Recycling of the Hepatic Asialoglycoprotein Receptor in Isolated Rat Hepatocytes

RECEPTOR-LIGAND COMPLEXES IN AN INTRACELLULAR SLOWLY DISSOCIATING POOL RETURN TO THE CELL SURFACE PRIOR TO DISSOCIATION*

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We recently reported that the dissociation of internalized receptor-125I-asialo-orosomucoid (ASOR) complexes by isolated hepatocytes is a biphasic process; most complexes dissociate rapidly but 25–50% dissociate slowly (Oka, J. A., and Weigel, P. H. J. Biol. Chem. 258, 10253–10262). Cells were allowed to endocytose a pulse of surface-bound 125I-ASOR, and were washed and then incubated at 37 °C in the presence or absence of ethylene glycol bis(β-aminoethyl ether)-N,N,N',N'-tetraacetic acid (EGTA). Without EGTA, very little intact ASOR appeared in the medium. With EGTA present, a large amount of intracellular ligand appeared undegraded in the medium in a time-dependent manner. N-Acetylgalactosamine, but not ASOR, in the medium also caused release of intact 125I-ASOR. Within 15 min, more than 50% and by completion at least 80% of the internalized ligand in the slow dissociation compartment was released into the medium. If cells containing internalized ligand were incubated at 37 °C for increasing times before the addition of EGTA, the amount of intact ligand decreased and the amount of intact ligand in the medium increased by essentially equal amounts. The temperature dependence for the return of internal 125I-ASOR to the cell surface was similar to that for endocytosis, with a cut-off temperature of about 12 °C. We conclude that a normal part of the endocytic process involves the return of receptor-ligand complexes to the cell surface from an internal slowly dissociating pool. This might reflect either an obligatory step or a reversible statistically random step in the endocytic/recycling pathway.

EXPERIMENTAL PROCEDURES

Materials—Human orosomucoid (α1 acid glycoprotein) was a gift from Dr. M. Wickerhauser of the Plasma Derivatives Laboratory, the American Red Cross. ASOR was desialylated (6) and iodinated (8) as described elsewhere. Sephadex G-75 (superfine) was from Pharmacia Chemical Co. Triton X-100, collagenase (type I), BSA (fraction V), phosphotungstic acid, EGTA, GallNac, and neuraminidase (type X) were from Sigma. 1,3,4,6-Tetrachloro-3a,6a-diphenylglycouril was from Pierce Chemical Co. Digitonin was from Matheson, Coleman, and Bell. Male Sprague-Dawley rats were obtained from Timco Breeding Laboratories, Houston, TX. Medium 1 and Medium 1/BSA have been described (6). All other chemicals were reagent grade.

Hepatocyte Preparation—Rat hepatocytes were prepared by the collagenase perfusion procedure of Seglen (9) as described previously (10). Final cell pellets were suspended in ice-cold Medium 1 and were usually ≥98% viable and single cells. Experiments were performed in the absence of serum. Prior to all experiments, fresh cell suspensions (2 × 10^6/ml in Medium 1/BSA; 10% of the Erlenmeyer flask volume) were incubated at 37 °C for 45 min at 100 rpm in a gyratory water bath to increase and stabilize the number of surface receptors/cell (10, 11).

Measurement of Degradation and Intracellular Total, Receptor-bound, and Nonbound 125I-ASOR—Cells (4 × 10^6/ml in Medium 1/BSA) were first allowed to bind 125I-ASOR (1.5 μg/ml) at 0 °C for 60 min, washed, and incubated at 37 °C. After 7 min (unless noted otherwise) the cells were then put on ice, washed with EGTA to remove any remaining surface-bound ligand, resuspended at 2 × 10^6/
ml in Medium 1/BSA, with or without 7.5 mM EGTA and put at the desired temperature to start the experiment. Samples were removed and diluted with 1 volume of Medium 1 on ice. One portion of the diluted suspension was used to determine degradation by measuring acid-soluble radioactivity. Other portions were centrifuged and radioactivity in the supernatant fluids was determined. This represents both intact and degraded $^{125}$I-ASOR released into the medium. Intact asporin in the medium was then calculated by subtracting the amount of degraded material. The cell pellets were resuspended in Medium 1 with or without 0.05% digitonin, incubated on ice for 10 min, and centrifuged. Radioactivity in these cell pellets and supernatants was determined to assess the total intracellular $^{125}$I-ASOR (i.e., the cell pellet without digitonin), the amount of intracellular $^{125}$I-ASOR still bound to receptor (i.e., the digitonin-treated cell pellet), and the amount of intracellular $^{125}$I-ASOR which had dissociated from receptor and was free (i.e., the digitonin-treated cell supernatant). The use of digitonin to quantify intracellular receptor activity and to differentiate intracellular free and receptor-bound ligand was recently described (12, 13). In any of these experiments, cell viability was not compromised by the presence of EGTA or GalNAc in the medium.

