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THE HEPATIC GALACTOSYL RECEPTOR SYSTEM: TWO DIFFERENT LIGAND DISSOCIATION PATHWAYS ARE MEDIATED BY DISTINCT RECEPTOR POPULATIONS

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After internalization of ^{125}I-asialo-orosomucoid (ASOR) by isolated rat hepatocytes, ligand dissociates by two kinetically distinct pathways (Oka and Weigel, J. Biol. Chem. 257, 10,253, 1983). These slow and fast dissociation pathways correspond to two functionally different subpopulations of cell surface galactosyl receptors designated, respectively, State 1 and State 2 receptors. Freshly isolated cells or cells equilibrated below 24°C express only State 1 receptors. Cells equilibrated at 37°C express both State 1 and State 2 receptors. Ligand dissociation after internalization of surface-bound ^{125}I-ASOR was measured using the permeabilizing detergent, digitonin. The slow dissociation pathway was mediated by State 1 receptors and was the only pathway expressed by cells which were freshly isolated or had been equilibrated at 24°C. State 2 receptors are expressed at temperatures above about 20°C, and both the fast and slow dissociation pathways occurred in cells equilibrated at 37°C. State 2 receptors therefore mediate the rapid dissociation pathway. Dissociation and subsequent degradation of specifically bound ligand routed in either pathway were complete, respectively, within 3 and 6 hrs.

The mammalian hepatic galactosyl (Gal) receptor mediates the removal of desialylated glycoproteins from plasma (1). The enormous endocytic capacity of this system is possible because receptors are recycled to the cell surface and reutilized thousands of times before they are degraded (1-3). Receptor recycling, which takes 3-7 min (1,4), requires that internalized ligand rapidly dissociate from receptor so that the two molecules can be segregated and directed along their respective intracellular routes. Surprisingly, receptor-ligand dissociation was previously found to occur via two kinetically and thermally distinct pathways with first order rate constants of 0.014 min^{-1} for the slow and 0.23 min^{-1} for the fast pathway (4,5). We have suggested that

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there are two separate pathways in hepatocytes for the uptake, dissociation and degradation of ligand (6-8). Hepatocytes equilibrated at 37°C express two types of cell surface Gal receptor activity. These two receptor activities are defined[1] by their differential modulation, in the absence of ligand, by a variety of treatments including low temperature (9), metabolic energy poisons (10,11), microtubule drugs (12,13), monensin (14,15), chloroquine (16) and hyperosmolarity[1]. These treatments reversibly decrease the expression of surface Gal receptor activity on cells at 37°C. However, only approximately 50% of the total receptor activity is affected and all of these treatments affect this same proportion of receptors (7). We have designated these modulatable and nonmodulatable receptors, respectively, as State 2 and State 1 receptors. Cells freshly isolated by collagenase perfusion or cells equilibrated first at 37°C and then at 24°C express essentially only State 1 receptor activity. Down modulation of State 2 receptor activity at 24°C occurs in 1-2 hrs (9). We show here that the slow dissociation pathway is mediated by State 1 Gal receptors and is distinct from the fast dissociation pathway which is mediated by State 2 Gal receptors.

METHODS

Materials. Orosomucoid, a gift from M. Wickerhauser, American Red Cross Protein Derivatives Laboratory, was desialylated and iodinated as described (17). Phosphotungstic acid and digitonin were from Sigma. Other chemicals were reagent grade. Freshly isolated hepatocytes were prepared from male Sprague-Dawley rats (Harlan, Houston, TX) by collagenase perfusion (17). Buffers and media were previously described (18). Cells were kept at room temperature during the filtration and differential centrifugation steps. They were then stored on ice after isolation and were at least 90% viable and single cells. Experiments were in the absence of serum. Cell suspensions were incubated at 37°C for 1 hr or 24°C for 2 hr in Erlenmeyer flasks at 100 rpm in a gyratory water bath to equilibrate cell surface receptor number (9,18).

Assays for ^{125}I-ASOR binding, dissociation and degradation. Assessment of specific binding of ^{125}I-ASOR by a centrifugation assay and the prebinding of ^{125}I-ASOR to surface receptors at 4°C have been described (18). Specific binding was >90%. Endocytosis at 37°C was determined by an EGTA washing procedure (18) and free, dissociated ligand was determined by the ability of digitonin to release intracellular ^{125}I-ASOR (4). Digitonin stock solutions dissolved with warming in absolute ethanol (1.4% w/v) were used within two weeks or less. Degradation of ^{125}I-ASOR was followed by the appearance of acid soluble radioactivity assessed with phosphotungstic acid in HCl (17).

