ATP-Dependent Inactivation and Reactivation of Constitutively Recycling Galactosyl Receptors in Isolated Rat Hepatocytes†

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ABSTRACT: Isolated rat hepatocytes depleted of ATP with NaN_3 without ligand lose galactosyl (Gal) receptors from the cell surface and accumulate inactive receptors within the cell [McAbee, D. D., & Weigel, P. H. (1987) *J. Biol. Chem. 262*, 1942–1945]. Here, we describe the kinetics of receptor redistribution and inactivation after ATP depletion with NaN_3 and of receptor redistribution and reactivation after ATP recovery. Only intact cells (>98% viable) isolated from Percoll gradients were assayed. Gal receptor activity and protein were measured by the binding of ^{125}I-asialoorosomucoid (^{125}I-ASOR) and ^{125}I-anti-Gal receptor IgG (^{125}I-IgG^R), respectively, at 4 °C. Surface and total (surface and intracellular) cellular Gal receptors were measured in the absence or presence, respectively, of digitonin. Following ATP depletion, 60–70% of Gal receptor activity and protein were lost from cell surfaces with first-order kinetics ($t_{1/2}$ = 6.5 min, k = 0.107 min^{-1}) at an initial rate of 11 000 ^{125}I-ASOR binding sites cell^{-1} min^{-1}. Lost cell-surface Gal receptors were transiently recovered still active inside the cell. After a short lag, total cellular receptor inactivation then proceeded with first-order kinetics ($t_{1/2}$ = 13 min, k = 0.053 min^{-1}) at an initial rate of 14 000 ^{125}I-ASOR binding sites cell^{-1} min^{-1}. Up to half of all cellular Gal receptors were inactivated by 40 min. ^{125}I-IgG^R binding to NaN_3-treated, permeable cells, however, was virtually constant. The distribution of total cellular receptors changed from 35% on the cell surface initially to 10% after 40 min of ATP depletion. Normal receptor location and activity were fully restored in the presence of cycloheximide when NaN_3 was removed by washing or dilution. Gal receptors were reactivated within the cell prior to their reexpression on the cell surface. We conclude that a subpopulation of Gal receptors constitutively recycles and is inactivated and then reactivated during transit through this pathway. Receptor activity is modulated within the cell, and both receptor reactivation and appearance at the cell surface require ATP.

Recycling during endocytosis is a common feature of several migrant receptors (Goldstein et al., 1985) including the galactosyl (Gal)[1] receptor in mammalian hepatocytes (Regoeczi et al., 1978; Tanabe et al., 1979; Steer & Ashwell, 1980; Warren & Doyle, 1981). In fact, the very large endocytic capacity of hepatocytes for desialylated glycoproteins is dependent on Gal receptor recycling (Pardridge et al., 1983). Treatment of isolated hepatocytes in the absence of ligand with various agents such as colchicine (Kolset et al., 1979), weak bases (Tolleshaug & Berg, 1979), monensin (Berg et al., 1983; Fiete et al., 1983), or metabolic energy poisons (Clarke & Weigel, 1985; Tolleshaug et al., 1985; Scarmato et al., 1986) depletes surface Gal receptor activity. Most investigators have concluded from such results that these receptors constitutively recycle and become trapped inside the cell. Loss of receptor activity from the cell surface, however, is only indirect evidence for recycling, since this result can be explained in other ways. For example, surface Gal receptor activity is lost following prolonged treatment of cultured rat hepatocytes with high concentrations of monensin without added ligand (Fiete et al., 1983). In this case, Gal receptors accumulated in an inactive form on the cell surface rather than intracellularly.

Recently, we reported direct evidence demonstrating constitutive recycling of Gal receptors. Following ATP depletion of isolated rat hepatocytes in the absence of ligand, a subpopulation of Gal receptors was lost from the cell surface and accumulated intracellularly (McAbee & Weigel, 1987). Similar results were obtained for human HepG2 cells treated with lysosomotropic amines without ligand (Schwartz et al., 1984; Zijderhand-Bleekemolen et al., 1987). A surprising observation in our study, however, was that up to half of all cellular Gal receptors were reversibly inactivated and trapped inside the cell following ATP depletion. These data suggested that an inactivation/reactivation cycle operates during receptor recycling and regulates receptor behavior in an ATP-dependent manner during receptor transit through the constitutive recycling pathway. To understand more fully the reversible inactivation of Gal receptors, we have now characterized the kinetics and site of inactivation and reactivation of Gal receptors in isolated rat hepatocytes depleted of ATP.

Experimental Procedures

Materials. Human orosomucoid (α_1-acid glycoprotein), a gift of Dr. M. Wickerhauser of The Plasma Derivatives Laboratory of the American Red Cross (Bethesda, MD), was desialylated with neuraminidase and iodinated as described previously (Weigel & Oka, 1982). Collagenase (type I), BSA (fraction V), CNBr-activated Sepharose 4B, cycloheximide, iodoacetamide, neuraminidase (type X), calf thymus DNA, ATP, Percoll, digitonin, SDS, Nonidet P-40, sodium deoxy-

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[1] Abbreviations: ASOR, asialoorosomucoid; BSA, bovine serum albumin; Gal, galactosyl; IgG^R, affinity-purified goat anti-receptor immunoglobulin G; K_d, dissociation constant; k, rate constant; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; Tris-HCl, tris(hydroxymethyl)aminomethane hydrochloride; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid; kDa, kilodalton(s); EDTA, ethylenediaminetetraacetic acid; PBS, phosphate-buffered saline.

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cholate, hydrogen peroxide (30%), and rabbit anti-goat IgG–peroxidase conjugate were from Sigma (St. Louis, MO). Collagenase (type IV) was also obtained from Serva (Westbury, NY). *N*-(2-Hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid and Triton X-100 were from Research Organics, Inc. (Cleveland, OH). Bisbenzimide (Hoechst dye 33258) was from Behring Diagnostics (La Jolla, CA). *N*,*N*′-Methylenebis(acrylamide), ammonium persulfate, SDS–PAGE molecular weight standards, and 4-chloro-1-naphthol were from Bio-Rad Laboratories (Richmond, CA). Acrylamide, twice recrystallized, was from United States Biochemical Corp. (Cleveland, OH). Dithiothreitol was from Boehringer Mannheim Biochemicals (Indianapolis, IN). Nitrocellulose paper (0.4 μm) was from Schleicher & Schuell (Keene, NH). 1,3,4,6-Tetrachloro-3a,6a-diphenylglycoluril and BCA protein assay reagent were obtained from Pierce Chemical Co. (Rockford, IL). $Na^{125}I$ (10–20 mCi/μg of iodine) was from Amersham Corp. (Arlington Heights, IL). All other chemicals were reagent grade. Medium 1 contains modified Eagle's medium (Grand Island Biological Co., Grand Island, NY; catalog no. 420–1400) supplemented with 2.4 g/L *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid, pH 7.4, and 0.22 g/L $NaHCO_3$. Medium 1/BSA is medium 1 containing 0.1% (w/v) BSA. Hanks' balanced salt solution was prepared according to the Grand Island Biological Co. catalog formulation.