**RESULTS AND DISCUSSION**

We recently described the use of digitonin to quantitate intracellular receptor-bound versus free ligand (12, 13). With this method, we demonstrated that the dissociation of internalized $^{125}$I-ASOR from its receptor in freshly isolated hepatocytes is biphasic (6). Virtually the same results were obtained by Harford et al. (7) using a detergent solubilization technique with cultured hepatocytes. The generation of free ligand inside the cell after the endocytosis of surface-bound $^{125}$I-ASOR is characterized by two rate constants ($\approx 0.28$ and 0.014 min$^{-1}$). Depending on the steady state status of the overall system, 25–50% of the internalized $^{125}$I-ASOR enters a slowly dissociating pool (6). Receptor-ligand complexes in this pool dissociate over a period of 120–150 min. At the start of the experiments presented here, cells contained a pulse of internalized $^{125}$I-ASOR (achieved by the brief endocytosis of surface-bound ligand). Internalized ligand committed to the rapidly dissociating pool was already dissociated. Intracellular $^{125}$I-ASOR was therefore either free or bound to receptor in a slow dissociation compartment. These cells were then incubated at 37 °C with or without EGTA in the medium.

In the absence of EGTA, very little intact $^{125}$I-ASOR was found in the medium, although degraded glycoprotein began to accumulate after 15 min (Fig. 1, A and B). In the presence of EGTA, the rate of degradation was lower and there was a rapid accumulation of intact $^{125}$I-ASOR in the medium. The rate and extent of the decrease in intracellular receptor-bound ligand was also greater in the presence of EGTA (Fig. 1C). The initial rate of $^{125}$I-ASOR release was 20-30-fold greater when EGTA or GalNAc (see below) was present. There was little change in the amount of free intracellular $^{125}$I-ASOR (± EGTA) within the first 20 min (Fig. 1D), whereas the receptor-bound ligand in the slow dissociation compartment decreased by 51 fmol/10$^6$ cells (Fig. 1C) and the amount of $^{125}$I-ASOR released into the medium increased by about 54 fmol/10$^6$ cells (Fig. 1A). These results suggest that the $^{125}$I-ASOR lost from cells in the presence of EGTA comes from the intracellular slowly dissociating receptor-bound pool and not the free pool. Without EGTA, the pool of free intracellular $^{125}$I-ASOR increased as the slow dissociation of receptor-ligand complexes occurred and then decreased when the rate of degradation reached a steady state. With EGTA present, the free internal $^{125}$I-ASOR pool was smaller (consistent with the loss of $^{125}$I-ASOR from the slowly dissociating pool to the medium) and the free ligand pool size decreased earlier because free ligand was degraded but was not replaced by ligand released from the slowly dissociating pool.

To summarize the results in Fig. 1, the intracellular ligand is either free or bound to receptor in a slowly dissociating kinetic compartment. With no extracellular EGTA, the size of the free ligand pool increases and then decreases, the size of the receptor-bound pool slowly decreases and degraded (but very little intact) $^{125}$I-ASOR is released into the medium. With EGTA present, the free ligand pool decreases sooner, the steady state rate of degradation is slower, the receptor-bound pool size decreases more rapidly and a large amount of intact $^{125}$I-ASOR appears in the medium. The same results were obtained if the system was first allowed to reach a steady state by incubating cells with ASOR for >90 min.