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RESULTS AND DISCUSSION

State 2 Gal receptor activity on the cell surface is operationally defined by the reversible down-modulation of this activity by low temperature (24-37°C), metabolic energy poisons, colchicine, monensin or hyperosmolar conditions. State 1 surface Gal receptor activity is not affected by these treatments. Cells containing only State 1 receptors (freshly isolated cells) or both State 1 and 2 receptors (37°C equilibrated cells) were rapidly chilled to 0°C, allowed to bind ^{125}I-ASOR, washed and incubated at 37°C. The rate for dissociating internalized ligand from receptors was then determined. Cells containing both modulatable (State 2) and nonmodulatable (State 1) receptor activity, exhibited both fast and slow ligand dissociation (Fig. 1A). Cells containing only the nonmodulatable (State 1) receptor population only showed the slow dissociation process; the fast process was absent (Fig. 1B). Data from the digitonin-treated samples in Fig. 1A and 1B were replotted on a log scale (Fig. 1C). The first order rate constants calculated by linear

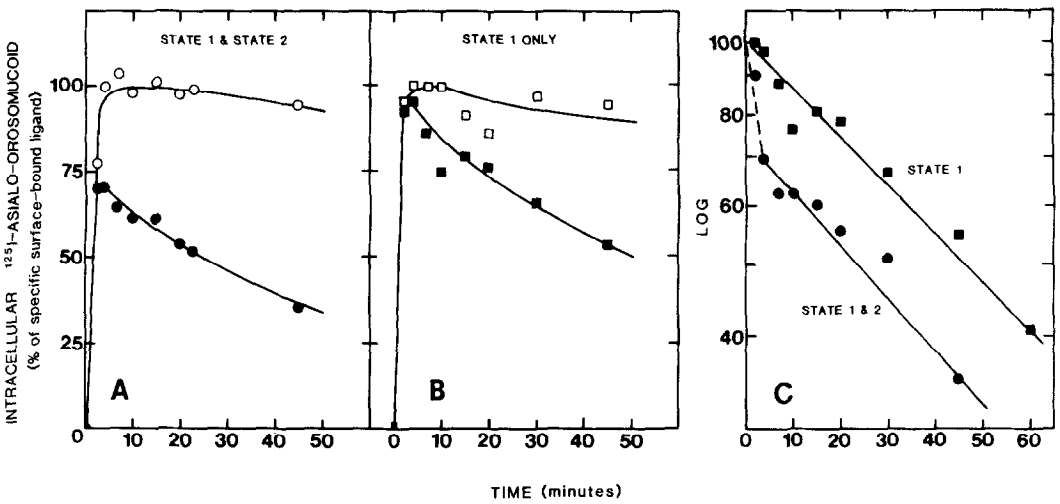

Figure 1. Dissociation of internalized ^{125}I-ASOR prebound only to State 1 receptors or to both State 1 and State 2 receptors. Freshly isolated (B) or 37°C equilibrated (A) cells containing, respectively, State 1 (□, ■) or both State 1 and 2 (○, ●) receptors were allowed to bind 1.5 ug/ml ^{125}I-ASOR at 4°C for 1 h. The cells were washed, incubated at 37°C and samples, chilled on ice, were washed once with media containing EGTA and then with media with (●, ■) or without (○, □) 0.055% digitonin. Radioactivity in the final cell pellet was determined and represents, respectively, the specific internal receptor-bound ligand and the total internal ligand. 100% values: 59 fmol/10^6 cells, State 1 cells; 311 fmol/10^6 cells, State 2 cells. The fast dissociation process, which is over within 5 min in Panel A, is absent in Panel B. C. Log plot of intracellular receptor-ligand complexes from Panel A (●) and Panel B (■).

