Rat Hepatocytes Bind to Synthetic Galactoside Surfaces Via a Patch of Asialoglycoprotein Receptors

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ABSTRACT The binding of rat hepatocytes to flat polyacrylamide surfaces containing galactose is sugar-specific, requires Ca^{+2}, and occurs only above a critical concentration of sugar in the substratum (Weigel et al., 1979, *J. Biol. Chem.*, 254, 10,830). Binding is completely inhibited by asialo-orosomucoid but not by orosomucoid or asialo-agalacto-orosomucoid, suggesting that cell binding is mediated by asialoglycoprotein receptors. Asialo-orosomucoid was labeled with fluorescein isothiocyanate and used as a direct fluorescent probe to monitor the distribution of cell surface asialoglycoprotein receptors before and after hepatocyte binding to galactoside or control substrata. Cells bound at 37°C were de-adhered at 4°C using the Ca^{+2} chelator EGTA. The released cells were then stained with fluorescein-asialo-orosomucoid, fixed, washed, and examined by fluorescence microscopy.

On freshly isolated cells before binding, the distribution of asialoglycoprotein receptors appears diffuse and nonclustered. However, more than half of the cells released intact from a galactoside surface had a single large (4 μm^2) fluorescent patch. The receptor patch cannot be detected on cells while they are bound to a galactoside surface but rather only on released cells, indicating that the cell-substratum junction is the site of the receptor patch. No asialoglycoprotein receptor patches ($\leq$1%) were observed on cells that were incubated on, but did not bind to, an underivatized polyacrylamide surface or to a surface with a galactose concentration below the critical concentration for binding. Furthermore, no receptor patches were present on cells that had bound to and were subsequently released from substrata that did not contain galactose, including glass, tissue culture plastic, nontissue culture plastic, and collagen. The distribution of asialoglycoprotein receptors is preserved at 4°C because at 37°C the patches disappear with a half-life of ~2.6 min. The results directly demonstrate that a large cluster of asialoglycoprotein receptors mediates the binding of rat hepatocytes to a galactoside surface.

The number of studies of the mechanisms by which cells attach to and spread on a substratum has been increasing steadily in recent years. Much progress has been made, particularly with respect to the role of cell and plasma fibronectins in mediating cell substratum adhesion (see reference 3 for review). However, knowledge of the chemical composition and structure of the adhesive surface being studied is often lacking or inferred and, therefore, may hamper interpretation of the results. We have been exploring the use of flat synthetic polyacrylamide surfaces containing covalently attached ligands as a model substratum to study cell adhesion. These surfaces can be synthesized in several ways (14) and can be controlled and manipulated experimentally with respect to their charge, ligand concentration, size, shape, and porosity. We are particularly interested in how cells respond to synthetic culture surfaces that contain well-defined carbohydrate ligands.

The asialoglycoprotein receptor was initially discovered and characterized in mammalian liver by Ashwell and Morell (1), and is believed to mediate the in vivo binding and endocytosis of desialylated serum glycoproteins, which are subsequently degraded. The receptor specifically binds glycoproteins containing terminal galactosyl groups on their oligosaccharide chains and requires Ca^{+2} for activity. The asialoglycoprotein receptor on the surface of isolated rat hepatocytes has been examined in several laboratories (16, 20). Rat hepatocytes, freshly isolated by a collagenase perfusion technique, will specifically bind to a polyacrylamide surface only if it contains galactose (23). In this system, binding is a dramatic threshold response (24) which occurs only at or above a critical concentration of galactose in the polyacrylamide matrix. Because this

threshold binding response has important physiological implications for both normal and abnormal cellular behavior, it is important to define the molecular events involved in the cell-substratum interaction. The asialoglycoprotein receptor present on the hepatocyte surface is a likely candidate to mediate this interaction because only a galactoside surface is recognized and Ca^{+2} is required for adhesion (24). We show here that hepatocyte binding to a galactoside surface is mediated by a large patch[1] of cell surface asialoglycoprotein receptors. A preliminary report of these results has already appeared (21).

MATERIALS AND METHODS

Materials

Human orosomucoid (α_1 acid glycoprotein) was a generous gift from Dr. M. Wickerhauser of the American National Red Cross. Asialo-orosomucoid and asialo-agalacto-orosomucoid were prepared as described by Schachter et al. (13). Triton-X-100, collagenase (Type I or Type IV), bovine serum albumin (BSA) (fraction V), and Hepes were obtained from Sigma Chemical Co., St. Louis, Mo. Sprague-Dawley rats (125–150 gm) were obtained from Timco Breeding Laboratories, Houston, Tex. Scintillation fluid (3a70B) and high specific activity NaB^3H_4 (10–20 Ci/mmol) obtained in 25-mCi ampules were purchased from Research Products International Corp., Elk Grove Village, Ill. Medium 1 contained a modified Eagle's medium (Grand Island Biological Co. [GIBCO], Grand Island, N. Y.) supplemented with 2.4 gm/liter Hepes, pH 7.4, and 0.22 gm/liter $NaHCO_3$. Formaldehyde was purchased from Polysciences Inc., Warrington, Pa., and fluorescein isothiocyanate (FITC) from GIBCO. All other chemicals were reagent grade.