Previously internalized $^{125}$I-ASOR returns to the cell surface in a time-dependent manner. If this reflects a normal
function of the endocytosis and/or receptor recycling machinery, then we would expect that, with increasing time, a pulse of internalized ligand would be more extensively processed by the cell (i.e. dissociated from receptor, delivered to lysosomes, and finally degraded) and that the amount of ligand which could return to the cell surface would decrease. This was tested by adding EGTA to the medium at increasingly later times after the initial internalization of surface-bound 125I-ASOR. The rate and extent of 125I-ASOR release was dependent on the time of EGTA addition (Fig. 2A), indicating that the return of receptor-ligand complexes to the cell surface may reflect a normal function of the endocytic machinery.

In a number of experiments, the percent of 125I-ASOR in the internal slowly dissociating pool which could be released with EGTA or GalNAc (see below) was at least 80%. Within the limitations of our experimental design, some of the receptor-bound ligand may already have been processed beyond the point of being able to return to the surface. Therefore, it is possible that all of the internal 125I-ASOR in the slow dissociation compartment can return to the cell surface. We do not know if this return is an obligatory step or a statistical step reflecting a random equilibrium process in the endocytic/recycling pathway.

GalNAc, the monosaccharide with the greatest affinity for the receptor (15, 16), very effectively caused the release of previously internalized 125I-ASOR into the medium (Fig. 2B). ASOR in the medium only caused a slight release of 125I-ASOR and was not as effective as either EGTA or GalNAc (Fig. 2B). It is therefore the intracellular receptor-bound 125I-ASOR which returned to the cell surface (or at least to a state of accessibility to these agents). Free 125I-ASOR did not return to the cell surface, get released, and then rebind to surface receptors. If this had occurred, then ASOR in the medium would have competed for rebinding of 125I-ASOR to surface receptors and would have caused a greater accumulation of ligand (also see Fig. 5 below).

We routinely employ acid precipitation in order to distinguish undegraded and degraded 125I-ASOR molecules. To show that the radioactivity released from cells in the presence of EGTA is intact 125I-ASOR, samples of medium were chromatographed on Sephadex G-75. Only two peaks were observed, one co-migrating with authentic 125I-ASOR and the other with Na125I (Fig. 3).

The temperature dependence for the rate of return of intracellular 125I-ASOR to the cell surface was examined to determine the cut-off temperature for this process and whether it could be correlated with that for endocytosis (i.e. 10 °C) (17), or degradation (i.e. 20 °C) (18). The release of internal 125I-ASOR into medium containing EGTA was very temperature-dependent (Fig. 4A). Below about 10–12 °C, the temperature dependence of the rate of release was low and the process was very slow (Fig. 4B). At or above about 12 °C, the temperature dependence dramatically increased in a manner similar to that for endocytosis (17).

We have shown that at 18 °C the slow dissociation of intracellular receptor-ligand complexes virtually ceases, whereas the fast dissociation process, although slower, proceeds to completion (6). Degradation is also blocked at 18 °C (8, 18). At 18 °C, the amount of free internal 125I-ASOR did not change over a period of 5 h (Fig. 5C). This was expected since the fast dissociation process was already complete and slow dissociation and degradation did not occur. During this time, the amount of receptor-bound ligand (Fig. 5A) decreased by 65% (52 fmol/10⁶ cells) and was matched, with closely

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**FIG. 2. Effect of GalNAc, ASOR, or the time of EGTA addition on the release of internalized 125I-ASOR.** A, cells were allowed to internalize surface-bound 125I-ASOR for 7 (Δ), 15 (○), or 30 (□) min at 37 °C, chilled, washed, and the release of intact 125I-ASOR into the medium when the cells were subsequently put at 37 °C was determined as in Fig. 1. B, in separate experiments, performed as in Fig. 1, cells containing internalized 125I-ASOR were put at 37 °C in Medium 1/BSA containing either no addition (▲), 25 mM GalNAc (●), or 5 μg/ml of nonradioactive ASOR (■).
FIG. 4. Effect of temperature on the rate of release of internalized \(^{125}\text{I}-\text{ASOR}\) in the presence of EGTA. A, cells containing internalized \(^{125}\text{I}-\text{ASOR}\), as in Fig. 1, were washed at 0 °C and then incubated in Medium 1/BSA plus EGTA at 4 °C (A), 8 °C (A), 12 °C (O), 18 °C (O), or 25 °C (O). Samples were taken at the indicated times to determine the amount of intact \(^{125}\text{I}-\text{ASOR}\) in the medium. B, the initial rate of release of internalized \(^{125}\text{I}-\text{ASOR}\) at various temperatures was calculated from A.

