Hepatocyte Adhesion to Carbohydrate-Derivatized Surfaces. I. Surface Topography of the Rat Hepatic Lectin

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Abstract. The rat hepatic lectins, galactose- and N-acetylgalactosamine-binding proteins found on the hepatocyte cell surface, mediate adhesion of isolated primary rat hepatocytes to artificial galactose-derivatized polyacrylamide gels. Biochemical and immunohistochemical techniques were used to examine the topographical redistribution of the rat hepatic lectins in response to galactose-mediated cell adhesion. Hepatocytes isolated from rat liver by collagenase perfusion had an average of 7×10^5 cell surface lectin molecules per cell, representing 30–50% of the total lectin molecules per cell, the remainder residing in intracellular pools. Hepatocytes incubated on galactose-derivatized surfaces, whether at 0-4°C or 37°C, rapidly lost >80% of their accessible cell surface lectin binding sites into an adhesive patch of characteristic morphology. The kinetics of rat hepatic lectin disappearance were used to estimate a lateral diffusion coefficient of $>9 \times 10^{-9}$ cm²/s at 37°C, suggesting rapid and unimpeded lectin diffusion in the plane of the membrane. Indirect immunofluorescence labeling of adherent cells using antihepatic lectin antibody revealed a structured ring of receptors surrounding an area of exclusion (patch) of reproducible size and shape which represented ~8% of the hepatocyte cell surface. Notably, adherent cells, which had lost >80% of their accessible surface binding sites, still endocytosed soluble galactose-terminated radioligand at >50% of the rate of nonadherent control cells. No net movement of rat hepatic lectin from intracellular pools to the cell surface was found on cells recovered after adhesion to galactose-derivatized surfaces at 37°C, suggesting that the physical size and/or lectin density of the patch was restricted by kinetic or topological constraints.

RAT hepatic lectins (RHLs)[1] are well-defined polypeptides (8, 12) found at the hepatocyte cell surface which bind galactose- (Gal) or N-acetylgalactosamine–terminated ligands in a calcium-dependent manner and have been a prototype for the study of receptor-mediated endocytosis (for reviews see 1, 7, 29). RHLs consist of three closely related polypeptides (designated RHLs 1, 2, and 3), organized as hetero-oligomers on the surface of hepatocytes or hepatoma cells (3, 13, 23, 24, 27). In addition to their role in receptor-mediated endocytosis, RHLs mediate hepatocyte adhesion specifically to Gal-derivatized surfaces (11, 21, 32–34). Initial Gal-mediated hepatocyte adhesion is rapidly followed, at 37°C, by energy-dependent strengthening of the adhesive bond. Weigel (32) reported that hepatocytes detached from Gal-derivatized surfaces after adhesion had a small patch of lectin on their membrane. We combined biochemical probes and cell adhesion methods to quantitate lectin redistribution on adherent hepatocytes, and applied indirect immunofluorescence and confocal microscopy to determine the morphology of the intact adhesion site. Cells were also recovered to determine whether RHLs from intracellular pools were recruited to the cell surface in response to Gal-mediated cell adhesion. The resulting data from this model system for ligand-mediated adhesion offer insight into how the surface distribution of receptors may be regulated during early events in cell adherence.

1. *Abbreviations used in this paper*: AEMAS, N-[2-[[3-[[4-[(2,5-dioxo-1-pyrrolidinyl)oxy]-4-oxobutyl]amino]-3-oxopropyl]dithio]ethyl]-2-propenamide; CMF-HBS, calcium- and magnesium-free HBS; D_L, lateral diffusion coefficient; Dabco, 1,4-diazabicyclo [2.2.2] octane; Gal, galactose; Glc, glucose; GlcNAc, N-acetylglucosamine; RHL, rat hepatic lectin.

Materials and Methods

Media and Buffers

Dulbecco's modified Eagle medium with Hepes (DMEM-H), Hepes-buffered saline (HBS), and calcium- and magnesium-free HBS (CMF-HBS) were prepared as described previously (4, 11). Dulbecco's formulation (9) was used for preparing PBS.