Preparation of Hepatocytes

Suspensions of rat hepatocytes were prepared by a modification of the collagenase perfusion procedure of Seglen (15) as described previously (24). Freshly isolated cell suspensions were kept on ice in Medium 1 and were usually 60–85% single cells and 80–90% viable, as assessed by trypan blue exclusion. Unless indicated otherwise, experiments were performed in the absence of serum.

Preparation of [^{3}H]Asialo-Orosomucoid

[^{3}H]Asialo-orosomucoid was prepared as described (20) by the reductive methylation procedure of Means and Feeney (8) using NaB^3H_4 with the highest available specific activity.

Preparation of Fluorescein-Asialo-Orosomucoid

FITC was dissolved in absolute ethanol, evaporated to dryness in a small round bottom flask, and dissolved in ice-cold 0.1 M sodium carbonate, pH 9.5, to give a final concentration of 5 mg/ml. 50 μl of the FITC solution was then added to 0.18 ml of a solution of asialo-orosomucoid (2.4 mg/ml) in phosphate-buffered saline (PBS) at 8°C. After 1 h, another 50 μl of the FITC solution was added and the incubation was continued for another hour at 8°C. The fluorescent protein was purified by chromatography over Sephadex G-25 superfine (Pharmacia Fine Chemicals, Div. of Pharmacia Inc., Piscataway, N. J.) and dialyzed extensively against PBS at 4°C. The preparations used in these experiments contained 1.3–1.5 mol of fluorescein/mol protein, as determined by the procedure of Wells et al. (25) and were stored at 0°C. At higher ratios of fluorescein to protein, activity of the protein decreased. Activity decreased after 2 wk, and attempts to stabilize or to store the derivative frozen at various temperatures longer than this time were not successful.

Hepatocyte Binding of [^{3}H]Asialo-Orosomucoid

Binding of [^{3}H]asialo-orosomucoid to suspensions of freshly isolated cells was determined at 4°C as recently described (20). Samples were diluted tenfold with cold Medium, nonbound radioactivity was removed by centrifugation, and radioactivity in the lysed cell pellet was determined on 10% aqueous samples with a Packard Tri-Carb model 2002 liquid scintillation spectrometer (Packard Instrument Co., Inc., Downers Grove, Ill.)

[1] The term patch, rather than cap, has been used here to describe the single large aggregate of receptors on a cell to avoid any implications related to its mechanism of formation.

Galactoside Ligands and Synthetic Culture Surfaces

The synthesis and characterization of 6-aminohexyl β-D-galactopyranoside (22) and its conversion (14) to an acryloyl derivative (i.e., an acrylamide monomer) have been described. *N*-Acrylamidomethyl 2-aminocarbonylethyl 1-deoxy-1-thiogalactopyranoside (24) was synthesized by the method of Lee et al. (6). These carbohydrate ligands were covalently incorporated into thin (0.25 mm) flat polyacrylamide gels, as described previously (24). Earlier work showed that rat hepatocytes interact in qualitatively similar ways with surfaces containing either of these galactoside ligands (24).

Hepatocyte Adhesion to Galactoside Surfaces

Hepatocyte adhesion to the synthetic galactoside surfaces was quantitated as already described (23). Briefly, 50–60 μl of ice-cold cell suspension (2×10^6 cells/ml) was placed on a gel piece ($8 \times 8 \times 0.25$ mm) lying flat in a 60-mm polystyrene petri dish. The dish, containing up to eight gel pieces in a circular array, was put in a water bath at 37°C. At the desired time, 10 ml of Medium 1 (at 37°C) was slowly pipetted into the center of the dish, which was then transferred to a gyratory platform shaker and swirled at 88 rpm for 24 s. The Medium and nonbound cells (which collect in the center of the dish) were aspirated off and each gel piece was put into 1 ml of 0.1 M potassium phosphate, pH 7.0, containing 0.5% Triton-X-100. The number of bound cells was determined by measuring the lactate dehydrogenase activity of the lysate (2, 14). In some experiments, after washing a dish, the medium and nonbound cells were removed with a pasteur pipette and the cells were collected by centrifugation and used in the remainder of the experiment.

Fluorescence Microscopy

Slides with cell samples suspended in PBS were air dried and mounted with no. 1 coverslips using a medium containing 9 mM Hepes, pH 7.4, 135 mM NaCl, 1.8 mM $CaCl_2$, and 10% glycerol. Fluorescence microscopy was performed with a Leitz Orthoplan microscope equipped with a ploemopak 2 epi-illuminator and an I_2 fluorescein filter cube. Photographs were taken with a ×63 objective (numerical aperature, 1.3) using Kodak Tri-X pan black and white 35-mm film which was pushed, in development with Ethol Blue, to ASA 1,600.

To compare samples within each experiment, the exposure times and developing and printing conditions were the same. Phase and fluorescence pictures were printed on Kodabromide no. 3 and 5 paper, respectively.

General

All centrifugations of cell suspensions were performed for 2 min at 800 rpm with a Sorvall GLC-1 table top centrifuge (DuPont Instruments-Sorvall, DuPont Co., Newton, Conn.). Protein was determined by the method of Lowry et al. (7).