FIG. 5. Release of internalized \(^{125}\text{I}-\text{ASOR}\) and changes in the intracellular free and receptor-bound \(^{125}\text{I}-\text{ASOR}\) pools at 18 °C. Cells containing \(^{125}\text{I}-\text{ASOR}\) which had been internalized at 37 °C for 7 min were chilled, washed with EGTA, and put at 18 °C in Medium 1/BSA with EGTA as described in Fig. 1. At the indicated times, samples were taken to determine the amount of intact \(^{125}\text{I}-\text{ASOR}\) in the medium (B, O), and the amount of intracellular receptor-bound (A, O) and free (C, O) \(^{125}\text{I}-\text{ASOR}\). Identical results were also obtained if the initial endocytosis of surface-bound ligand was at 18 °C, rather than 37 °C.

correlated kinetics, by the appearance of intact \(^{125}\text{I}-\text{ASOR}\) (55 fmol/10^6 cells) in the medium (Fig. 5B). This confirms the conclusion that it is not the free \(^{125}\text{I}-\text{ASOR}\) pool, but rather the receptor-bound \(^{125}\text{I}-\text{ASOR}\) in the slowly dissociating pool, which can return to the cell surface.

The observation that internal \(^{125}\text{I}-\text{ASOR}\) in a slow dissociation compartment can return to the cell surface before it dissociates is unexpected. As we recently proposed, there are at least three major classes of models which can explain the kinetics of the asialoglycoprotein receptor system (6). Return of internalized receptor-ligand complexes to the cell surface could occur (i) due to an early reversible step in endocytosis prior to the point at which receptor and ligand normally dissociate or (ii) because some of the internalized receptor-ligand complexes escape the dissociation step but nonetheless go through the recycling pathway. Preliminary experiments suggest that metabolic energy poisons, which do not inhibit endocytosis per se but do block receptor recycling (19), do not inhibit the return of internalized \(^{125}\text{I}-\text{ASOR}\) to the cell surface. This finding makes explanation (ii) unlikely. The similar temperature dependences of the rate of ligand release (Fig. 4) and of the endocytosis of prebound ligand (17) is also consistent with (i). Furthermore, other investigators have provided evidence for a reversible internalization/externalization step in the pinocytosis of sucrose (20), or mannose-containing molecules by alveolar macrophages (21), of chemotactic peptide by polymorphonuclear leukocytes (22), and of galactosylated membrane glycoproteins by a macrophage cell line (23). In these studies, two kinetic compartments have been characterized which are very similar to what we (5, 6) and Harford et al. (7) have reported for the asialoglycoprotein receptor.

The occurrence of a reversible step in endocytosis, in which internalization/externalization of receptor-bound ligand occurs, may explain the findings reported by us (11, 24), Zeitlin and Hubbard (25), and Fiete et al. (26) that the surface asialoglycoprotein receptor content can be modulated by temperature, ionophores, or a variety of metabolic and microtubule drugs. These agents might perturb an internalization/externalization process, thereby altering its kinetics and the steady state distribution of receptor between the cell interior and surface. The cellular structures in which the kinetically defined fast and slow dissociation processes occur must be identified before the role of these processes in endocytosis and in the return of ligand to the cell surface can be elucidated. The morphological description by Hubbard and Wall of several intracellular membrane compartments (termed Endosomes) involved in the endocytosis of asialoglycoproteins by hepatocytes should be valuable in this effort (27).

3 B. L. Clarke, J. A. Oka, and P. H. Weigel, unpublished results.
Return of Internalized Ligand to the Cell Surface

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Recycling of the hepatic asialoglycoprotein receptor in isolated rat hepatocytes. Receptor-ligand complexes in an intracellular slowly dissociating pool return to the cell surface prior to dissociation.

P H Weigel and J A Oka

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