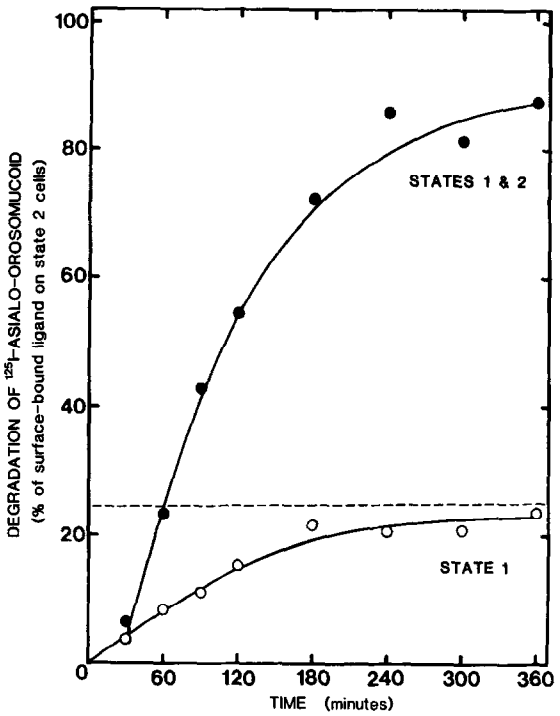

Figure 2. Kinetics and extent of degradation of ^{125}I-ASOR prebound only to State 1 receptors or to both State 1 and State 2 receptors. State 1 cells (○; freshly isolated) and State 2 cells (●; equilibrated at 37°C) were pre-bound at 4°C with ^{125}I-ASOR, washed and transferred to 37°C to initiate a synchronous round of endocytosis. The release of acid soluble radioactivity relative to the specifically bound ^{125}I-ASOR on State 2 cells at the start of the experiment (148 fmol/10^6 cells) was determined. The dashed line represents the amount of surface-bound ^{125}I-ASOR on State 1 cells (37 fmol/10^6 cells).

regression analyses for the slow dissociation were 0.016 min^{-1} ($r = -0.989$) for the 37°C equilibrated cells and 0.015 min^{-1} ($r = -0.977$) for the freshly isolated cells. These two rates are not significantly different as analyzed by an F variance ratio test ($p > 0.95$). The same results as in Fig. 1B were obtained when cells were first equilibrated at 37°C and then equilibrated at 24°C. Intracellular dissociation of ligand from either State 1 or State 2 receptors was complete in 3 hrs (not shown).

Despite different rates of dissociation, ligand is processed to the same ultimate fate in either pathway; all of the bound ^{125}I-ASOR is degraded (Fig. 2). We have recently shown[2] that hepatocytes have two degradative pathways for asialoglycoproteins differentiated by the rate at which they degrade ligand, the presence (State 2 receptors) or absence (State 1 receptors) of a lag before degradation begins and by their differential sensitivity to colchicine (6,7).

Hepatocytes therefore appear to have two distinct pathways by which Gal receptors can mediate ligand uptake, dissociation and degradation.

The following results support the conclusion that the slow and fast dissociation pathways are functionally different and that receptors in one pathway do not enter the other pathway. (i) At 18°C the number of internalized complexes in the slow dissociation compartment saturates but dissociation is completely blocked (4). The fast dissociation process, however, continues at 18°C and receptor recycling still occurs; ASOR uptake reaches 6 times the surface (or 2 times the total) receptor number/cell within 5 hrs. (ii) Internalized State 1 receptor-ligand complexes committed to the slow dissociation compartment can return to the cell surface before they dissociate (19). With increasing time at 37°C they are either further processed or translocated along the endocytic pathway until they pass a point from which they cannot return to the surface. At 18°C slow dissociation ceases (4) and there is no time dependent loss of the ability of complexes to return to the surface (8). Although complexes committed to the slow dissociation compartment return to the cell surface at 18°C, further processing and dissociation does not occur. These State 1 receptor complexes, once having returned to the surface, do not re-enter the cell via the State 2 pathway; they cannot follow the route of the rapidly dissociating complexes. The fast pathway can still function since ASOR added to these cells is processed.

The results from these and other studies are summarized in a schematic model of the Gal receptor system (Fig. 3). Two separate receptor pathways are proposed to function either in parallel in the same cell or in different subpopulations of hepatocytes. State 1 and State 2 receptors function in two different pathways; the State 1 receptor pathway and the State 2 receptor pathway. Endocytosis in either pathway has at least nine Stages, designated by numbers in the Model: 1) Ligand binding, 2) Receptor-ligand complex internalization, 3) Entry of complexes into two different Gal receptor pathways, 4) Dissociation of complexes by either a fast or a slow process to give free ligand and receptor, 5) Segregation (spatial separation) of free