RESULTS

Asialoglycoprotein Receptors Mediate Cell Adhesion to Galactoside Surfaces

We have previously shown that freshly isolated rat hepatocytes bind in a sugar-specific (23) and Ca^{+2}-dependent manner (24) to polyacrylamide surfaces containing galactose. These requirements for successful cell binding are the same as those for the binding of desialylated proteins by the asialoglycoprotein receptor in mammalian liver (11). Cell binding to these surfaces is specifically inhibited by asialo-orosomucoid (Fig. 1), for which this receptor has the highest known affinity (17). Complete inhibition of binding occurred at ~6 μg/ml (150 nM). Binding is not affected by orosomucoid or by asialo-agalacto-orosomucoid, which is also consistent with the specificity of the asialoglycoprotein receptor in plasma membranes (17) and on freshly isolated hepatocytes (20). Cells previously bound to a surface could be released at 37°C when incubated with asialo-orosomucoid (6 μg/ml, 30 min) but not with asialo-agalacto-orosomucoid or orosomucoid (not shown). Initial experiments suggest that the effective concentration of asialo-orosomucoid needed to inhibit cell binding increases as the

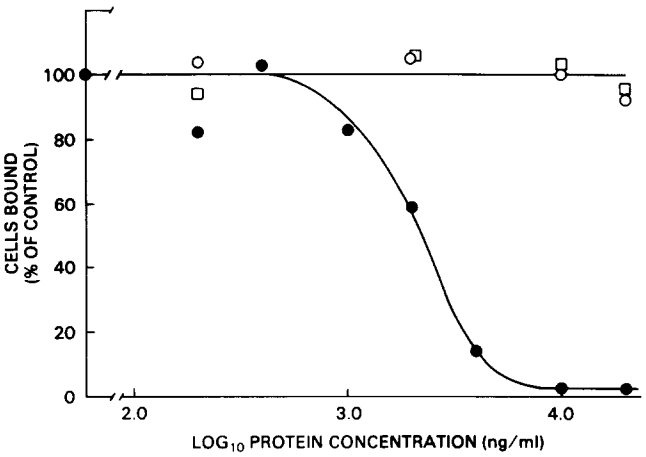

FIGURE 1 Effect of asialo-orosomucoid on the specific adhesion of rat hepatocytes to galactoside surfaces. Cell suspensions (2×10^6/ml in Medium 1) were incubated on ice for 30 min with the indicated concentrations of orosomucoid (□), asialo-orosomucoid (●), or asialo-agalacto-orosomucoid (○). Samples (55 μl) were then placed on galactoside surfaces, equilibrated in Medium 1, and incubated at 37°C for 60 min. The polyacrylamide gel pieces contained 3,359 nmol of *N*-acrylamidomethyl 2-aminocarbonylethyl 1-deoxy-1-thiogalactopyranoside per $cm^2 \times 0.25$ mm gel piece (see reference 24 for details on the structure and synthesis of this ligand). The surfaces were washed to remove nonbound cells, and the number of bound cells was determined as described in Materials and Methods. The control value of 100% represents binding of 90% of the added cells.

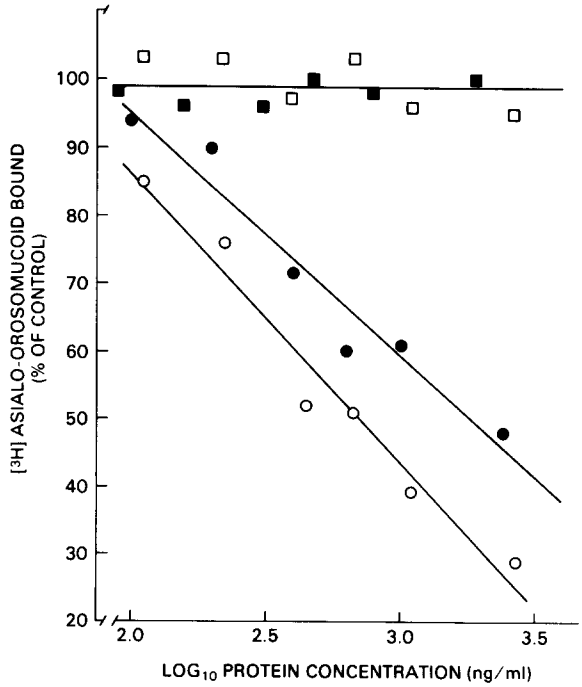

FIGURE 2 Effect of fluorescein-asialo-orosomucoid on the binding of [^{3}H]asialo-orosomucoid by rat hepatocytes. Cells (2.4×10^6/ml in Medium 1) were incubated at 4°C for 2.5 h with 0.6 μg [^{3}H]asialo-orosomucoid/ml and the indicated concentrations of unlabeled orosomucoid (■), asialo-orosomucoid (○), fluorescein-asialo-orosomucoid, containing 1.4 mol fluorescein/mol protein (●), or asialo-agalacto-orosomucoid (□). Bound [^{3}H]asialo-orosomucoid was determined on washed cell pellets as described in Materials and Methods.

galactoside concentration in the surface increases. This effect was observed earlier for the inhibition of cell binding by free galactose (24). Also, experiments with antiserum to the rat receptor (the generous gift of Dr. Gilbert Ashwell) have shown that the antiserum completely abolishes cell binding to the galactoside surfaces (21). All the available information, therefore, is consistent with the conclusion that the asialoglycoprotein receptor mediates this cell binding response.