Carbohydrate-derivatized Polyacrylamide Gels

Aminohexyl β-glycosides of galactose (Gal), glucose (Glc), and N-acetylglucosamine (GlcNAc) were immobilized on polyacrylamide gel disks using either the noncleavable acrylic linker N-6 (the N-succinimidyl ester of 6-acrylamidohexanoic acid, [22]) or a disulfide-containing cleavable acrylic linker, AEMAS (26). Glycoside derivatization and immobilization were essentially as described previously (4). Carbohydrate-derivatized gel

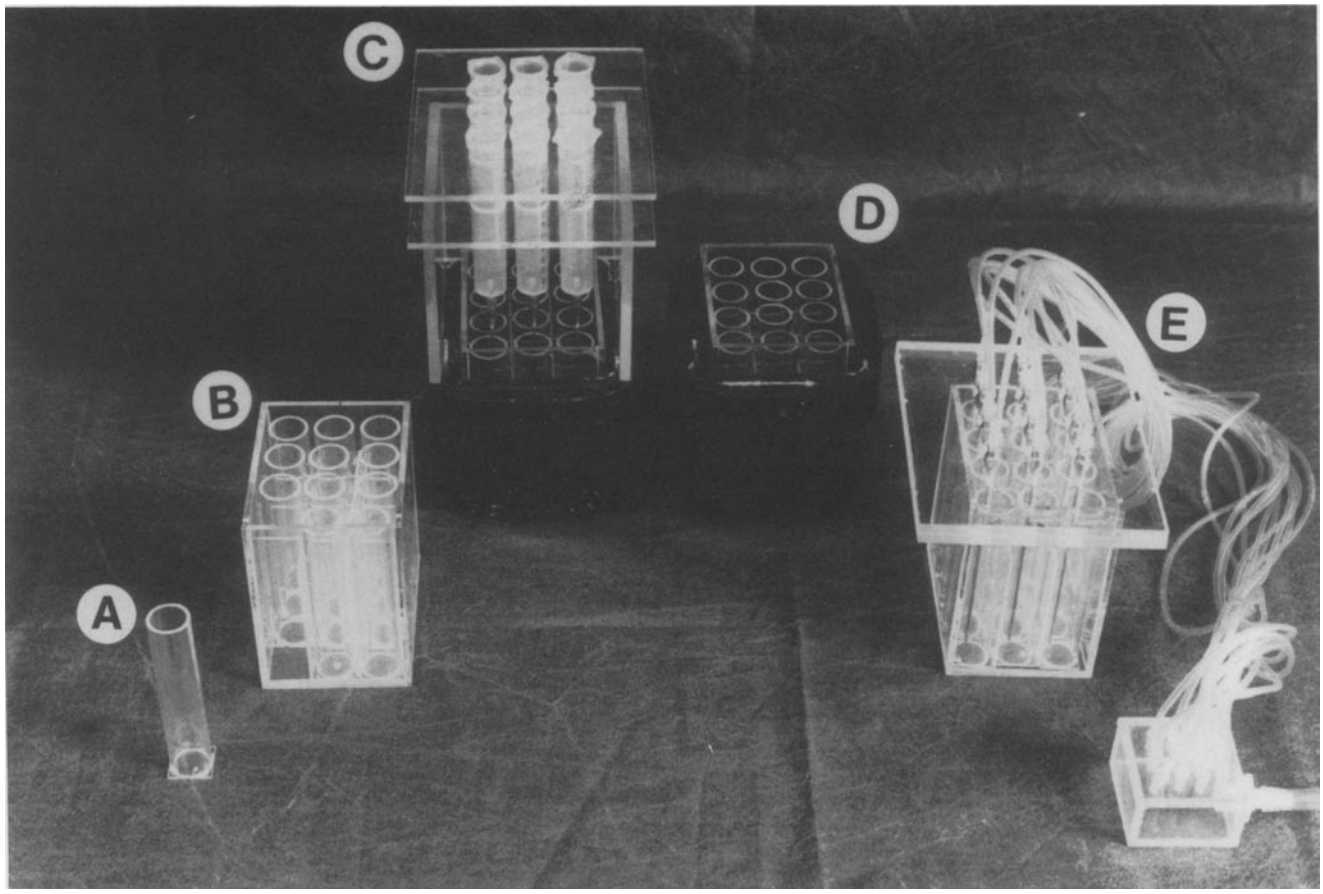

Figure 1. Apparatus to measure radioligand binding to cells incubated on covalently derivatized polyacrylamide surfaces. Gel disks (14 mm) were placed in the bottom of individual cylinders (*A*), which were fitted into plexiglass boxes (*B*), and cells and radioligand were added. The outer boxes had holes in the side to allow rapid temperature equilibration. At the end of the incubation, the boxes were placed in centrifuge carriers (Sorvall PN 11053), and fitted with a collar which supported a plexiglass manifold (*C*) containing appropriately spaced syringe bodies fitted with 19-gauge needles. Using this device, cold PBS (~14 ml) was added to each chamber slowly and without disturbing the cell layer. The manifold was then removed and the cylinders centrifuged at 4°C, 67 *g* for 3 min (*D*) to ensure that all of the cells (whether adherent or not) were flush to the gel surface. Most of the medium above the gels was then removed by vacuum using a second manifold (*E*) containing appropriately spaced Luer hub needles (16 gauge × 4 in) which reached to within 3 mm of the gel surface. The chambers were then refilled with PBS (*C*) for subsequent washing. The wash procedure was performed a total of four times before recovery of the cells and quantitation of radiolabel as described in the text.

