Differential effects of leupeptin, monensin and colchicine on ligand degradation mediated by the two asialoglycoprotein receptor pathways in isolated rat hepatocytes

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We have shown that degradation of asialo-orosomucoid (ASOR) in isolated rat hepatocytes occurs by two different intracellular pathways [Clarke, Oka & Weigel (1987) *J. Biol. Chem.* **262**, 17384–17392] mediated by two subpopulations of cell surface galactosyl (Gal) receptors, designated State 1 or State 2 receptors. In the present study, several inhibitors were tested for their effects on ligand degradation by the State 1 or State 2 pathway. Leupeptin, monensin and chloroquine completely inhibited degradation of ¹²⁵I-labelled ASOR in both pathways. Dose–response studies showed, however, that the State 2 pathway was more sensitive to leupeptin or monensin than the State 1 pathway. No differences were observed with chloroquine. For example, the onset of inhibition in the State 2 and State 1 pathways occurred at about 0.05 and 0.3 μ M-leupeptin respectively, a 6-fold difference. At 3.5 μ M-monensin, ¹²⁵I-ASOR degradation in the State 2 pathway was completely blocked, whereas degradation in the State 1 pathway was essentially unaffected. Colchicine was observed to give the largest differential sensitivity between the two pathways. The State 2 degradation pathway was about 30-fold more sensitive to colchicine than the State 1 pathway. Lumicolchicine had no effect. The onset of inhibition of the rate of ¹²⁵I-ASOR degradation in the State 2 and State 1 pathways occurred at $\sim$ 0.1 and 3.0 μ M-colchicine respectively. At very high concentrations (> 0.1 mM), the State 1 pathway could be completely inhibited. We conclude that intracellular ¹²⁵I-ASOR processing or delivery to degradative compartments in both the State 1 and State 2 Gal receptor pathways requires low pH. Ligand delivery to the degradative compartment does not require microtubules in the State 1 pathway, consistent with the very rapid onset of degradation in this pathway. The State 2 degradation pathway does require microtubules.

INTRODUCTION

Endocytosed proteins can be directed along different cellular pathways to different fates depending on the cell type and the ligand [1]. In the case of transferrin, the protein is endocytosed, delivers iron to the endosome intracellularly and is subsequently recycled to the cell surface and released as apotransferrin without any permanent changes in the protein structure [2,3]. In contrast, some protein hormones, such as insulin [4] and epidermal growth factor [5], are proteolytically processed very rapidly following endocytosis. Other ligands such as low-density lipoproteins [1] or asialoglycoproteins [6–8] are dissociated from their receptors after internalization and routed to a lysosomal compartment to be degraded, while the receptor is recycled to the cell surface and reutilized. In these latter cases the ligand appears to remain intact for up to 20–30 min in many cell types prior to the appearance of degradation products.

Hepatocytes endocytose and process internalized asialo-orosomucoid (ASOR) by two parallel and distinct pathways [8]. These two pathways are associated with two functionally distinct surface galactosyl (Gal) receptor populations that have different thermal and kinetic properties. Table 1 summarizes the characteristics that

distinguish and define these two receptor pathways. For example, the total cell surface Gal receptor activity can vary depending on the conditions. One subpopulation of receptors present on the cell surface, designated State 2 Gal receptors, is dynamically modulated by a variety of agents such as metabolic energy poisons, acidotropic amines, microtubule depolymerizing agents (D. D. McAbee, B. L. Clarke, M. Lear, J. A. Oka & P. H. Weigel, unpublished work) and low temperatures between 24 and 37 °C [9]. The other subpopulation of receptors on the cell surface is not modulated by these treatments and is designated the State 1 Gal receptor population. In both pathways receptor–ASOR complexes are endocytosed and subsequently dissociate so that ligands can be routed to lysosomes while the receptor is recycled to the cell surface. Although the rate of internalization is the same in both pathways, receptor–ligand dissociation after internalization and ligand delivery to lysosomes proceeds at very different rates [10–12]. State 2 receptor–ASOR complexes rapidly dissociate ($k = 0.28 \text{ min}^{-1}$), while State 1 complexes dissociate slowly ($k = 0.014 \text{ min}^{-1}$). The dissociation rate for State 1 receptor–ASOR complexes is much slower than the internalization rate. Therefore, during continuous endocytosis the surface and intracellular State 1

Abbreviations used: ASOR, asialo-orosomucoid; BSA, bovine serum albumin; Gal, galactosyl; PBS, phosphate-buffered saline.