Hepatocytes. Male Sprague-Dawley rats (150–200 g, Harlan Breeding Laboratories, Houston, TX) were maintained with standard laboratory chow and water, ad libitum. Hepatocytes were prepared by a modification of the collagenase perfusion procedure of Seglen (1973) as described previously (Weigel, 1980; Oka & Weigel, 1987). Cells were kept at room temperature during the filtration and differential centrifugation steps. Final cell pellets suspended in ice-cold medium 1 were >90% viable and single cells. Experiments were performed in medium 1/BSA in the absence of serum. Prior to experiments, cell suspensions (2×10^6 cells/mL in medium 1/BSA, 10% of the flask volume) were incubated at 37 °C for 60 min to increase and stabilize the number of surface receptors per cell (Weigel & Oka, 1983). Cell viability was determined by trypan blue exclusion.

Antibody Preparation. A male goat was inoculated subcutaneously at multiple sites with 100 μg of affinity-purified Gal receptor (Ray & Weigel, 1985). Inoculations, emulsified with either Freund's complete (initial inoculation) or incomplete (subsequent inoculations) adjuvant, and bleedings were alternated every 10 days. Whole IgG fraction was isolated from antiserum by two sequential precipitations with 14% (w/v) Na_2SO_4 at room temperature (Johnstone & Thorpe, 1982) and then dissolved in and dialyzed exhaustively against 0.9% NaCl, 10 mM Tris-HCl, pH 7.8, and 5 mM EGTA. Crude IgG (~40 mg/mL) was applied to a Gal receptor–Sepharose 4B column preequilibrated with 0.9% NaCl, 10 mM Tris-HCl, pH 7.8, and 5 mM EGTA. Bound IgG^R was eluted with 0.1 M glycine, pH 2.5, and 5 mM EGTA directly into 1 M Tris, pH 9.0. Affinity-purified IgG^R was dialyzed against 0.9% NaCl/50 mM Tris-HCl, pH 7.8, and stored at −70 °C. ^{125}I-IgG^R prepared by the method of Fraker and Speck (1978) had specific activities of 100–650 dpm/fmol.

SDS–PAGE/Electroblotting. Affinity-purified Gal receptor (Ray & Weigel, 1985) was subjected to SDS–PAGE (Laemmli, 1970). Samples in 63 mM Tris-HCl, pH 6.8, 2% SDS, 10% glycerol, and 0.01% Bromophenol Blue were reduced with 11 mM dithiothreitol, heated for 5 min in a boiling water bath, and then alkylated with 50 mM iodoacetamide. Protein samples (1–20 μg of protein) were separated on a 10% polyacrylamide gel containing 0.1% SDS using a MINI-SLAB gel apparatus (Idea Scientific, Corvalis, OR) and a Hoeffer PS 1200 direct-current power supply (San Francisco, CA) and then electrophoretically transferred to nitrocellulose paper at 10 V, 4 °C, overnight, in 150 mM NaCl, 20 mM Tris-HCl, pH 7.5, and 20% methanol, using a GENIE electrophoretic blot apparatus (Idea Scientific) and a Bio-Rad Model 160/1.6 power supply. Protein transferred onto nitrocellulose paper was detected by amido black staining (1 mg/mL in 7% acetic acid). Western blot analysis using IgG^R as the primary antibody was performed by the method of Burnette (1981) with modification. Following incubation with either preimmune serum (1:200 dilution) or IgG^R (5 μg/mL), the protein-laden nitrocellulose paper was incubated with rabbit anti-goat IgG–peroxidase conjugate (5 μg/mL) for 2 h at room temperature in 150 mM NaCl/10 mM Tris-HCl, pH 7.8, containing 5% (w/v) BSA and 0.2% (v/v) Nonidet P-40. The nitrocellulose paper was washed twice for 10 min with 150 mM NaCl/10 mM Tris-HCl, pH 7.8, containing 0.2% (v/v) Nonidet P-40, 0.25% (w/v) sodium deoxycholate, and 0.1% (w/v) SDS and then rinsed thoroughly with 150 mM NaCl/10 mM Tris-HCl, pH 7.8. 4-Chloro-1-naphthol (40 mg) was dissolved in 10 mL of anhydrous methanol at −20 °C and then mixed with 50 mL of 150 mM NaCl, 10 mM Tris-HCl, pH 7.8, containing 0.03% hydrogen peroxide at 50 °C. The nitrocellulose paper was immediately incubated in this mixture at room temperature until staining was attained, then washed thoroughly with water, and air-dried.

ATP Depletion. Hepatocytes were depleted of ATP prior to measurement of the binding of ^{125}I-ASOR and ^{125}I-IgG^R. Cells were treated at 37 °C with 10–15 mM NaN_3 using freshly prepared 500 mM stock solutions in phosphate-buffered saline. Samples were rapidly chilled by addition of 3–4 volumes of ice-cold Hanks' solution, overlaid on discontinuous Percoll gradients (Clarke & Weigel, 1985), and centrifuged at 450*g* for 10 min at 4 °C. For suspensions containing $>10^7$ cells, the gradients were composed of 0.5 mL of 50%, 10 mL of 40%, and 10 mL of 30% Percoll in phosphate-buffered saline in a 50-mL 28 × 112 mm polycarbonate round-bottom centrifuge tube. For suspensions containing $<10^7$ cells, the gradients were composed of 2 mL of 40% and 2 mL of 30% Percoll in phosphate-buffered saline in a 10-mL 13 × 100 mm glass tube. Only intact cells (>98% viable), isolated from the bottom of the 40% layer, were used in binding assays. A modification of the luciferin–luciferase procedure of Stanley and Williams (1969) was used to quantitate ATP (Weigel & Englund, 1975), employing a Beckman LS 7500 scintillation counter (Fullerton, CA) for photon detection.

Binding Assays. Viable control or ATP-depleted hepatocytes [$(0.5–1) \times 10^6$ cells/sample] isolated from Percoll gradients were incubated in Hanks' solution containing (unless specified otherwise) either 1.5 μg/mL ^{125}I-ASOR or 13 μg/mL ^{125}I-IgG^R at 4 °C for 60 min, with occasional mixing. Total (surface and intracellular) binding was measured in the presence of 0.055% digitonin (Weigel et al., 1983) added 10 min prior to the addition of ^{125}I-ASOR or ^{125}I-IgG^R. Surface binding only was measured in the absence of digitonin. Digitonin at 0.055% permeabilizes cells without solubilizing Gal receptors, releases cytosolic proteins of ≥200 kDa, and makes intracellular receptors accessible to added ligand (Weigel et al., 1983). The cells, kept on ice, were then washed twice by centrifugation with Hanks' solution, resuspended in 0.5 mL of 100 mM NaCl, 50 mM sodium phosphate, pH 7.4, and 5 mM EDTA, sonicated for 60–120 s at 80 W in a water bath