disks of 14-mm diameter were cut from derivatized polyacrylamide sheets using a cork borer, washed extensively, and stored in 0.9% NaCl in 7.5% (vol/vol) isopropyl alcohol at 4°C before use. The density of immobilized carbohydrate was determined on each gel preparation after hydrolysis of the carbohydrate as described previously (11). Gels containing 2.5–60 μmol of immobilized galactoside or control glycosides per ml of gel were used. The threshold density of immobilized carbohydrate required to support strong cell adhesion (11) depended on the linker used, and varied during the course of the experiments. Therefore, the density of immobilized saccharides was adjusted to ensure strong carbohydrate-directed adhesion.

Hepatocyte Preparation

Male Sprague-Dawley rats (150–250 g, Harlan Labs, Indianapolis, IN) were fed standard laboratory chow and water, ad libitum. Hepatocytes were prepared by noncirculating perfusion of rat livers with a collagenase-containing buffer essentially as described previously (20, 28). The preparation was enriched for viable cells by Percoll density centrifugation (15). The resulting cell suspensions were routinely >95% viable and consisted of predominantly single cells. Cells were incubated in rotation culture (115–125 rpm) at 37°C for 60 min before use.

Quantitation of Rat Hepatocyte Adhesion to Gal-derivatized Gel Disks

Derivatized gel disks were placed in the bottom of 24-well tissue culture dish wells (Costar Nucleopore Corp., Cambridge, MA) and anchored in place with teflon cylinders (11 mm diameter × 2 mm wall thickness × 15 mm high) made by cutting teflon tubing in 15-mm segments. After equilibration of the gels with DMEM-H, 1 ml of cell suspension was pipetted onto each gel. After the desired incubation time, the plate was completely immersed in ice-cold PBS and inverted, allowing the nonadherent cells to settle away from the derivatized surface without subjecting the adherent cells to an air–liquid interface. After 10 min, the plate was righted while immersed and a portion of the PBS was removed and replaced with 0.5% Triton X-100 in 0.1 M potassium phosphate buffer (pH 7) to lyse the adherent cells. Aliquots of the lysate were used for determination of released lactate dehydrogenase activity, which was directly related to the number of adherent cells (25).

Radioligands

Two high-affinity ligands were used to quantitate RHLs on isolated hepatocytes. $Gal_{39}BSA$ (GalBSA) was prepared using the method of Lee et al. (17). The low molecular weight, high-affinity ligand Di-GalNAc consists of the dipeptide N-acetyl-tyr-asp in which both of the aspartate carboxylic acids are derivatized via an amide linkage to glycines which are further derivatized at their carboxylic acids via amide linkage with aminohexyl-GalNAc (16). GalBSA was radioiodinated using carrier-free $Na^{125}I$ and Iodobeads (Pierce Chemical Co., Rockford, IL), and the iodinated product was purified by Sephadex G-25 gel filtration chromatography. Initial specific activities ranged from 27–120 Ci/mmol. Scatchard analysis of ^{125}I-

GalBSA binding to isolated rat hepatocytes was consistent with a single high-affinity binding site with a K_D of 0.4 nM (data not shown). ^{125}I-EGF (220–240 Ci/mmol) was the kind gift of Dr. Carol Renfrew (The Johns Hopkins School of Medicine). Di-GalNAc, the kind gift of Drs. Y. C. and R. T. Lee (The Johns Hopkins University), was radioiodinated as described above, then separated from free iodine by Sephadex G-10 column chromatography. The initial specific activity was 4.9 Ci/mmol.