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Table 1. Characteristics of the two galactosyl receptor pathways

Two functionally distinct Gal receptor subpopulations and pathways are defined by nine characteristics. These features demonstrate differential effects on Gal receptor activity or function and ASOR processing in isolated rat hepatocytes in suspension and in culture. Some of these data have been reviewed previously [8].

Feature	State 1 pathway (minor)	State 2 pathway (major)	Reference
Surface receptor modulation by temperature, monensin, ATP depletion, microtubule drugs, chloroquine	No	Yes	[9]*
Receptors constitutively recycle	No	Yes	[13,14]
Receptors undergo an inactivation-reativation cycle	No?	Yes	[13,14]
Diacytosis of internalized receptor-ligand complexes	Yes	No	[39]
Dissociation of receptor-ligand complexes	Slow	Fast	[6,10,11]
Ligand dissociation blocked by monensin	No	Yes	[23,31]
Ligand dissociation blocked at 18 °C	Yes	No	[10]
Lag period before ligand degradation	No	Yes	[12]
Colchicine (1 μ M) inhibits ligand degradation	No	Yes	This report

* D. D. McAbee, B. L. Clarke, M. Lear, J. A. Oka & P. H. Weigel, unpublished work.

Gal receptors become occupied with ligand and after several rounds of internalization they do not contribute significantly to the steady state rate of continuous ASOR endocytosis [10]. In the absence of ligand, the State 2 Gal receptors recycle constitutively and undergo a reversible inactivation/reativation cycle as they pass through their constitutive recycling itinerary [13,14]. Presumably the same inactivation/reativation cycle in this pathway occurs when ligand is present.

In addition to differences in receptor recycling, the cellular processing of ligand depends on which Gal receptor pathway is involved. In the State 1 receptor pathway, ligand degradation occurs immediately following internalization, whereas in the State 2 pathway, ligand degradation occurs only after a lag period of 15–20 min [12]. The simplest conceptual model to explain all these and other results requires two functionally distinct parallel pathways for ligand processing and receptor recycling. Our proposed model does not necessarily require the presence of two structurally different receptors, but it does predict the existence of separate endocytic compartments [8]. In this study we examined the effects of various inhibitors to determine if degradation of 125 I-labelled ASOR in both Gal receptor pathways occurs by similar mechanisms.

EXPERIMENTAL

Materials

Human orosomucoid (α_1 acid glycoprotein), obtained from Sigma Chemical Co., was desialylated with neuraminidase and iodinated as described [15]. Sephadex G-25 and G-75 were from Pharmacia. Triton X-100, collagenase (type 1), bovine serum albumin (BSA) (fraction V), neuraminidase (type X), colchicine, lumicolchicine, monensin, bacitracin, leupeptin hemisulphate,

chloroquine diphosphate salt, phosphotungstic acid and EDTA were from Sigma. HEPES was obtained from Research Organics, Inc. Bradford reagent was from Pierce. Na^{125}I (10–20 mCi/ μ g of iodine) was from Amersham Corp. All other chemicals were reagent grade.

Media and buffers

Medium 1 contained modified Eagle's medium (Grand Island Biological Co., catalogue no. 420–1400) supplemented with 2.4 g of HEPES/l, pH 7.4, and 0.22 g of NaHCO_3 /l. Medium 1/BSA is Medium 1 with 0.1% (w/v) BSA. Medium 1/EDTA is Medium 1 containing 20 mM-EDTA. Hanks' balanced salt solution contained 1.26 mM- CaCl_2 , 5.36 mM-KCl, 0.3 mM- KH_2PO_4 , 0.5 mM- MgCl_2 , 0.4 mM- MgSO_4 , 137 mM-NaCl, 0.33 mM- Na_2HPO_4 , 5.5 mM-glucose, 0.001% (w/v) Phenol Red and 4.2 mM- NaHCO_3 , pH 7.4. Phosphate-buffered saline (PBS) contained 137 mM-NaCl, 8 mM-sodium phosphate, 2.7 mM-KCl and 1.5 mM-potassium phosphate, pH 7.4.

Hepatocyte preparation

Male Sprague-Dawley rats (160–200 g) were obtained from Timco Breeding Laboratories, Houston, TX, U.S.A. Hepatocytes were prepared by the collagenase perfusion procedure of Seglen [16] with several modifications as described previously [12]. Final cell pellets were suspended in ice-cold Medium 1/BSA and were > 85% viable and single cells. Experiments were performed in the absence of serum. Cell viability was determined by Trypan Blue exclusion. Cells containing both State 1 and State 2 receptor activity on the cell surface were prepared by equilibrating suspensions at 37 °C for 60 min [11,12]. Freshly isolated cells kept on ice contain only the State 1 receptor population on the cell surface.