Specific Recognition of Fluorescein-Asialo-Orosomucoid by Hepatocytes

A fluorescein-labeled derivative of asialo-orosomucoid was used to monitor changes in the cell surface distribution of asialoglycoprotein receptors when cell binding occurs. This derivative retained at least 85% of its binding activity, as judged by its ability to compete specifically and effectively for the binding of [^{3}H]asialo-orosomucoid by isolated rat hepatocytes (Fig. 2). Fig. 3*B* shows that at 37°C the fluorescein-asialo-orosomucoid was endocytosed into cells. We estimated that a typical cell can have well over 100 fluorescent vesicles. This uptake was mediated by the asialoglycoprotein receptor because virtually no fluorescent vesicles were observed in the presence of excess unlabeled asialo-orosomucoid (Fig. 3*A*).

Distribution of Asialoglycoprotein Receptors on Freshly Isolated Hepatocytes

Experiments to assess the initial distribution of surface receptors on cells before binding to the galactoside surfaces were performed using direct fluorescence microscopy with the fluorescein-asialo-orosomucoid derivative. These cells displayed a diffuse, apparently random distribution of fluorescence, as in Fig. 3*A*, with no detectable clusters or patches. These hepatocytes had a lot of interfering autofluorescence and, because they were spherical, it was difficult to focus on the cell surface. Therefore, we could not assess whether the receptor was in small clusters, as would be expected if, for example, it resides in coated pits.

Distribution of Asialoglycoprotein Receptors on Hepatocytes Bound to Galactoside Surfaces

Rat hepatocytes were allowed to bind to polyacrylamide galactoside surfaces at 37°C for 30 min and, after removing nonbound cells, the polyacrylamide surfaces with adherent cells were put on ice. To release the cells from the surfaces, while preserving the cell surface distribution of asialoglycoprotein receptors, the cells were incubated in the cold with medium containing EGTA. The released cells were then collected, washed, and incubated on ice with fluorescein-asialo-orosomucoid. Because successful cell adhesion to galactoside surfaces requires Ca^{2+} ions, the cells will de-adhere in the presence of the divalent chelator even in the cold. Cells bound to galactoside surfaces, then treated in this manner, and fixed for fluorescence microscopy, exhibited a single large bright fluorescent patch (Fig. 4). Approximately 50% of the released cells, which were judged to be intact, displayed a patch, whereas none or usually <1% of the control cells had a patch. Table I summarizes the results from four experiments in which various surfaces and conditions were examined. Fluorescence photomicrographs of control cells without patches were identical to that shown in Fig. 3*A*. Because the cells are not spread or flattened, but rather spherical, there are many focal planes

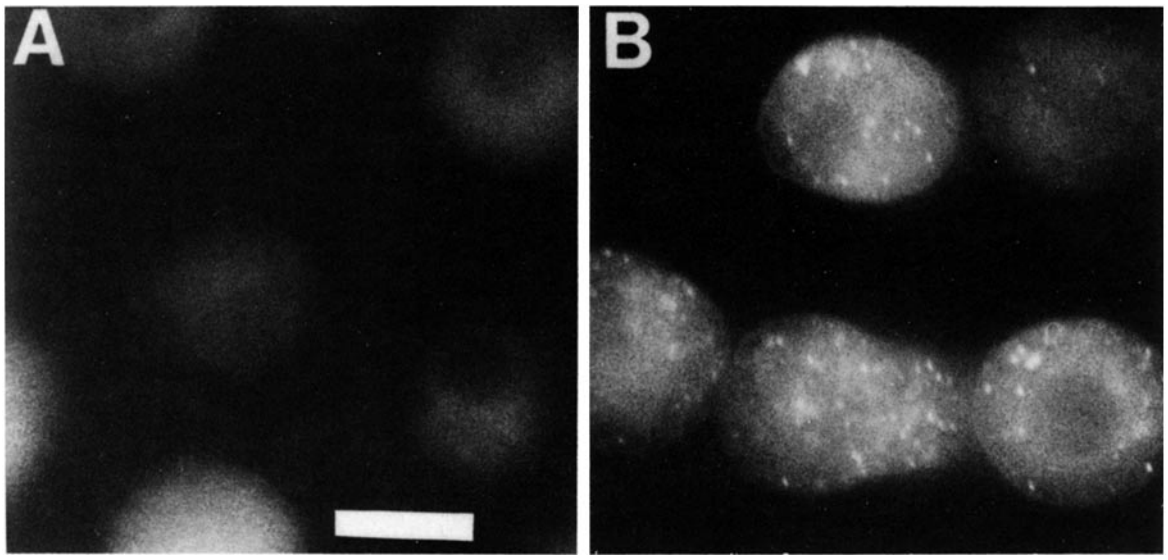

FIGURE 3 Internalization of fluorescein-asialo-orosomucoid by rat hepatocytes. Cell suspensions (4 ml, 2.2 × 10^6/ml in Medium 1 plus 0.1% BSA) were incubated at 37°C in 17-ml polystyrene-capped tubes on a rotator at 6 rpm (23) in medium containing 3.7 μg fluorescein-asialo-orosomucoid/ml with (*A*) or without (*B*) 187 μg/ml of unlabeled asialo-orosomucoid. After 40 min, the cell suspensions were centrifuged, the cell pellets were resuspended in 4 ml of Medium 1 plus 0.1% BSA, and 1 ml of a solution containing 16% formaldehyde in PBS was added to each suspension. After incubation at 37°C for 30 min, the cells were washed twice by centrifugation in Medium 1 plus 0.1% BSA, resuspended in PBS, and prepared for fluorescence microscopy as described in Materials and Methods. Exposure, developing, and printing times and conditions were the same for both samples. Bar, 5 μm. × 4330.