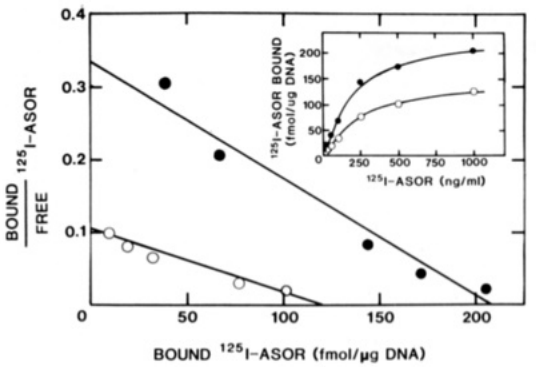

FIGURE 1: Saturation binding of ^{125}I-ASOR to permeable control and NaN_3-treated hepatocytes. Hepatocytes were treated with 15 mM NaN_3 (○) or PBS (●) for 30 min at 37 °C, after which the cells were chilled to 4 °C, washed, and permeabilized with digitonin as described under Experimental Procedures. Cells were incubated with various concentrations of ^{125}I-ASOR for 60 min at 4 °C in the absence or presence of excess unlabeled ASOR (100 μg/mL). The cells were centrifuged, and the amount of unbound ^{125}I-ASOR in the supernatants was determined. The cells were washed twice, and radioactivity and DNA were determined. Specific binding of ^{125}I-ASOR to both types of cells exceeded 95%. The inset shows the binding isotherm. Values for specifically bound and free ^{125}I-ASOR were plotted according to Scatchard (1949). Solid lines were generated by least-squares linear regression analysis ($r = -0.97$ for control, $r = -0.99$ for NaN_3-treated cells). Symbols represent the mean of duplicates.

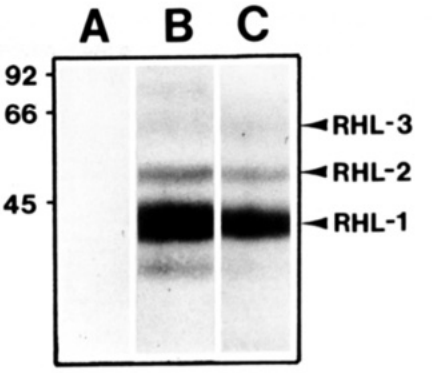

FIGURE 2: Immunoblot analysis of purified Gal receptor with IgG^R. SDS–PAGE of purified Gal receptor (lanes A–C) and immunoblotting with either preimmune serum (lane A) or IgG^R (lane B) were performed as described under Experimental Procedures. Receptor protein transferred to nitrocellulose paper was detected by amido black staining (lane C).

sonicator (Laboratory Supplies Co., Hicksville, NY), and assayed for DNA and radioactivity. Nonspecific binding of both ^{125}I-ASOR and ^{125}I-IgG^R, as determined in the presence of a 50-fold excess of unlabeled probe, was routinely below 15% of the total binding. Binding of ^{125}I-ASOR or ^{125}I-IgG^R to intact and permeable cells was linear under the conditions used. ^{125}I-ASOR or ^{125}I-IgG^R bound by intracellular Gal receptors was determined as the difference between binding to permeable and intact hepatocytes.

General Procedures. Cellular DNA was determined by the method of Labarca and Paigen (1980) using calf thymus DNA as standard. Protein was determined by the BCA protein assay procedure (Smith et al., 1985; Pierce Chemical Co.) using BSA as standard. Centrifugation of cell suspensions was at 800 rpm for 2 min at 4 °C in a refrigerated TJ-6 table-top centrifuge (Beckman Instruments). ^{125}I radioactivity was determined by using a Packard Multiprias 2 γ spectrometer (Downers Grove, IL).

RESULTS

In the following experiments, *only* intact cells (>98% viable) isolated from discontinuous Percoll gradients for each time point were used in binding assays after the experiment. This procedure is absolutely necessary when working with severely ATP-depleted hepatocytes in suspension (Clarke & Weigel, 1985). Isolated rat hepatocytes treated with NaN_3 at 37 °C without added ligand lose up to 50% of their total (surface and intracellular) Gal receptor activity when the cells are subsequently assayed at 4 °C for ^{125}I-ASOR binding (McAbee & Weigel, 1987). Decreased ^{125}I-ASOR binding could be due to either a loss in the number of binding sites or a decreased affinity between ^{125}I-ASOR and Gal receptors. To distinguish between these two possibilities, equilibrium ^{125}I-ASOR binding studies were performed at 4 °C on control and NaN_3-treated hepatocytes in the presence of digitonin (Figure 1). Treated hepatocytes bound ~40% less ^{125}I-ASOR than did untreated control cells. In eight experiments, this decrease ranged from 35% to 50%. At saturation, control cells bound 204 fmol of ^{125}I-ASOR/μg of DNA with a $K_d = 1.2 \times 10^{-9}$ M, and NaN_3-treated cells bound 123 fmol of ^{125}I-ASOR/μg of DNA with a $K_d = 2.5 \times 10^{-9}$ M. These dissociation constants were not significantly different ($p = 0.05$). Since NaN_3 itself does not affect ^{125}I-ASOR binding (Clarke & Weigel, 1985; Tolleshaug et al., 1985; Scarmato et al., 1986), we conclude that ATP depletion in live cells diminishes the number of ASOR binding sites without significantly changing the affinity of the remaining Gal receptors.

Characterization of IgG^R. ^{125}I-ASOR is an effective probe for cellular Gal receptor localization only when receptor activity does not change. In view of the previous result, however, it was apparent that to measure accurately changes in Gal receptor distribution on cells treated with NaN_3, it was necessary to develop a probe to measure cellular Gal receptors independent of their ligand binding activity. To achieve this, we raised, isolated, and affinity-purified a polyclonal goat IgG directed against the affinity-purified rat hepatic Gal receptor, which is composed of three polypeptide chains designated RHL-1, RHL-2, and RHL-3 (Drickamer et al., 1984; Figure 2, lane C). Western blot analysis of purified Gal receptors showed that IgG^R recognized all three Gal receptor subunits in proportion to the amount of receptor protein present on the nitrocellulose paper (Figure 2, lane B). No staining was observed when preimmune serum was substituted for IgG^R (Figure 2, lane A). In addition, IgG^R stained only Gal receptor subunits when Triton X-100 extracts of hepatocytes were analyzed by electroblot (data not shown). Thus, IgG^R was specific for the Gal receptor.