Endocytosis and degradation of ^{125}I -ASOR

All experiments involved a single round of surface-bound ligand. Freshly isolated cells (3×10^6 cells/ml) in Medium 1/BSA were left on ice (State 1 cells) or preincubated at 37°C (State 2 cells) for 60 min to increase surface receptor activity [9]. The cells at 37°C were chilled to 4°C by adding an equal volume of ice-cold Medium 1 and placing them on ice for 10 min. Hepatocytes were then centrifuged, resuspended in Medium 1/BSA plus $1.5 \mu\text{g}$ of ^{125}I -ASOR/ml, incubated for 60 min at 4°C , and then washed three times by centrifugation with ice-cold Hanks' to remove non-bound radioactivity. Endocytosis of ^{125}I -ASOR was initiated by transferring the cell suspensions to 37°C . When necessary, endocytosis was determined by adding an equal volume of ice-cold Medium 1/EDTA and incubating the cells for 10 min at 4°C . The cells were then washed three times with Hanks', and internalization was assessed by measuring the amount of cell-associated radioactivity that was not released by EDTA.

The rate of ^{125}I -ASOR degradation was measured as the increase in acid-soluble radioactivity with time [15]. Samples of cell suspensions were mixed with equal volumes of ice-cold 10% phosphotungstic acid in 2 M-HCl. After at least 15 min on ice, the precipitates were centrifuged at 3000 rev./min ($r_{\text{av.}} = 13 \text{ cm}$) for 10 min and radioactivity in the supernatant fluids was determined. Approx. 99% of intact ^{125}I -ASOR is precipitated, while free iodide and iodotyrosine remain in solution under these conditions. In the dose-response experiments, each point represents the steady-state rate of degradation of surface-bound ^{125}I -ASOR. Each slope (rate) was calculated by least-squares linear regression analysis of four to five kinetic data points. The average of all correlation coefficients in these determinations was > 0.96 . Rates were calculated as (increase in acid soluble radioactivity)/(time), and were normalized within an experiment to the control rate of degradation (set to 100% in the absence of inhibitor).

Inhibitor treatments

Experiments using inhibitors to block proteolysis, microtubule function, or to disrupt pH or ion gradients, followed a similar protocol. Cells containing surface-bound ^{125}I -ASOR were incubated with the inhibitor for 10 min at 4°C before transferring the cells to 37°C . Stock solutions of 25 mM-leupeptin and 40 mM-chloroquine (both in PBS), and 20 mM-colchicine, 20 mM-lumicolchicine and 1 mM-monensin (all in dimethyl sulphoxide) were prepared and stored at -20°C .

General

Protein was determined by the method of Bradford [17]. Centrifugations were at 800 rev./min ($r_{\text{av.}} = 13 \text{ cm}$) for 2 min in a refrigerated centrifuge (Beckman model TJ-6 table-top). ^{125}I radioactivity was determined using a Packard Multiprias 2 gamma spectrometer. Cell incubations were in Erlenmeyer flasks in a gyratory water bath shaking at 100 rev./min at densities of 3×10^6 cells/ml. One flask was used per time point, and the starting times for the incubations were staggered so that all flasks finished together.

Fig. 1. Effect of leupeptin on the kinetics of ^{125}I -ASOR degradation

Freshly isolated State 1 (○, ●) or 37°C -equilibrated State 2 (△, ▲) cells were allowed to prebind ^{125}I -ASOR for 1 h, washed, incubated with 0.1 mg of leupeptin/ml (●, ▲) or PBS (○, △) on ice for 10 min and then transferred to 37°C to initiate a synchronous round of endocytosis and degradation. Samples were taken at the indicated times and acid-soluble radioactivity was determined. The 100% values for prebound ^{125}I -ASOR in State 1 and State 2 cells were 60 and 240 fmol/ 10^6 cells respectively

RESULTS

When freshly isolated hepatocytes are incubated at 37°C for 1 h, they increase their surface Gal receptor activity 2–4-fold when compared with cells that have been kept on ice [9]. In order to discuss these two situations and relate the properties of Gal receptors on these isolated hepatocytes we have used the following nomenclature [8,11]. State 2 cells refer to those cells that have been previously equilibrated at 37°C . State 1 cells are those cells that have been freshly isolated from the collagenase perfusion and stored at 4°C . State 1 cells initially possess only one functional Gal receptor pathway, referred to as the State 1 pathway. State 2 cells contain two functional receptor pathways, both the State 1 and the State 2 pathways.