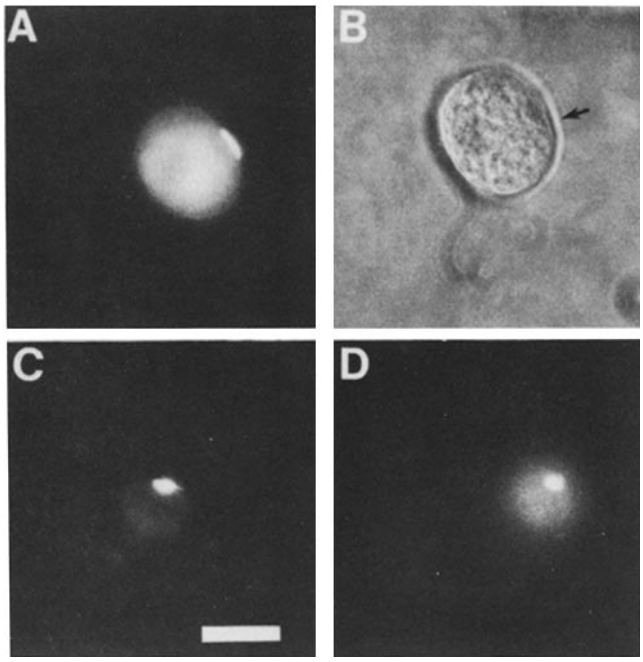

FIGURE 4 Use of fluorescein-asialo-orosomucoid to visualize asialoglycoprotein receptor patches on hepatocytes released from galactoside surfaces. Photomicrographs of fluorescent cells (see Table I, exp 1) were made as described in Materials and Methods. Exposure times, development, and printing conditions were the same. Panel *B* is a phase micrograph of the cell shown in panel *A*. The arrow in *B* points to the middle of the fluorescent patch. The patches shown in panels *A*, *C*, and *D* are representative of the appearance of these fluorescent patches on most cells. Bar, 5 μm. × 3464.

throughout each cell. In most cases, therefore, the phase contrast photomicrograph of a cell exhibiting a patch is not in focus (as in Fig. 4*B*). This is even more pronounced because the photomicrographs were taken without changing focus from the fluorescent patch. For this reason, the phase contrast pictures do not provide additional information and most are not shown.

The controls, which were treated in the same way as the experimental, except as noted in the legend to Table I, included the following: cells treated in suspension with or without EGTA, cells on plain polyacrylamide surfaces, cells on galactoside surfaces with sugar concentrations below the critical concentration necessary for successful cell binding, or cells released from galactoside surfaces but incubated with fluorescein-asialo-orosomucoid and an excess of unlabeled glycoprotein (Table I, exp 1 and 2). In addition, controls were also performed in which the distribution of asialoglycoprotein receptors on cells attached to nongalactose containing surfaces was assessed. Attachment of rat hepatocytes to glass, tissue culture plastic, nontissue culture plastic, or to collagen-coated dishes also did not cause the formation of asialoglycoprotein receptor patches (Table I, exp 3 and 4).

Location of the Asialoglycoprotein Receptor Patch

Cells were incubated, while they were bound to galactoside surfaces, with fluorescein-asialo-orosomucoid in complete medium at 4°C for 1 h. Although the fluorescein conjugate will cause the rapid release of bound cells at 37°C, at 4°C this competitive release is extremely slow. It was possible, therefore, to fix and examine stained cells still bound to a galactoside surface with the fluorescence microscope. No patches were observed on any of the bound cells, whereas cells released first and then stained had patches as described above. This indicates that the receptors are not accessible to the fluorescent glycoprotein unless the cells are first released. We therefore conclude that the patch of asialoglycoprotein receptors is at the junction between the cell and the galactoside surface.

TABLE I

Occurrence of Asialoglycoprotein Receptor Patches on Hepatocytes under Various Conditions and on Different Culture Surfaces

Culture surface and experimental condition	Percent of cells with a fluorescent patch			
	exp 1*	exp 2‡	exp 3‡	exp 4‡
Polyacrylamide surface; no galactose (dish A)	0			
Galactoside surface; below threshold (dish B)	<1			
Galactoside surface (dish C)	55	64	52	40
Galactoside surface; minus fluorescein-asialo-orosomucoid		0		
Galactoside surface; plus 50-fold excess asialo-orosomucoid		3		
Galactoside surface; 37°C, 15 min before staining			5	
Cells in suspension; EGTA treated	0			
Cells in suspension; not EGTA treated	<1			
Tissue culture plastic; no serum			<1	
Tissue culture plastic; plus serum				0
Nontissue culture plastic; no serum			<1	
Glass coverslips; no serum			<1	
Collagen-coated plastic; plus serum				<1

The results are summarized from four binding experiments using different cell preparations.