Equilibrium ^{125}I-IgG^R binding to cells at 4 °C was achieved after 60 min for both intact (Figure 3A) and permeable (Figure 3B) hepatocytes. Greater than 85% of the ^{125}I-IgG^R binding was abolished by a 100-fold excess of unlabeled IgG^R but not by nonimmune goat IgG. Moreover, permeable cells bound up to 5 times more ^{125}I-IgG^R than did intact cells, similar to the previously reported increase of ^{125}I-ASOR binding (Weigel et al., 1983). This result is consistent with the conclusion that 50–90% of all Gal receptors are located intracellularly (Pricer & Ashwell, 1971; Steer & Ashwell, 1980; Warren & Doyle, 1981; Weigel et al., 1983). Intact hepatocytes bound ^{125}I-IgG^R in a saturating manner and with moderately high affinity (Figure 4). At saturation, intact cells bound 4.9 pmol of ^{125}I-$IgG^R/10^6$ cells with a $K_d = 9.2 \times 10^{-8}$ M. At saturation, permeable cells bound 19 pmol of ^{125}I-$IgG^R/10^6$ cells with a $K_d = 7.7 \times 10^{-8}$ M (data not shown). Routinely, different preparations of ^{125}I-IgG^R exhibited K_d's between 6×10^{-8} and 9×10^{-8} M for both intact and permeable cells. Therefore, while causing up to a 5-fold increase in ^{125}I-IgG^R binding due to exposure of intracellular

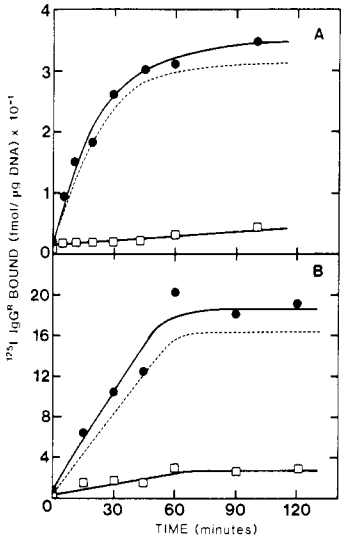

FIGURE 3: Kinetics of ^{125}I-IgG^R binding to intact and permeable hepatocytes. After equilibration at 37 °C, hepatocytes were chilled to 4 °C and isolated over discontinuous Percoll gradients, as described under Experimental Procedures. The cells treated (B) or untreated (A) with digitonin were then incubated with 2.5 μg/mL ^{125}I-IgG^R at 4 °C for the specified times in the absence (●) or presence (□) of 250 μg/mL unlabeled IgG^R. The cells were then washed, and radioactivity and DNA were determined. Dashed lines represent specifically bound ^{125}I-IgG^R. Symbols represent the mean of duplicates.

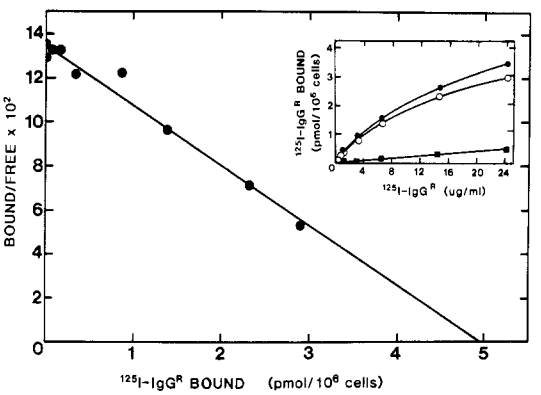

FIGURE 4: Saturation binding of ^{125}I-IgG^R to intact hepatocytes. Cells were incubated with various concentrations of ^{125}I-IgG^R at 4 °C for 60 min. The cells were washed, and cell-associated radioactivity and DNA were determined as described in Figure 1. Free ^{125}I-IgG^R and specifically bound ^{125}I-IgG^R were determined after equilibrium binding and analyzed according to Scatchard (1949) as in Figure 1. The solid line was generated by least-squares linear regression analysis (r = −0.99). Inset: specific ^{125}I-IgG^R binding (○) was calculated by subtracting ^{125}I-IgG^R bound to cells in the presence of a 100-fold excess of unlabeled IgG^R (■) from total bound ^{125}I-IgG^R (●). Symbols represent the mean of duplicates.

receptors, digitonin does not affect the binding affinity between ^{125}I-IgG^R and cell-associated Gal receptors. In addition, ^{125}I-IgG^R recognizes active and inactive Gal receptors equally well and with similar affinities (McAbee & Weigel, 1987), averaging about four IgG^R molecules bound per ASOR binding site. These results indicate that ^{125}I-IgG^R is a suitable probe for measuring Gal receptors in both intact and permeable hepatocytes.

Kinetics of Surface Gal Receptor Redistribution following ATP Depletion. We then examined the kinetic changes in cell surface binding of ^{125}I-ASOR and ^{125}I-IgG^R after ATP depletion of hepatocytes (Figure 5). When cells were treated with NaN_3, the kinetics of ATP depletion were biphasic with an initial rapid phase (50% depletion in 10 s) followed by a more prolonged slow phase. After 20 min, 15 mM NaN_3

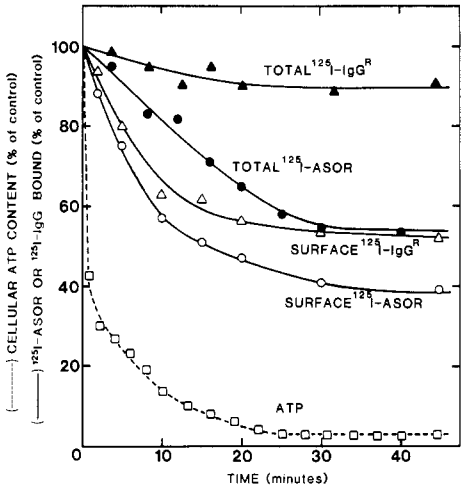

FIGURE 5: Effect of time of exposure to NaN_3 on the binding of ^{125}I-ASOR and ^{125}I-IgG^R to intact and permeable hepatocytes. After equilibration at 37 °C, cells were treated with 15 mM NaN_3 at 37 °C for various times, then rapidly chilled, isolated over discontinuous Percoll gradients, and assayed at 4 °C for binding of ^{125}I-ASOR (○, ●) or ^{125}I-IgG^R (△, ▲) in the absence (○, △) or presence (●, ▲) of digitonin, as described under Experimental Procedures. In parallel incubations, NaN_3-treated cells were assayed for ATP (□). Symbols represent the mean of duplicates, and binding of ^{125}I-ASOR and ^{125}I-IgG^R is presented as the percent bound relative to untreated cells.

maximally reduced cellular ATP by >95%. Depletion of cellular ATP by >25%, which blocks Gal receptor recycling (Clarke & Weigel, 1985), occurred prior to any measurable alteration in either ^{125}I-ASOR or ^{125}I-IgG^R binding. Thus, due to its rapidity, the rate of ATP depletion in NaN_3-treated hepatocytes was essentially independent of the rates of Gal receptor redistribution and inactivation. After ATP depletion, the number of surface binding sites for ^{125}I-ASOR and ^{125}I-IgG^R decreased rapidly (Figure 5). Semi-log plots of these data indicated that the number of binding sites for both probes decreased with virtually identical first-order kinetics (for ^{125}I-ASOR, $t_{1/2}$ = 6.4 min, k = 0.109 min^{-1}, and r = −0.996; for ^{125}I-IgG^R, $t_{1/2}$ = 6.5 min, k = 0.106 min^{-1}, and r = 0.977). Surface ^{125}I-ASOR binding activity was lost at an initial rate of 11 000 sites cell^{-1} min^{-1}. After 30 min of treatment, surface binding of ^{125}I-ASOR and ^{125}I-IgG^R was maximally decreased by, respectively, 65% and 55%. Severe ATP loss down to 1–2% of control with either high concentrations of NaN_3 (25 mM), a combination of 10 mM NaN_3 and 2 mM NaF, or prolonged NaN_3 treatment (15 mM, 60 min) caused no further loss of surface ^{125}I-ASOR and ^{125}I-IgG^R binding. Hence, only a subpopulation of surface Gal receptors redistributed following ATP depletion.