Effects of protease inhibitors on the degradation of ^{125}I -ASOR

State 1 or State 2 cells were treated with a variety of inhibitors to assess their effects on the degradation of surface-bound ligand. Leupeptin is a thiol protease inhibitor known to cause ligand accumulation in lysosomes [18–20]. In cells treated with 0.1 mg of leupeptin

Fig. 2. Effect of leupeptin concentration on the rate of ^{125}I -ASOR degradation

The rate of degradation of surface-bound ^{125}I -ASOR was determined as described in the legend to Fig. 1 (open symbols) or State 2 (closed symbols) cells in the presence of the indicated concentrations of leupeptin. The different symbols represent different independent experiments.

/ml (0.2 mM), ^{125}I -ASOR degradation was blocked in both State 1 and State 2 cells (Fig. 1). A dose-response curve for leupeptin showed a threshold for the onset of inhibition beginning at about $0.05\ \mu\text{M}$ for State 2 cells and $0.3\ \mu\text{M}$ for State 1 cells (Fig. 2). Complete cessation of degradation in both State 1 and 2 cells occurred at $300\ \mu\text{M}$ -leupeptin. This result suggests that ASOR degradation in the State 2 Gal receptor pathway is slightly more sensitive to leupeptin than degradation in the State 1 pathway.

Several experiments were performed to test for the presence of cell-surface proteases and, in particular, whether the no-lag State 1 degradation pathway might depend on such surface proteases (Table 2). An EDTA wash was used to remove divalent cations or proteases from the cell surface before binding ^{125}I -ASOR to the cells at 4°C . The prior EDTA stripping did not inhibit degradation of surface-bound ^{125}I -ASOR at 37°C in the immediate, State 1 pathway (Table 2), or in the delayed-phase, State 2 pathway (results not shown). In fact, degradation was enhanced, since more ^{125}I -ASOR could be bound to the treated cells. In this experiment, however, Ca^{2+} was present during the ^{125}I -ASOR binding step so that Ca^{2+} -activated cell surface proteases cannot be ruled out. Cells were also washed at 4°C with a pH 5.0 buffer to release possible receptor-bound proteases on the cell surface. After the low-pH stripping, the rate of surface-bound ^{125}I -ASOR degradation by State 1 cells was increased (Table 2). Treatment of cells with EDTA or low pH at 4°C exposes cryptic Gal receptors and increases the amount of ^{125}I -ASOR prebound to the cell surface [8] and its subsequent degradation. Regardless, the immediate degradation of ^{125}I -ASOR in the State 1 pathway was not inhibited by prior low-pH or EDTA stripping of cell surface proteins.

The State 1 degradation process was also not blocked by up to 1 mM-bacitracin (Table 2) which inhibits

Table 2. Effect of bacitracin or low-pH or EDTA washes on degradation of surface-bound ^{125}I -ASOR in the State 1 pathway

Freshly isolated (State 1) cells were incubated at 4°C for 10 min in Medium 1/EDTA or Buffer 1/BSA adjusted to pH 5.0 with 2-(*N*-morpholino)ethanesulphonic acid instead of Hepes. Control cells were incubated in Medium 1/BSA. Cells were then washed three times at 4°C with Hanks', and allowed to bind ^{125}I -ASOR ($1.5\ \mu\text{g}/\text{ml}$) at 4°C for 1 h. Non-bound ligand was removed by washing 3 times with Hanks', and the cells were resuspended in Medium 1/BSA and placed at 37°C . Release of acid-soluble radioactivity into the medium was determined between 0 and 30 min (four or five points) as described in the Experimental section. In another experiment, State 1 cells, containing surface-bound ^{125}I -ASOR, were incubated at 37°C in the presence of the protease inhibitor bacitracin. The rates of ^{125}I -ASOR degradation were calculated as described above. Slopes were calculated by least-squares linear regression analysis, and in all cases $r \geq 0.97$. In experiments 1 and 2, the numbers in parentheses are the rates of degradation normalized for the initial amount of specific surface-bound ^{125}I -ASOR (i.e. % of surface-bound ligand/min per 10^6 cells). In experiment 3, the numbers in parentheses are the percentages of the control rate without inhibitor.