* Cell binding to surfaces with 0, 871, and 1,628 nmol of 6-aminohexyl β-D-galactoside per cm^2 × 0.25-mm gel piece was, respectively, 0, 10, and 90% of the added cells. The petri dish with cells bound to the high galactose-containing surfaces (dish C) was placed on ice and 5 ml of ice-cold Medium 1 plus 0.1% BSA containing 6 mM EGTA were added (Medium 1 contains 1.8 mM Ca^{+2}). The nonbound cells from the other surfaces (dishes A and B) were collected and also resuspended on ice in this medium. Two portions of the initial cell suspension (0.44 ml) were centrifuged and resuspended at 4°C in 5 ml of Medium 1 plus 0.1% BSA, with and without 6 mM EGTA. After 80 min, virtually all the cells were released from the galactoside surfaces in dish C. These cells were collected and centrifuged along with the other four cell suspensions. Each cell pellet was resuspended at 4°C with 0.5 ml of Medium 1 plus 0.1% BSA containing 13 μg of fluorescein-asialo-orosomucoid and incubated for 60 min. Formaldehyde was added (final concentration, 4%) and the cell suspensions were incubated for 30 min at 4°C followed by 20 min at room temperature. Cell pellets were washed, resuspended in PBS, and slides for fluorescence microscopy were prepared as described in Materials and Methods. Intact cells (100–200/sample) were scored for the presence or absence of fluorescent patches (see Fig. 4).

‡ These experiments were performed as described above for the bound cells using the indicated surfaces or galactoside surfaces with 1,628 nmol of galactose/cm^2 × 0.25-mm gel piece. The samples designated plus serum were incubated with 5% fetal bovine serum. Cells on collagen-coated plastic petri dishes (5), which were a gift from Dr. R. Klebe, were released on ice with collagenase (35 mg/ml in Medium 1 + 0.1% BSA) rather than EGTA.

Effect of Incubation at 37°C on the Asialoglycoprotein Receptor Patch

To verify that low temperature does in fact maintain the surface distribution of receptors on the de-adhered cells, the effect of temperature on the appearance of fluorescent patches was examined. Cells were released from galactoside surfaces at 4°C, and then incubated at 37°C for various times before being incubated at 4°C with the fluorescein-asialo-orosomucoid. After 15 min at 37°C, the percent of cells with fluorescent patches decreased by 90% (Table I, exp 3). Fig. 5 shows the time-course for the loss of asialoglycoprotein receptor patches

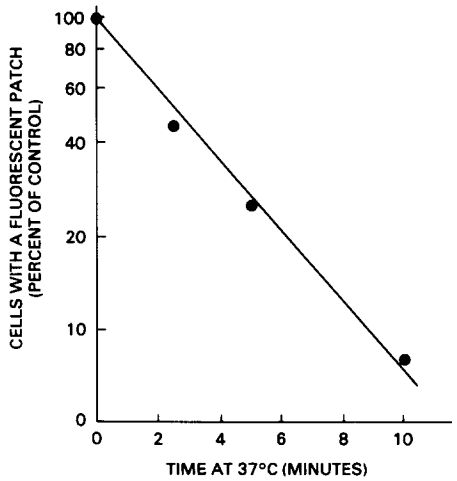

FIGURE 5 Effect of 37°C incubation on asialoglycoprotein receptor patches. The cell suspension shown in exp 4 of Table I was used. Cells were allowed to adhere to galactoside surfaces and bound cells were released on ice with EGTA, washed by centrifugation, and suspended in the cold, as described in the legend to Table I. At this point, however, the cell suspension was divided into four equal portions and each one was incubated at 37°C for a different length of time, as indicated in the figure. The samples were then put on ice, stained with fluorescein-asialo-orosomucoid, and prepared for fluorescence microscopy as described in Table I. The percent of intact cells displaying fluorescent patches was then determined relative to the control which was kept on ice after releasing the cells. Note that the ordinate is a logarithmic scale.

at 37°C. The half time for the disappearance of patches in this experiment was ~2.6 min. This is a first-order process and is consistent with the possibility that the receptors diffuse away from the region of the patch or that the receptor patch is internalized. However, preliminary experiments in which released cells have been stained with fluorescein-asialo-orosomucoid at 4°C, then washed and incubated at 37°C before fixation, show that the occupied receptor patch region is not internalized as a whole at 37°C. The results substantiate the assumption that low temperature (0–4°C) preserves the cell surface distribution of asialoglycoprotein receptors on cells that had bound to and were subsequently released with EGTA from the galactoside surface.[2]

DISCUSSION

The threshold nature of the binding response of freshly isolated rat hepatocytes to a synthetic culture surface containing covalently linked galactosyl groups is striking (24) and suggests that some event(s) involving the receptor mediating this response may occur at the cell surface. The available evidence strongly indicates that surface asialoglycoprotein receptors mediate hepatocyte binding to a galactoside surface. In the present study, we have, therefore, used fluorescein-asialo-orosomucoid as a direct fluorescent probe to determine the distribution of asialoglycoprotein receptors before and after cell binding. The fluorescent conjugate is specifically recognized by the receptor (Fig. 2) and is endocytosed as though it were authentic asialo-orosomucoid (Fig. 3).

[2] In an earlier report of this study, defined fixation conditions were used to stabilize the asialoglycoprotein receptor distribution (21). The procedure reported here using low temperature, however, proved easier to control and offered the advantage of being reversible.

Fluorescence microscopy using the fluorescein-asialo-orosomucoid indicates that on freshly isolated cells in suspension these receptors are in a diffuse distribution. Wall et al. (19) recently showed in the perfused rat liver that endocytosis of lactosyl ferritin, which is recognized by the asialoglycoprotein receptor, occurs via coated pits. These workers also concluded that the receptor is asymmetrically distributed on the hepatocyte surface in vivo; it is localized in sinusoidal and lateral but not bile canalicular membranes (18). Freshly isolated hepatocytes, which still effectively endocytose [^{3}H]asialo-orosomucoid (12), are no longer polar and we surmise that the asialoglycoprotein receptor distribution is also no longer asymmetric. It is likely that these surface receptors are either nonclustered or localized in small coated pit regions. However, the binding of a hepatocyte to a galactoside surface is accompanied by the formation of a single large receptor patch. The area of this receptor zone is ~4 μm^2 or ~2–3% of the surface area of the cell (assuming the cell is spherical and smooth). Asialoglycoprotein receptor patches are not found in any of the circumstances examined in which hepatocytes are either unattached to a substratum or attached to substrata that do not contain galactose.