If different mechanisms were responsible for the loss of surface receptor activity and surface receptor protein, then ATP depletion at different temperatures might uncouple the two processes. As seen in Table I, however, the first-order rate constants for the loss of surface binding sites for ^{125}I-ASOR and ^{125}I-IgG^R after ATP depletion at 37, 31, and 27 °C were indistinguishable. It should be noted that between 4 and 37 °C, the rates of ATP depletion mediated by NaN_3 are very rapid and essentially temperature independent (Clarke & Weigel, 1985). Thus, the kinetic loss of surface receptor activity correlates with the kinetic loss of surface receptor protein and suggests that inactivation of Gal receptors does not take place on the plasma membrane.

Kinetics of Total Cellular Gal Receptor Inactivation following ATP Depletion. Gal receptors were rapidly inactivated after the onset of ATP depletion, as measured by ^{125}I-ASOR binding to permeable cells (Figure 5). By 30 min, total cellular

Table I: Loss of Cell-Surface Gal Receptor Protein and Activity following ATP Depletion at Different Temperatures[a]

	^{125}I-ASOR binding		^{125}I-IgG^{R} binding	
temp (°C)	k^b (min^{-1})	$t_{1/2}{}^c$ (min)	k (min^{-1})	$t_{1/2}$ (min)
37	0.108	6.4	0.105	6.6
31	0.073	9.5	0.083	8.4
27	0.061	11.3	0.060	11.6

[a] Hepatocytes were incubated at the specified temperatures for 90 min, and then NaN_3 was added (t = 0) to a final concentration of 15 mM NaN_3 at the same temperature. At various times for up to 60 min, samples were chilled, and intact cells were isolated and assayed for binding of ^{125}I-ASOR and ^{125}I-IgG^{R} as described under Experimental Procedures. [b] First-order rate constants were determined graphically by least-squares linear regression analysis using the equation $\ln [(X_t - X_{min})/(X_{max} - X_{min})] = -kt$ where t = time in minutes, X_{max} is the amount of probe bound at t = 0, X_{min} is the amount of probe bound after attainment of the new steady state, and X_t is the amount of probe bound at various times during Na_3N_3 treatment. The average correlation coefficient calculated for these plots was −0.988 ± 0.007 (n = 6). [c] Half-times were calculated from the equation $\ln 0.5 = -kt_{1/2}$, where k is the rate constant calculated above.

^{125}I-ASOR binding activity decreased by 45%. A semi-log plot of these data indicated that the loss of ^{125}I-ASOR binding sites was first order with a $t_{1/2}$ = 13 min (k = 0.053 min^{-1}, r = −0.964). Total ^{125}I-ASOR binding decreased at a rate of 14 200 sites cell^{-1} min^{-1}, similar to the rate of loss of surface binding sites. Total ^{125}I-IgG^{R} binding, however, remained essentially constant throughout the experiment. As was the case for surface receptors, a subpopulation of ^{125}I-ASOR binding sites in permeable cells remained active despite severe ATP depletion. Since the number of surface binding sites for ^{125}I-IgG^{R} decreased following NaN_3 treatment, the results indicate that ATP depletion redistributed Gal receptors from the cell surface to an intracellular compartment.

Since the removal of Gal receptor protein and activity from the cell surface are kinetically indistinguishable, it is possible that constitutively recycling Gal receptors are inactivated inside the cell after leaving the cell surface. If so, then intracellular Gal receptor activity may transiently increase shortly after the onset of ATP depletion. To test this possibility, hepatocytes were treated with NaN_3 at 37 °C and then assayed at 4 °C for ^{125}I-ASOR binding in the presence and absence of digitonin (Figure 6). Surface binding sites for ^{125}I-ASOR diminished immediately with no detectable lag and progressively decreased through the first 10 min of NaN_3 treatment at a linear rate of 14 500 sites cell^{-1} min^{-1} (r = −0.98; Figure 6A). The number of ^{125}I-ASOR binding sites in permeable cells, however, remained constant during the first 3 min of incubation and then decreased. Calculation of the amount of ^{125}I-ASOR bound to intracellular Gal receptors within the first 3 min following ATP depletion indicated that for every unit of Gal receptor activity (femtomoles of ^{125}I-ASOR bound per microgram of DNA) lost from the cell surface, there was a concomitant rise in intracellular Gal receptor activity. Thus, during this interval, ~50 000 ^{125}I-ASOR binding sites were shifted from the cell surface to an intracellular compartment prior to the onset of inactivation. The distribution and activity of Gal receptors achieved a new steady state after additional incubation (Figure 6B). After 40 min, 401 000 ^{125}I-ASOR binding sites/cell (47% of all cellular Gal receptors) were inactivated, a value equivalent to about twice the number of Gal receptors lost from the cell surface (206 000 ^{125}I-ASOR binding sites/cell). Although the loss in the number of surface ^{125}I-ASOR binding sites preceded the loss in the total number of ^{125}I-ASOR binding sites, the initial absolute rates of loss were similar in the linear portions of each curve (13 000 sites cell^{-1} min^{-1} for intact cells; 14 100 sites cell^{-1} min^{-1} for

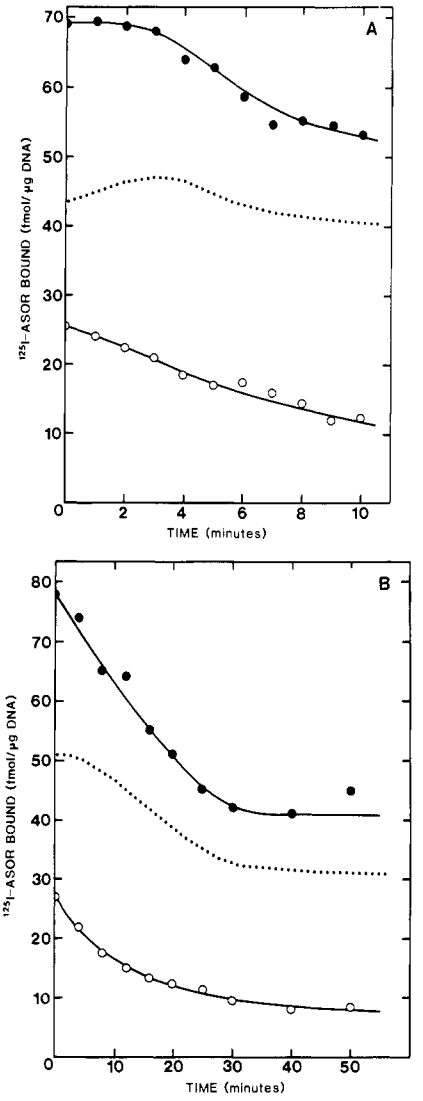

FIGURE 6: Kinetics of Gal receptor inactivation in the presence of NaN_3. Hepatocytes were treated with NaN_3 as described in Figure 5 and then assayed for binding of ^{125}I-ASOR in the absence (○) or presence (●) of digitonin as described under Experimental Procedures. Symbols represent the mean of duplicates. Intracellular ^{125}I-ASOR binding (dotted lines) was calculated as the difference between surface and total cellular ^{125}I-ASOR binding.

permeable cells). Thus, these kinetic data confirm that continuously recycling Gal receptors are inactivated inside the cell after internalization, not on the cell surface.