Experiment	Treatment	Rate of ^{125}I -ASOR degradation (c.p.m./min per 10^6 cells)	
		Treated cells	Untreated cells
1	Low pH wash	19.54 (0.051 %)	13.20 (0.076 %)
2	EDTA wash	36.90 (0.097 %)	17.38 (0.093 %)
3	0 μM -Bacitracin	—	5.68 (100 %)
	1 μM -Bacitracin	4.86 (86 %)	—
	10 μM -Bacitracin	5.82 (102 %)	—
	0.1 mM-Bacitracin	4.40 (77 %)	—
	1 mM-Bacitracin	4.72 (83 %)	—

Fig. 3. Effect of chloroquine on the degradation of ^{125}I -ASOR by State 1 hepatocytes

State 1 cells containing surface-bound ^{125}I -ASOR were incubated with PBS (a) or 10 mM-chloroquine (b) and transferred to 37 °C to initiate a synchronous round of endocytosis (●, ■) and degradation (○, □). Samples were taken at the indicated times, chilled to 4 °C, washed in medium 1/EDTA to remove surface-bound ligand, and cell-associated radioactivity was determined. Acid-soluble radioactivity in parallel samples was determined as described in the Experimental section.

glutathione-dependent proteases on the cell surface [21]. The amount of degradation due to soluble or released proteases was tested by incubating cells at 37 °C for 3 h, removing the cells by centrifugation and then incubating ^{125}I -ASOR with the conditioned medium at 37 °C. No significant proteolysis was detected by this mechanism.

Effect of chloroquine on ^{125}I -ASOR degradation

Chloroquine is an acidotropic amine that neutralizes acidic intracellular compartments and interferes with normal ligand processing [22]. Chloroquine does not inhibit the endocytosis of surface-bound ^{125}I -ASOR in State 2 rat hepatocytes but does completely stop ligand dissociation [8]. State 1 cells treated with 10 mM-chloroquine showed no release of ^{125}I -ASOR degradation products (Fig. 3b). A substantial amount of the initial cell-associated radioactivity is lost from untreated State 1 cells by 90 min and appears in the media as degradation products (Fig. 3a). State 1 cells treated with chloroquine retained all the radioactivity within the cell as intact ASOR. A titration curve for the effect of chloroquine on ^{125}I -ASOR degradation in State 1 or State 2 cells shows

Fig. 4. Effect of chloroquine concentration on the rate of ^{125}I -ASOR degradation

The initial steady-state rates of degradation of one round of surface-bound ^{125}I -ASOR were determined as described in the legend to Fig. 1 in the presence of increasing concentrations of chloroquine for both State 1 cells (open symbols) and State 2 cells (closed symbols). Each different symbol represents an independent experiment.

that both degradation pathways have very similar sensitivity to the inhibitor (Fig. 4).

Effect of monensin on ^{125}I -ASOR degradation

Monensin is an ionophore that mediates an electro-neutral exchange of Na^+/K^+ for H^+ across membranes and can disrupt these ion gradients and the dissociation of internalized receptor-ligand complexes [23]. Dissociation is an obligatory step before ligand degradation in class II recycling receptor systems [24]. Monensin blocked ^{125}I -ASOR degradation mediated by both State 1 and State 2 cells in a similar manner in a narrow concentration range between 2 and 8 μM (Fig. 5). However, a small but significant difference was observed in the sensitivities of the two pathways, particularly in the region of 2–4 μM -monensin. The State 2 pathway was more sensitive than the State 1 pathway (Fig. 5a). ^{125}I -ASOR degradation in the State 2 pathway was essentially completely blocked by 3.5 μM -monensin, whereas degradation in the State 1 pathway was largely unaffected (Fig. 5b).

Effect of colchicine on ^{125}I -ASOR degradation

Delivery of ligand to lysosomes is often sensitive to colchicine and other microtubule depolymerizing drugs that will therefore indirectly inhibit degradation of ligand

Fig. 5. Effect of monensin concentration on ^{125}I -ASOR degradation

The rates of degradation of surface-bound ^{125}I -ASOR were determined as described in Fig. 1 in the presence of various monensin concentrations using State 1 (open symbols represent different experiments). (a) The results were normalized to the rate of degradation in the absence of monensin in State 2 cells that contain both State 1 and 2 pathways or to the rate in State 1 cells that contain only the State 1 receptor pathway. (b) Results were normalized to the control rate of degradation in State 2 cells.