The results clearly indicate that a large cluster of asialoglycoprotein receptors forms at the cell-galactoside surface junction when the cell binds. That the receptor patch on a released cell is lost at 37°C (Fig. 5) further supports the conclusion that its formation and maintenance is in response to the galactoside surface. Movement or modulation of receptors to the region(s) of cell-substratum contact is the most likely explanation for the formation of the patch, and experiments are in progress to determine this. Hepatocytes that have been fixed with formaldehyde under conditions that do not affect the level of specific binding of [^{3}H]asialo-orosomucoid but that greatly decrease endocytosis of the bound glycoprotein will not adhere to a galactoside surface (21). This observation suggests that receptor mobility is involved in the cell binding process.

Approximately 50% of the released cells, which appeared intact by light microscopy, had a patch (Table I). There are several possible reasons why this percentage was not higher. (*a*) The receptor patch disappears with a half-life of ~2.6 min at 37°C and at 4°C this process may still occur, although at a slower rate. Also, during the various experimental manipulations the cell suspensions may have warmed slightly with the consequent loss of some patches. (*b*) The fluorescence of the fluorescein-asialo-orosomucoid fades rapidly at room temperature. Under the conditions reported here, the half time for fluorescence decay in the fluorescence microscope is on the order of 20 s. Therefore, although this work was performed with minimal background lighting, some patches may have already been bleached by the time of observation. (*c*) We have preliminary evidence that bound cells are extremely sensitive to shear forces and, in fact, are easily ripped from the galactoside surface. These cells appear to leave behind a membrane patch on the surface and would be scored as not having a patch if they still appeared to be intact after the staining and fixation steps. Many cells have a bright fluorescent region which is irregular and hanging off the cell surface. These cells were not scored as having a patch but our interpretation is that these are cells whose receptor patch regions have partially pulled away from the cell surface during the EGTA incubation. Although the cells were manipulated as little as possible, it is nonetheless likely that a significant portion of the released cells that appear intact may have left their receptor patch on the substratum. We, therefore, believe that all the cells bound to the galactoside surface have a patch of asialoglycoprotein receptors mediating that adhesion.

To our knowledge, this is the first report that directly shows a patch (or cap) of cell surface receptors to be involved in adhesion of the cell to a substratum. In several other systems, investigators have inferred from indirect evidence that a caplike region forms during binding because the receptor, which is capable of recognizing the ligand adsorbed to the particular culture substratum, disappears from the exposed surface of a bound cell. For example, Michl et al. (9, 10) have reported that, when mouse peritoneal macrophages bind to a substratum coated with antigen-antibody complexes, there is a rapid and large decrease in the cell surface binding of soluble antibody against trypsin-resistant Fc receptors. Also, Grinnell (4) recently showed that baby hamster kidney cells in suspension will bind small latex beads coated with plasma fibronectin but that, when spread on a substratum coated with plasma fibronectin, these cells can no longer bind such beads. In the above cases, the bound cells apparently cannot be de-adhered in a relatively nondestructive way which also preserves the distribution of cell surface receptors, as could be done in the hepatocyte-galactoside surface system.

The present study was undertaken to learn more about the threshold binding response of rat hepatocytes triggered by a critical concentration of galactose in the substratum. A threshold binding response has also been demonstrated for the binding of chicken hepatocytes to *N*-acetylglucosamine surfaces (24), the only other cell type so far examined for this response. Threshold responses may have very important implications for the normal and abnormal interactions of cells with other cell surfaces or constituents of the extracellular matrix. The adhesion of rat hepatocytes to synthetic galactoside surfaces promises to be a good model system in which to study receptor-related events and cell-substratum interactions, particularly because the overall system can be manipulated experimentally in several ways.[3] The experiments reported here demonstrate that the asialoglycoprotein receptor mediates the hepatocyte-galactoside surface interaction and indicates that to do so it undergoes substantial rearrangement on the cell surface. Studies are in progress to determine the relationship between the threshold binding response and the formation of asialoglycoprotein receptor patches.

I am very grateful to Betty Jackson for help in preparing this manuscript, Janet A. Oka for very capable technical assistance, and Drs. Gerald Fuller and William Snell for helpful comments and discussions.

This work was supported by a grant from the National Institutes of Health, GM 26228.

Received for publication 4 August 1980, and in revised form 15 September 1980.

REFERENCES

1. Ashwell, G., and A. G. Morell. 1974. The role of surface carbohydrates in the hepatic recognition and transport of circulating glycoproteins. *Adv. Enzymol.* 41:99–128.
2. Berg, T., D. Bowman, and P. O. Seglen. 1972. Induction of tryptophan oxygenase in primary rat liver cell suspensions by glucocorticoid hormone. *Exp. Cell Res.* 72:571–574.
3. Grinnell, F. 1978. Cellular adhesiveness and extracellular substrata. *Int. Rev. Cytol.* 53:65–144.