It is important to note that relocation and inactivation of Gal receptors are dependent on cellular ATP levels and are not due to an unrelated effect of NaN_3. Incubation of hepatocytes for 90 min in medium 1/BSA under N_2 atmosphere, to cause anoxia, depleted cellular ATP and reversibly reduced surface and total ^{125}I-ASOR binding activity by 70% and 45%, respectively (data not shown). These experiments, however, were more cumbersome to perform, and the kinetics of these changes were lower than seen with NaN_3, as expected for the low efficiency of gas exchange.

Recovery of ATP and Kinetics of Gal Receptor Distribution and Reactivation. The normal activity and distribution of Gal receptors were fully restored when hepatocytes were washed free of NaN_3 (Figure 7). The recovery of cellular ATP was biphasic (Figure 7, inset). During the wash at 4 °C (~5 min), cells regained 65% of their normal ATP content, and complete recovery of cellular ATP required up to 3 h of incubation at 37 °C. Surface ^{125}I-ASOR binding activity completely re-

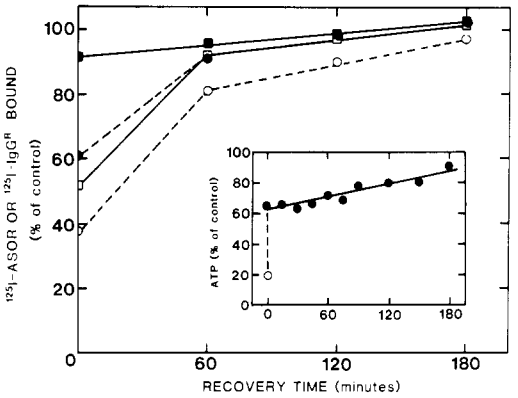

FIGURE 7: Recovery of cellular ATP and binding of ^{125}I-ASOR and ^{125}I-IgG^R to cells after removal of NaN_3. Hepatocytes were treated as described in Figure 1, then chilled to 4 °C, and washed free of NaN_3 by centrifugation (400*g*, 2 min). The cells were resuspended in medium 1/BSA containing 2×10^{-5} M cycloheximide (which blocks protein synthesis by >98%; Weigel & Oka, 1983) and incubated at 37 °C. At the designated times, samples were chilled to 4 °C, and viable cells were assayed for binding of ^{125}I-ASOR (○, ●) or ^{125}I-IgG^R (□, ■) in the absence (○, □) or presence (●, ■) of digitonin, as described under Experimental Procedures. In parallel incubations, the recovery of cellular ATP was determined (inset). Symbols represent the mean of duplicates, and binding of ^{125}I-ASOR and ^{125}I-IgG^R is presented as the percent bound relative to untreated cells.

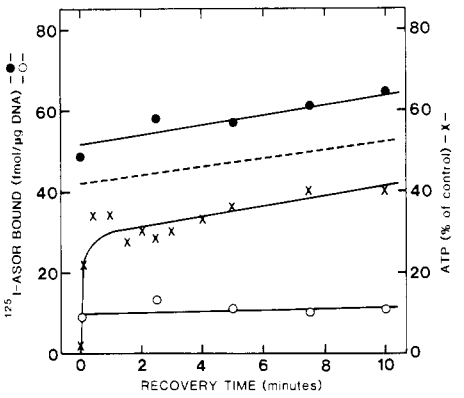

FIGURE 8: Kinetics of Gal receptor reactivation and ATP restoration. Hepatocytes were treated with 10 mM NaN_3 for 60 min at 37 °C and then at time zero were diluted 10-fold into 37 °C equilibrated medium 1/BSA. At the specified times, samples were chilled to 4 °C, and viable cells were isolated and assayed for ^{125}I-ASOR binding in the absence (○) or presence (●) of digitonin, as described under Experimental Procedures. In parallel incubations, cellular ATP content was determined (×) and is presented as the percent of ATP relative to untreated cells. Intracellular ^{125}I-ASOR binding (dashed line) was calculated as the difference between surface and total binding. Symbols represent the mean of duplicates. Lines were generated by least-squares linear regression analysis (r = 0.986 for total bound ^{125}I-ASOR; r = 0.997 for intracellular-bound ^{125}I-ASOR; r = 0.997 for surface-bound ^{125}I-ASOR).

covered after about 3 h, while restoration of total ^{125}I-ASOR and surface ^{125}I-IgG^R binding was complete between 1 and 2 h. In addition, recovery of normal Gal receptor distribution and activity was independent of protein synthesis, since they occurred in the presence of cycloheximide. Qualitatively similar results were also obtained when hepatocytes recovered from N_2-induced anoxia. Hepatocytes regained their normal receptor activity and distribution, albeit at a slower rate, when incubation flasks were reoxygenated (data not shown).

Although total cellular receptor activity appeared to recover before surface receptor activity, some inactive receptors were nonetheless found at the cell surface (Figure 7). To determine whether receptors were reactivated inside the cell or on the cell surface, we examined the initial kinetic changes in active receptor content in these two compartments. Hepatocytes treated with 10 mM NaN_3 were allowed to recover at 37 °C by diluting the cell suspension 10-fold into fresh, 37 °C medium 1/BSA (Figure 8). NaN_3 below 2 mM affects neither the activity nor the distribution of hepatic Gal receptors (McAbee & Weigel, 1987). The dilution technique avoids the complication of the 4 °C temperature perturbation during the centrifugal washing procedure. Under these conditions, cellular ATP recovered with biphasic kinetics to approximately 40% of control after 10 min. Surface ^{125}I-ASOR binding activity increased at an initial linear rate of 1500 sites cell^{-1} min^{-1}, while intracellular ^{125}I-ASOR binding activity increased at a linear rate of 11 300 sites cell^{-1} min^{-1}. We conclude, therefore, that reactivation of Gal receptors occurs inside the cell prior to receptor reexpression on the cell surface.

Discussion

Despite extensive investigation of the Gal receptor system, little is known about the behavior of Gal receptors during their transit through the recycling pathway. It is known that the passage of some Gal receptors through a recycling pathway in hepatocytes does not require ligand but is constitutive. This conclusion is based on direct evidence in human hepatoma cells (Schwartz et al., 1984; Zijerhand-Bleekemolen et al., 1987) and isolated rat hepatocytes (McAbee & Weigel, 1987) showing that, respectively, lysosomotropic amines and NaN_3 diminish surface Gal receptor activity and protein in the absence of ligand. In these studies, receptor protein was recovered inside the cells. We also found that Gal receptors were reversibly inactivated and trapped within the cell when constitutive recycling was disrupted by ATP depletion (McAbee & Weigel, 1987). This suggests that under normal circumstances, even without added ligand, Gal receptors are inactivated and then reactivated in a cyclic manner during migration through the recycling pathway. This could represent an important mechanism regulating the function of these receptors.