and receptor recycling [25]. The effect of colchicine on ^{125}I -ASOR degradation in State 2 cells (Fig. 6a) showed a biphasic titration curve, whereas the State 1 cells (Fig. 6b) showed a monophasic titration curve representing only one of the two events seen in the State 2 cells. Lumicolchicine had no effect [results not shown; 25]. In the region of 0.1 – $1 \mu\text{M}$ -colchicine, degradation in the State 2 cells was inhibited by about 50%, whereas under the same conditions there was no inhibition of degradation in the State 1 cells. Colchicine preferentially inhibits degradation in the State 2 pathway and has minimal effects on degradation in the State 1 pathway. Degradation of ^{125}I -ASOR in the State 2 Gal receptor pathway was approximately 30-fold more sensitive to colchicine than degradation in the State 1 pathway. 50% inhibition of degradation in the State 2 and State 1 cells was achieved at approx. 0.1 and $3 \mu\text{M}$ -colchicine respectively (Fig. 6).

DISCUSSION

We previously hypothesized that the Gal receptor system in isolated hepatocytes involves two parallel pathways for ligand processing and receptor recycling [8,11]. The evidence in support of this two-pathway model is summarized in Table 1. Ultimately, in either

Fig. 6. Effect of colchicine concentration on ^{125}I -ASOR degradation

The steady-state rates of degradation of surface-bound ^{125}I -ASOR were determined as described in the legend to Fig. 1 in the presence of increasing concentration of colchicine in either State 2 cells (a) or State 1 cells (b). Each different symbol represents a separate experiment.

pathway, internalized ligand (^{125}I -ASOR) is degraded to the level of amino acids and monosaccharides within a lysosomal compartment, while the receptor is spared destruction, presumably bypasses the lysosome and returns to the cell surface [7,8,26]. In a previous study we demonstrated that under conditions of a synchronous wave of internalization, the bound ligand is dissociated by two kinetically distinct processes [10]. Starting with one round of endocytosis of surface-bound ^{125}I -ASOR, the rate of ASOR degradation is a first-order process that is directly dependent on the number of active surface Gal receptors [12]. Surprisingly, approx. 25–50% of the endocytosed ^{125}I -ASOR was degraded immediately after internalization without a lag period. The remaining ^{125}I -ASOR was degraded following a lag of 15–30 min. Earlier studies by other investigators [20,27,28] as well as this laboratory [25] demonstrated that the intracellular transport of the majority of internalized asialoglycoproteins to lysosomes requires between 15 and 30 min. However, all of these studies employed conditions of continuous ligand uptake.

The proportion of surface-bound ^{125}I -ASOR degraded by either the immediate-phase or the delayed-phase pathways [12] was dependent on the relative expression of two functionally distinct populations of Gal receptors on the cell surface, previously designated as State 1 and State 2 receptors [11]. We interpret the different onset times for ligand degradation as the transit times for

ligand delivery to different lysosomal compartments in the two different pathways.

These previous degradation studies have shown that there are different pathways for delivery of ligand to a lysosomal compartment(s). The objective in the present study was to determine whether these two degradative pathways displayed the same sensitivity to a variety of inhibitors that block degradation of internalized proteins. Both Gal receptor pathways apparently require the ligand to be in or pass through a low pH compartment prior to degradation. The simplest explanation is that lysosomal function requires an acidic pH. This conclusion is based on the observation that both chloroquine and monensin, which collapses intracellular pH gradients, inhibit degradation of ^{125}I -ASOR in both pathways. Chloroquine destroys the low pH gradient in acidic compartments such as lysosomes and endosomes [29]. In the presence of chloroquine ^{125}I -ASOR is internalized but then is not dissociated from either State 1 or State 2 Gal receptors and is not degraded. Both the State 1 and State 2 pathways are similarly sensitive to chloroquine.

Monensin can disrupt both Na^+ and H^+ gradients and also increases the pH of intracellular organelles [30]. Monensin does not inhibit the endocytosis of surface-bound ^{125}I -ASOR in either the State 1 or the State 2 pathway [31]. Degradation of ^{125}I -ASOR in both Gal receptor pathways was sensitive to monensin. There was a slight but significant difference which might reflect the presence of discrete endosome/lysosome populations dedicated to different endocytosis pathways [27,32–34]. The State 2 degradative pathway was more sensitive to inhibition by monensin than the State 1 pathway. In a previous study [31], we showed that monensin blocks dissociation of internalized ^{125}I -ASOR from State 2 receptors, in agreement with the results of Harford *et al.* [23]. However, dissociation of State 1 Gal receptor-ASOR complexes still occurred in the presence of monensin, suggesting that ligand dissociation in the State 1 pathway may not be mediated by low pH.