[3] For example, one can show with this system that there is also a distinct critical concentration of galactose in the polyacrylamide matrix required for cell spreading. This threshold spreading response occurs only at or above a sugar concentration which is much greater than that required for cell adhesion to a galactoside surface (P. Weigel, unpublished results).

4. Grinnell, F. 1980. Fibroblast receptor for cell-substratum adhesion: studies on the interaction of baby hamster kidney cells with latex beads coated by cold insoluble globulin (plasma fibronectin). *J. Cell Biol.* 86:104–112.
5. Klebe, R. J. 1974. Isolation of a collagen-dependent cell attachment factor. *Nature* (*Lond.*). 250:248–251.
6. Lee, R. T., S. Cascio, and Y. C. Lee. 1979. A simple method for the preparation of polyacrylamide gels containing thioglycoside ligands. *Anal. Biochem.* 95:260–269.
7. Lowry, O. H., N. J. Rosebrough, A. L. Farr, and R. J. Randall. 1951. Protein measurements with the Folin phenol reagent. *J. Biol. Chem.* 193:265–275.
8. Means, G. E., and R. E. Feeney. 1968. Reductive alkylation of amino groups in proteins. *Biochemistry.* 7:2192–2201.
9. Michl, J., M. M. Pieczonka, J. C. Unkeless, and S. C. Silverstein. 1979. Effects of immobilized immune complexes on Fc- and complement-receptor function in resident and thioglycollate-elicited mouse peritoneal macrophages. *J. Exp. Med.* 150:607–621.
10. Michl, J., J. C. Unkeless, M. M. Pieczonka, and S. C. Silverstein. 1979. Mechanism of macrophage Fc receptor modulation by immobilized antigen-antibody complexes. *J. Cell Biol.* 83(2, Pt. 2):295*a* (Abstr.).
11. Morell, A. G., R. A. Irvine, I. Sternlieb, I. H. Scheinberg, and G. A. Ashwell. 1968. Physical and chemical studies on ceruloplasmin. V. Metabolic studies on sialic acid-free ceruloplasmin in vivo. *J. Biol. Chem.* 243:155–159.
12. Oka, J., and P. Weigel. 1980. Endocytosis mediated by the asialoglycoprotein receptor in isolated rat hepatocytes. *Fed. Proc.* 39:2101.
13. Schachter, H., I. Jabbal, R. L. Hudgin, L. Pinteric, E. J. McGuire, and S. Roseman. 1970. Intracellular localization of liver sugar nucleotide glycoprotein glycosyltransferases in a Golgi-rich fraction. *J. Biol. Chem.* 245:1090–1100.
14. Schnaar, R. L., P. H. Weigel, M. S. Kuhlenschmidt, Y. C. Lee, and S. Roseman. 1978. Adhesion of chicken hepatocytes to polyacrylamide gels derivatized with *N*-acetylglucosamine. *J. Biol. Chem.* 253:7940–7951.
15. Seglen, P. O. 1973. Preparation of rat liver cells. III. Enzymatic requirements for tissue dispersion. *Exp. Cell Res.* 82:391–398.
16. Steer, C. J., and G. Ashwell. 1980. Studies on a mammalian hepatic binding protein specific for asialoglycoproteins: evidence for receptor recycling in isolated rat hepatocytes. *J. Biol. Chem.* 255:3008–3013.
17. Van Lenten, L., and G. Ashwell. 1972. The binding of desialylated glycoproteins by plasma membranes of rat liver; development of a quantitative inhibition assay. *J. Biol. Chem.* 247:4633–4640.
18. Wall, D. A., G. Wilson, and A. L. Hubbard. 1979. Distribution of asialoglycoprotein binding and internalization sites on rat hepatocyte cell surfaces. *J. Cell Biol.* 83(2, Pt. 2): 68*a* (Abstr.).
19. Wall, D. A., G. Wilson, and A. L. Hubbard. 1980. The galactose-specific recognition system of mammalian liver: the route of ligand internalization in rat hepatocytes. *Cell.* 21: 79–93.
20. Weigel, P. H. 1980. Characterization of the asialoglycoprotein receptor on isolated rat hepatocytes. *J. Biol. Chem.* 255:6111–6120.
21. Weigel, P. H. 1980. The binding of rat hepatocytes to galactoside surfaces involves clusters of asialoglycoprotein receptors. *Fed. Proc.* 39:2101.
22. Weigel, P. H., M. Naoi, S. Roseman, and Y. C. Lee. 1979. Preparation of 6-aminohexyl D-aldopyranosides. *Carbohydr. Res.* 70:83–91.
23. Weigel, P. H., E. Schmell, Y. C. Lee, and S. Roseman. 1978. Specific adhesion of rat hepatocytes to β-galactosides linked to polyacrylamide gels. *J. Biol. Chem.* 253:330–333.
24. Weigel, P. H., R. L. Schnaar, M. S. Kuhlenschmidt, E. Schmell, R. T. Lee, Y. C. Lee, and S. Roseman. 1979. Adhesion of hepatocytes to immobilized sugars; a threshold phenomenon. *J. Biol. Chem.* 254:10830–10838.
25. Wells, A. F., C. E. Miller, and M. K. Nadel. 1966. Rapid fluorescein and protein assay method for fluorescent-antibody conjugates. *Appl. Microbiol.* 14:271–275.