As we have reported here, cellular ATP depletion at 37 °C caused a redistribution of 60–70% of the surface Gal receptors to an intracellular compartment(s) with first-order kinetics (k = 0.107 min^{-1}, $t_{1/2}$ = 6.5 min). Binding sites for ^{125}I-ASOR and ^{125}I-IgG^R on the surfaces of NaN_3-treated cells decreased with virtually indistinguishable kinetics, indicating that the loss of surface Gal receptor activity and the loss of protein were coincident. Lost surface receptors were intracellular since these ^{125}I-IgG^R binding sites were recovered in permeable hepatocytes. Shortly after ATP depletion, lost surface receptor activity was transiently recovered inside the cell, as assessed in permeable cells by ^{125}I-ASOR binding. After a 3-min lag, receptor inactivation then proceeded with first-order kinetics (k = 0.053 min^{-1}, $t_{1/2}$ = 13 min), and 45–50% of all cellular Gal receptors were eventually inactivated. Inactivation occurred intracellularly and reflected a bona fide loss in the number of cellular ligand binding sites rather than a decrease in receptor affinity. Following cellular ATP recovery, Gal receptors were reactivated within the cell and then delivered to the plasma membrane. Relocation and inactivation of Gal receptors in hepatocytes are due to alterations in cellular ATP levels rather than to effects of NaN_3, per se. Incubation of hepatocytes under an N_2 atmosphere results in ATP depletion similar to that obtained by metabolic energy poisons, although at a lower rate. The same effects on reversible receptor inactivation and redistribution were observed under these conditions.

On the basis of the present results, we propose the following model to describe the constitutively recycling Gal receptor

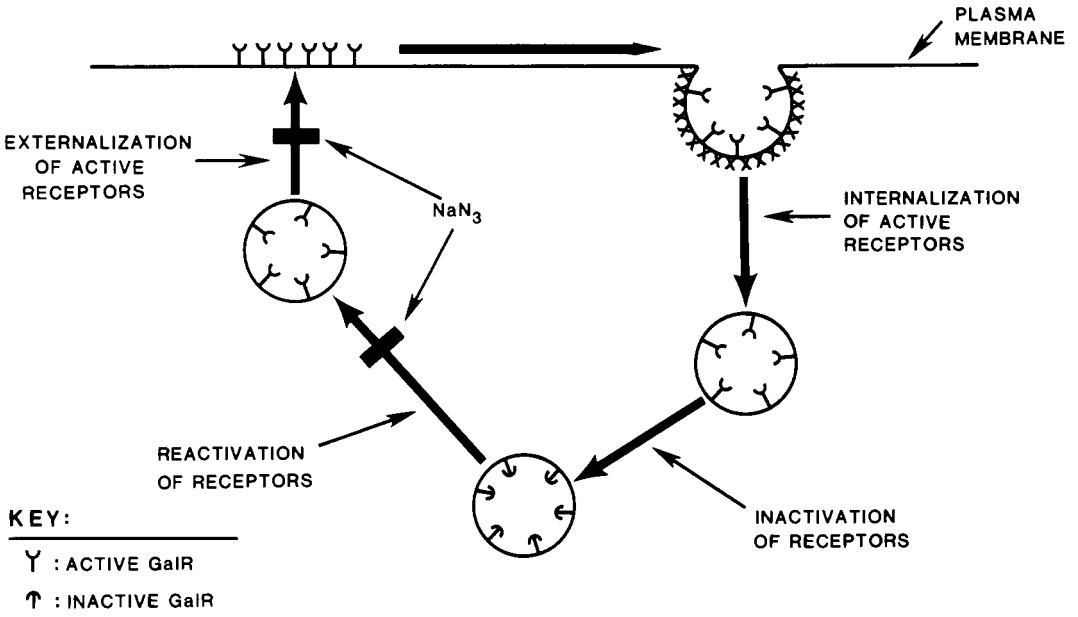

FIGURE 9: Model for the inactivation/reactivation of constitutively recycling Gal receptors. Description of the model is provided in the text.

pathway (Figure 9). Under normal circumstances, a subpopulation of Gal receptors recycles even in the absence of ligand, presumably following a coated pit pathway (Wall & Hubbard, 1981; Matsuura et al., 1982; Deschuyteneer et al., 1984). After internalization, receptors are inactivated in an intracellular compartment. Then, by ATP-dependent processes, inactive receptors are reactivated and delivered back to the plasma membrane, ready to undergo another cycle. Depletion of cellular ATP has at least two effects. First, it blocks all internalized Gal receptors from returning to the cell surface. Second, it inhibits receptor reactivation. As a result, inactive receptors accumulate intracellularly. After restoration of ATP, receptors are reactivated within the cell and regain their normal distribution.

Despite severe ATP depletion, only a portion of surface Gal receptors relocated to the cell interior, and not all cellular Gal receptors were inactivated. We have previously reported that there exist two functionally distinct subpopulations of Gal receptors on isolated rat hepatocytes, which we designate state 1 and state 2 Gal receptors (Weigel et al., 1986; Weigel, 1987). State 1 and state 2 Gal receptors are functionally distinct since they internalize, dissociate (Oka & Weigel, 1983, 1987; Weigel et al., 1986), and degrade (Clarke & Weigel, 1987) desialylated glycoproteins in isolated hepatocytes by two distinct parallel pathways (Weigel, 1987). Multiple receptor pools and recycling pathways have also been described for the transferrin receptor (Stein & Sussman, 1986; Hopkins, 1983) and the mannose receptor systems (Tietze et al., 1982). State 2, but not state 1, surface Gal receptor activity is lost when hepatocytes are treated with metabolic poisons, chloroquine, colchicine, monensin, hyperosmotic medium, or incubated at temperatures below 37 °C (Weigel, 1987; Oka & Weigel, 1987b; Weigel & Oka, 1983).[2] Moreover, each of these perturbants reduces surface state 2 receptor activity to a similar extent, and their effects are not additive.[2] By these criteria, we conclude that state 2 Gal receptors are the receptors which constitutively recycle and are reversibly inactivated. Thus far, we have not detected inactivation of state 1 Gal receptors in experiments performed in the absence of added ligand. It is possible that state 1 Gal receptors are capable of being inactivated but are not delivered to the appropriate intracellular compartment without ligand.

The number of cellular Gal receptors inactivated after ATP depletion was about twice the net number of receptors shifted from the cell surface to the cell interior. Apparently, under steady-state conditions, about 50% of the state 2 Gal receptors are present inside the cell, migrating along the intracellular loop of the constitutive recycling pathway (Figure 9). It is possible that at the onset of ATP depletion, the majority of these intracellular receptors have already passed the ATP-sensitive block(s) in the cycle en route back to the cell surface, are reexpressed at the plasma membrane, and then reenter the cell where they are subsequently inactivated and trapped. Alternatively, since maximum inactivation was achieved only after 30 min of incubation at 37 °C, some state 2 Gal receptors may make multiple passes through the recycling pathway before being inactivated and trapped.