The tripeptide protease inhibitor, leupeptin, inhibits cathepsins and has been used to demonstrate lysosomal-specific proteolysis. This peptide inhibitor blocked ^{125}I -ASOR degradation in both the State 1 and State 2 pathways. Again, there was a difference in the sensitivities between the two pathways. The State 2 pathway was approximately 6-fold more sensitive than the State 1 pathway.

Transport of internalized ^{125}I -ASOR to the degradative compartment is the most apparent difference between the State 1 and the State 2 degradative pathways. Colchicine preferentially blocks degradation in the State 2 Gal pathway. The concentration of colchicine required for inhibition in this pathway is approximately 30-fold less than that required to inhibit degradation in the State 1 pathway. Cytoplasmic dyneins use microtubules to move secretory vesicles in neural axons [35] and microtubules can direct counterflow migration of vesicles by the action of kinesins [36]. Microtubules are important during endocytosis for both intracellular organization of vesicles and their routing between sequential compartments [37].

The results are consistent with the conclusion that ligand delivery in the State 2 degradative pathway requires microtubules and that the 15–30 min lag time represents the time needed to transport ligand from the cell surface to lysosomal compartments in the cell interior. The resistance of the State 1 degradative pathway to

microtubule depolymerizing agents indicates that delivery of ligand to these degradative compartments does not require microtubules. Furthermore, since the onset of ^{125}I -ASOR degradation in the State 1 pathway is immediate, the lysosomes or similar degradative compartments in which the ligand in this pathway is processed must be much closer to the cell surface and not far into the cell interior. Possibly a subclass of lysosomes available at the cell surface is involved in processing ligand entering the cells by the State 1 pathway and these lysosomes are functionally distinct from those in the State 2 pathway. Subpopulations of lysosomes involved in asialoglycoprotein metabolism in hepatocytes have been reported by Berg *et al.* [27].

The major pathway for endocytosis and degradation of asialoglycoproteins is mediated by the State 2 Gal receptor population and probably accounts for 80 % of the steady-state rate of ligand processing [8]. Colchicine ($1\text{ }\mu\text{M}$) blocks continuous steady-state ^{125}I -ASOR uptake and degradation by 80 % [25]. The effects of colchicine are not unique and are also found with other microtubule drugs such as vinblastine sulphate, colcemide and podophyllotoxin. Lumicolchicine does not cause an inhibition. Similar studies by others using colchicine also typically show a residual insensitive degree of degradation ranging from 10–20 % of the control steady-state degradative rate. The colchicine-resistant degradation is probably due to the State 1 Gal receptor pathway.

The results do not support the idea that the source of the proteolytic activity responsible for degrading the internalized ^{125}I -ASOR in either of the two Gal receptor pathways is a cell surface protease. In particular, removal of surface proteins by treatment with pH 5.0 or EDTA-containing buffers or use of the surface protease inhibitor bacitracin [21] did not diminish the no-lag State 1 degradation process. Degradation is probably due to an intracellular protease residing in a low pH compartment. Delivery of the internalized ligand to the compartments with the protease(s) requires a pH gradient and/or the protease requires low pH for activity.

The present results provide further evidence for the two-pathway model to explain how the hepatic Gal receptor system functions [8,11]. Further studies are needed to determine whether the two pathways reside in the same cell or in two discrete hepatocyte subpopulations. Asialoglycoprotein degradation in the major State 2 Gal receptor pathway is more sensitive than the minor State 1 Gal receptor pathway to inhibition by leupeptin, monensin and particularly colchicine. Since the 'State 2 cell suspensions' contain both the State 1 and State 2 Gal receptor pathways, the actual difference in sensitivity between the State 1 pathway and the State 2 pathway is greater than the observed difference between State 1 and State 2 cells. We do not know if there is a structural basis for the two functionally distinct Gal receptor populations. However, we recently proposed a new structural model for the receptor, in which three different heterohexamers can be formed from the three rat Gal receptor polypeptides [38].

