independent endocytosis seems to give rise to vesicles that are able to fuse with each other and not with transferrin-receptor-containing endosomes [10]. Becich et al. [45] compared the endocytosis of wheat germ agglutinin (WGA) (conjugated to iron-dextran particles) and AOM in Hep G2 cells. Both WGA and ricin bind to glycoconjugates expressed on the cell surface. The finding that WGA and AOM are internalised via different sites is, therefore, in agreement with the data obtained in our study. We found, however, that ricin and AOM meet in late endocytic vesicles and that ricin is degraded (albeit at a much slower rate than AOM). The WGA and the AOM did not seem to meet in the HepG2 cells. Moreover, the WGA was not degraded. The reasons for the differences between rat hepatocytes and the liver cell line are not clear.

By using ^{125}I-TC-labelled probes we found that ricin was degraded primarily in endosomes whereas AOM was degraded mainly in lysosomes. Degradation of either ligand was inhibited by leupeptin, indicating that the process required thiol proteinases. Degradation products from ricin also accumulated in lysosomes. The kinetics of degradation in endosomes and lysosomes suggested that these two degradative compartments were connected in series rather than in parallel. Endosomal/prelysosomal degradation has earlier been demonstrated, particularly well, in macrophages [46,47], in which processing of antigens, hormones, and other protein ligands may take place in endosomes [48]. Blum et al. [46] showed that ricin was particularly susceptible to endosomal degradation in macrophages. In hepatocytes, both insulin and glucagon have been found to be degraded in endosomes [48–50]. Even a small proportion of AOM seems to be degraded in endosomes [29]. Maybe the fraction of AOM that is available for endosomal degradation is that taken up by the group of galactose receptors that are termed state I receptors by Weigel [18]. He showed that ligands internalised via the state I receptors started to be degraded without a lag phase, similar to what we found with ricin.

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