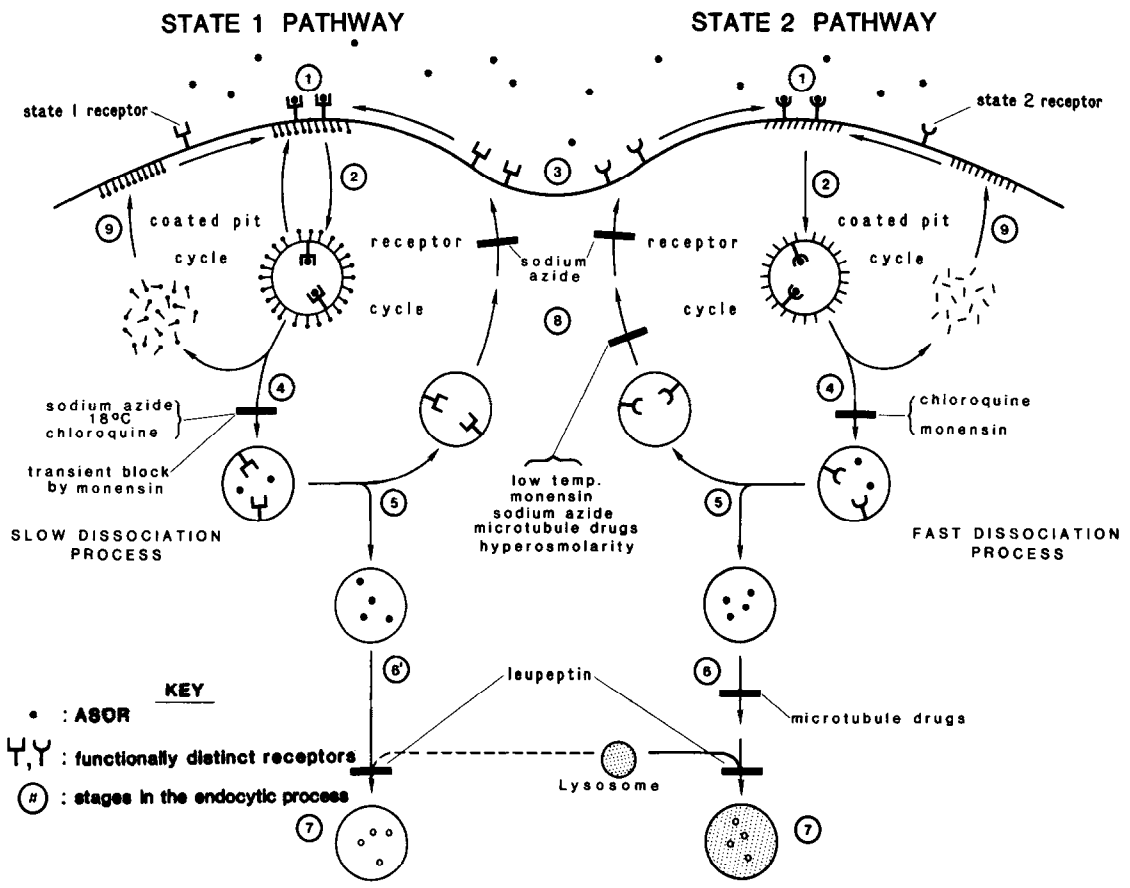

Figure 3. A functional model of the hepatic Gal receptor system. Two parallel pathways for internalization and ligand dissociation and degradation mediated by State 1 and State 2 Gal receptors are shown. Points at which various inhibitors or treatments block the system are indicated by solid bars.

ligand and receptor into different subcellular compartments, 6) Ligand delivery to lysosomes or other degradative compartments, 7) Ligand degradation, 8) Receptor processing and recycling to the cell surface, 9) Re-incorporation of receptor into coated pits and integration of the coated pit cycle(s) with the receptor cycle(s).

The major endocytic pathway is mediated by State 2 receptors, which are sensitive to modulation by a variety of agents; this reflects constitutive recycling. State 2 receptors mediate fast dissociation, and a 20 min lag prior to ligand degradation. The minor pathway is mediated by State 1 receptors, which are not modulated and do not recycle in the absence of ligand. The State

1 receptor pathway is characterized by slow dissociation of ligand and the immediate onset of degradation[2]. The State 1, slow dissociation pathway is the pathway responsible for diacytosis (20), the return of intact internalized ligand to the cell surface (19). Although the State 1 Gal receptor pathway is the minor pathway in terms of endocytic and degradative capability, it is likely that it has another unrecognized important nonendocytic function (e.g. intracellular routing of hepatocyte-specific glycoproteins).

Dissociation presumably occurs in two morphologically distinct compartments, different in the rate at which they release free ligand. During endocytosis, ligand bound to State 1 or State 2 receptors is routed into either the State 1 or State 2 pathway. About 1/2 of the complexes follow the State 2 pathway and this partition is unaffected by receptor occupancy (4). Stage 3 does not occur as a separate partition event after internalization. Entry or commitment of receptor-ligand complexes into either the slow or fast dissociation pathway is inherent in the system because State 1 and State 2 receptors are functionally distinct and/or in different types of coated pits.

The two functionally distinct receptor types could be due to structural differences (21), represented by the two different symbols, which determine whether or not the receptors are in coated pits or if one type is in a coated and the other a non-coated endocytic pathway. State 2 rapidly dissociating and recycling receptors would follow the coated pit endocytic pathway. Alternatively, as shown, both endocytic pathways could be mediated by different types of coated pits. In this case the receptors might be structurally different and thereby directed into the proper coated pit or they could be identical and randomly partitioned into functionally different coated pit pathways. Since the activation energies for endocytosis by State 1 and State 2 receptors is identical[2], the mechanisms of internalization may also be identical. The two pathway model is in agreement with recent studies showing two subpopulations of lysosomes in isolated rat hepatocytes (22).

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2. Clarke, B.L. and Weigel, P.H. manuscript in preparation.

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