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REFERENCES

1. Goldstein, J. L., Brown, M. S., Anderson, R. G. W., Russell, D. W. & Schneider, W. J. (1985) *Annu. Rev. Cell Biol.* **1**, 1–39
2. Klausner, R. D., Ashwell, G., van Renswoude, J., Harford, J. B. & Bridges, K. R. (1983) *Proc. Natl. Acad. Sci. U.S.A.* **80**, 2263–2266
3. Dautry-Varsat, A., Ciechanover, A. & Lodish, H. R. (1983) *Proc. Natl. Acad. Sci. U.S.A.* **80**, 2258–2262
4. Berhanu, P., Olefsky, J. M., Tsai, P., Thamm, P., Saunders, D. & Brandenburg, D. (1982) *Proc. Natl. Acad. Sci. U.S.A.* **79**, 4069–4073
5. Planck, S. R., Finch, J. S. & Magun, B. E. (1984) *J. Biol. Chem.* **259**, 3053–3057
6. Harford, J., Bridges, K., Ashwell, G. & Klausner, R. D. (1983) *J. Biol. Chem.* **258**, 3191–3197
7. Schwartz, A. L. (1984) *CRC Crit. Rev. Biochem.* **16**, 207–233
8. Weigel, P. H. (1987) in *Vertebrate Lectins* (Parent, J. B. & Olden, K., eds.), pp. 65–91, Van Nostrand Reinhold, NY
9. Weigel, P. H. & Oka, J. A. (1983) *J. Biol. Chem.* **258**, 5089–5094
10. Oka, J. A. & Weigel, P. H. (1983) *J. Biol. Chem.* **258**, 10253–10262
11. Weigel, P. H., Clarke, B. L. & Oka, J. A. (1986) *Biochem. Biophys. Res. Commun.* **140**, 43–50
12. Clarke, B. L., Oka, J. A. & Weigel, P. H. (1987) *J. Biol. Chem.* **262**, 17384–17392
13. McAbee, D. D. & Weigel, P. H. (1987) *J. Biol. Chem.* **262**, 1942–1945
14. McAbee, D. D. & Weigel, P. H. (1988) *Biochemistry* **27**, 2061–2069
15. Weigel, P. H. & Oka, J. A. (1982) *J. Biol. Chem.* **257**, 1201–1207
16. Seglen, P. O. (1976) *Methods Cell Biol.* **13**, 29–83
17. Bradford, M. M. (1976) *Anal. Biochem.* **72**, 248–254
18. Aulinskas, T. H., Coetzee, G. A. & Van der Westhuyzen, D. R. (1981) *Biochim. Biophys. Acta* **663**, 421–431
19. Tolleshaug, H. & Berg, T. (1981) *Exp. Cell Res.* **134**, 207–217
20. Harford, J., Klausner, R. D. & Ashwell, G. (1984) *Biol. Cell* **51**, 173–180
21. Roth, R. A. (1981) *Biochem. Biophys. Res. Commun.* **98**, 431–438
22. Schwartz, A. L., Bolognesi, A. & Fridovich, S. E. (1984) *J. Cell Biol.* **98**, 732–738
23. Harford, J., Wolkoff, A. W., Ashwell, G. & Klausner, R. D. (1983) *J. Cell Biol.* **96**, 1824–1828
24. Kaplan, J. (1981) *Science* **212**, 14–20
25. Oka, J. A. & Weigel, P. H. (1983) *Biochim. Biophys. Acta* **763**, 368–376
26. Ashwell, G. & Harford, J. (1982) *Annu. Rev. Biochem.* **51**, 531–554
27. Berg, T., Ford, T., Kindberg, G., Blomhoff, R. & Drevon, C. (1985) *Exp. Cell Res.* **156**, 570–574
28. Zeitlin, P. L. & Hubbard, A. L. (1982) *J. Cell Biol.* **92**, 634–647
29. deDuve, C., de Barsey, T., Poole, B., Trouet, A., Tulkens, P. & Van Hoof, F. (1974) *Biochem. Pharmacol.* **23**, 2495–2531
30. Maxfield, F. R. (1982) *J. Cell Biol.* **95**, 676–681
31. Oka, J. A. & Weigel, P. H. (1987) *J. Cell. Physiol.* **133**, 243–252
32. Marshall, S. (1985) *J. Biol. Chem.* **260**, 13524–13531
33. Yamashiro, D. J. & Maxfield, F. R. (1987) *J. Cell Biol.* **105**, 2723–2733
34. Schmid, S. L., Fuchs, R., Male, P. & Mellman, I. (1988) *Cell* **52**, 73–83
35. Gibbons, I. R. (1988) *J. Biol. Chem.* **263**, 15837–15840
36. Vale, R. D. (1987) *Annu. Rev. Cell Biol.* **3**, 347–378
37. Herman, B. & Albertini, D. F. (1984) *J. Cell Biol.* **98**, 565–576
38. Herzig, M. C. S. & Weigel, P. H. (1989) *Biochemistry* **28**, 600–610
39. Oka, J. A. & Weigel, P. H. (1984) *J. Biol. Chem.* **259**, 1150–1154

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