The recovery of normal receptor distribution and activity following ATP depletion was rather complex. Under conditions where cellular ATP recovered within 10 min to 40% of normal after dilution of NaN_3-treated cells, substantial intracellular receptor reactivation occurred without appreciable recovery of either surface receptor activity (Figure 8) or protein (data not shown), indicating that state 2 Gal receptors are reactivated within the cell, not on the plasma membrane. Receptor reactivation and reexpression at the cell surface are likely governed by different ATP-dependent mechanisms. It appeared, however, that not all inactive state 2 Gal receptors trapped within the cell during ATP depletion were reactivated in a single wave following removal of inhibitor. It is likely that some inactive state 2 Gal receptors completed multiple rounds of the recycling circuit before being reactivated, since normal levels of surface receptor antigen were detected after about 1 h of incubation while normal surface receptor activity was not attained until after 3 h (Figure 7). During later stages of recovery, a small fraction of surface Gal receptors was inactive, and this fraction progressively decreased as recovery became more complete.

The mechanism of Gal receptor inactivation and reactivation is unknown. One possibility is that receptor activity is regulated by a reversible covalent modification such as phosphorylation/dephosphorylation. For example, epidermal growth factor receptors lose their ligand binding capacity following

[2] J. A. Oka, D. D. McAbee, B. L. Clarke, and P. H. Weigel, unpublished experiments.

phosphorylation by protein kinase C (Fearn & King, 1985; Davis & Czech, 1986). Since cellular ATP loss blocks receptor reactivation, presumably active Gal receptors would be phosphorylated, and receptor inactivation would require dephosphorylation. Both the mammalian Gal receptor (Schwartz, 1984; Takahashi et al., 1985) and the avian *N*-acetylglucosamine receptor (Drickamer & Mamon, 1982) are phosphoproteins, although the function of this phosphorylation in either case remains to be discovered. Schwartz (1984) concluded that the extent of Gal receptor phosphorylation is not markedly altered in HepG2 cells endocytosing asialoglycoproteins. Clathrin-coated vesicles contain both kinases (Bar-Zvi & Branton, 1986; Pauloin et al., 1982; Kishimoto et al., 1987) and phosphatases (Pauloin & Jolles, 1986) which could modify receptors present in endosomes. Inactivation might be due to a change in the oligomeric structure or valency of the Gal receptor. Receptor clustering has been shown to be an important determinant of Gal receptor–carbohydrate ligand binding affinity (Hardy et al., 1985).

The role of inactivation in the normal Gal receptor cycle is unclear. Since the cell performs work during constitutive recycling, the involvement of ATP in one or more steps in the recycling pathway is required so that the first and second laws of thermodynamics remain inviolate. The cell may have coupled this energy requirement to the regulation of receptor activity in order to exert more control over the system. At present, we do not know if the receptor inactivation/reactivation cycle functions during the endocytosis of ligand. If it does operate during endocytosis, then it is likely that receptor inactivation occurs after, but not simultaneous with, receptor–ligand dissociation, since newly dissociated complexes can reassociate (Harford et al., 1983). The decreased ability of receptor to rebind dissociated ligand observed by Harford et al. (1983), however, may reflect receptor inactivation in addition to or rather than segregation of receptor and ligand molecules into separate compartments. Receptor inactivation may ensure efficient dissociation and segregation of receptor and ligand into their respective intracellular routes.

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Antibodies to the N-Terminus of Calpactin II (p35) Affect Ca^{2+} Binding and Phosphorylation by the Epidermal Growth Factor Receptor in Vitro†

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ABSTRACT: Calpactins I and II are related 39-kilodalton (kDa) proteins that interact with phospholipids and actin in a calcium-dependent manner and are substrates of tyrosine protein kinases. They contain a short amino-terminal tail attached to a 36-kDa core domain. Monoclonal antibodies (Mabs) were raised to bovine calpactin II and used as site-specific probes of its structure and function. All of the antibodies reacted with native calpactin II and gave rise to a single band of 39 kDa among total cell protein displayed on Western blots. Most of the antibodies (9/14) reacted with determinants on the tail as shown by Western blots and competition with a synthetic tail peptide. Four antibodies reacted with determinants on the core and a 10-kDa tryptic fragment. Antibody–calpactin II complexes were tested for their ability to interact with lipid, actin, and Ca^{2+} and to serve as substrates of the epidermal growth factor (EGF) receptor tyrosine protein kinase. Whereas none of the antibodies had a detectable effect on actin binding, two anticore antibodies reduced calpactin's affinity for phospholipid. Ca^{2+}-binding sites are known to reside within the core region, yet most antitail antibodies markedly increased the affinity of calpactin II for Ca^{2+}, with four Ca^{2+}-binding sites observed. Antitail antibodies either (i) abolished or (ii) greatly stimulated (10-fold) the phosphorylation of calpactin II by the EGF receptor. These results suggest that the interactions between calpactin II and Ca^{2+}, phospholipid, or the EGF receptor are more complex than previously thought and can be modulated by interactions occurring in the tail.

Calpactins are Ca^{2+}-binding proteins which interact with phospholipid and actin in vitro and have been suggested to be components of the cytoskeletal matrix under the plasma membrane in cells (Gerke & Weber, 1984; Greenberg & Edelman, 1983; Radke et al., 1983; Nigg et al., 1983; Glenney, 1986b; Glenney et al., 1987). Considerable interest has focused on these proteins because they are substrates of the oncogene and growth factor receptor tyrosine kinases (Erikson & Erikson, 1980; Sawyer & Cohen, 1985; Fava & Cohen, 1984; Pepinsky & Sinclair, 1986; Radke & Martin, 1979). Although originally thought to be a single 39-kilodalton (kDa) protein, more recent evidence has demonstrated the existence of at least three forms of calpactin (Glenney, 1986b; Glenney et al., 1987). Calpactin I is found in cells as either a 38-kDa monomer or a tetramer with an associated 11-kDa light chain (Gerke & Weber, 1984; Erikson et al., 1984; Zokas & Glenney, 1987). Calpactin II, on the other hand, is found only as a monomer (Glenney, 1986b; Fava & Cohen, 1984).

The cDNAs encoding calpactins I and II have been molecularly cloned and sequenced (Saris et al., 1986; Kristensen et al., 1986; Wallner et al., 1986; Huang et al., 1986), which allows correlation between structure and function. Calpactins had been suggested to be lipocortins (Pepinsky & Sinclair, 1986; Huang et al., 1986), proteins thought to be potent and specific inhibitors of phospholipase A_2. Subsequent investigations, however, revealed that this apparent A_2 inhibition by calpactin was not due to direct interaction between calpactin and phospholipase but was due to the shielding of the phospholipid substrate (Davidson et al., 1987; Haigler et al., 1987), in accordance with the known lipid-binding properties of calpactin (Glenney, 1985, 1986a,b; Davidson et al., 1987; Geisow et al., 1986; Johnsson et al., 1986; Haigler et al., 1987; Schlaepfer & Haigler, 1987). Thus, recent studies on frag-

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