Quantitation of Cell Surface RHLs on Adherent Hepatocytes

Freshly isolated rat hepatocytes were allowed to equilibrate in DMEM-H in rotation culture for 60 min at 37°C at 120 rpm. The cells were collected by centrifugation and resuspended in fresh medium at a final concentration of 8×10^5 cells/ml. Carbohydrate-derivatized gel disks were placed in the bottom of specially designed 15-mm diameter plexiglas cylinders (Fig. 1) and preequilibrated in DMEM-H for at least 1 h before addition of cells. Cells were added (0.25 ml/well, ~70% confluence) to gels incubating in an equal volume of DMEM-H at the desired temperature. After the chosen incubation time, the cylinders were rapidly chilled to 0–4°C, radiolabeled ^{125}I-GalBSA (12.5–25 nM final concentration) was added from a 25-fold concentrated stock, and the chambers were gently agitated. After 60 min at 0–4°C, the cells in each cylinder were washed free of unbound radioligand without disturbing the cell layer. Chambers were slowly filled with cold PBS (~14 ml per chamber) using the syringe apparatus pictured in Fig. 1, and centrifuged at 4°C at 67 *g* for 3 min. The medium above the gels was removed by aspiration using a specially designed manifold, leaving less than 1 ml of liquid in each chamber, and the chambers were refilled with fresh PBS for a second wash. The chambers were washed in this manner a total of four times. After aspiration of the final wash medium, 50 μl of 100 mM EDTA, (pH 7.4) was added to each chamber, the chambers were agitated and incubated at room temperature for several minutes. The liquid in each chamber, which included dislodged cells, was triturated vigorously, transferred to a test tube, and radioactivity quantitated using a gamma radiation counter. To normalize for cell recovery, 1 ml of 0.5% Triton X-100 in 0.1 M potassium phosphate buffer (pH 7) was added to each tube, and lactate dehydrogenase activity determined. Typically, 60–90% of the input cells were recovered (the chambers had no significant residual radioactivity or LDH activity, indicating efficient removal of the cells from the derivatized surfaces). Nonspecific binding, measured in the presence of 25 mM GalNAc (a competitive inhibitor), was generally <1% of the input radiolabel and was independent of cell recovery. The data are presented as the number of radioligand molecules associated per cell, calculated by subtracting the average background from each data point and normalizing for cell recovery. Data are presented as the mean of triplicate determinations ± SD.

Endocytosis of ^{125}I-GalBSA by Adherent Hepatocytes

Freshly isolated rat hepatocytes were equilibrated for 60 min at 37°C, collected, resuspended, and placed on carbohydrate-derivatized gels in cylinders as described above. After 60 min at 0–4°C, radiolabeled ^{125}I-GalBSA was added (as above) for an additional 60 min at 0–4°C. Endocytosis was initiated by transferring the cylinders to 37°C. At the end of the desired incubation time, the cells were washed and cell-associated radioactivity and cell recovery were quantitated as described above.

Immunohistochemistry

Affinity-purified polyclonal α-RHL antibody (19) and α-EGF-receptor antibody (10) were the kind gifts of Drs. Ann Hubbard and Carol Renfrew (The Johns Hopkins School of Medicine). Cells were incubated on Gal-derivatized gels for 60 min at the desired temperature. The medium above the gels was gently removed by aspiration, and the cells were fixed for 20 min at room temperature by addition of 4% (wt/vol) paraformaldehyde in a lysine-phosphate buffer containing 25 mM sodium periodate (18). The cells were washed briefly three times with PBS, and then incubated for 5 min in 0.25% ammonium chloride in PBS. Gels were preblocked with 0.2% gelatin in PBS for 5 min before addition of antibody (75–150 μl of 10 μg/ml per well). After 30 min of gentle rotation, the medium was removed, the gels were washed three times, and the appropriate second antibody was added for 15 min. The gels were again washed three times, the buffer removed, and 15 mg/ml Dabco (Aldrich Chemical Co., Milwaukee, WI) in 3:1 PBS/glycerol was added. After transferring the gels to microscope slides, a drop of glycerol was added, and coverslips were placed on top and secured with nail polish. The cells were viewed using a Zeiss Axioplan Universal microscope. Confocal microscopy (using an MRC500 Scanning Confocal Imaging System; Bio-Rad Laboratories, Cambridge, MA) was used to generate optical sections of cells, and photographs were taken from a high-resolution monitor.

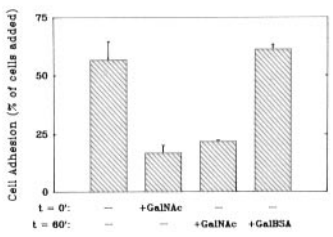

Figure 2. GalBSA does not reverse adhesion to Gal-derivatized polyacrylamide gel disks. Cells were incubated on polyacrylamide gel disks derivatized with Gal and secured in 24-well plates. GalNAc (25 mM) was included in some wells throughout the incubation to define background adhesion (t = 0'). After 1 h at 4°C, GalNAc (25 mM) or GalBSA (12.5 nM) was added (t = 60'), the incubation continued for 1 h at 4°C, and weak cell adhesion to the gels was determined as described in Materials and Methods.

Results

GalBSA Is Excluded from the Hepatocyte Adhesion Site

The technical key to quantitating RHL redistribution into an adhesive "patch" on cells adhering to Gal-derivatized gels was the use of a macromolecular radioligand that was sterically excluded from the cell–gel attachment site. Data demonstrating that ^{125}I-GalBSA could serve this purpose came from adhesion inhibition experiments (Fig. 2). Gal-derivatized gel disks were anchored in 24-well dishes and an aliquot of hepatocyte suspension was added at 4°C. After 60 min, the low molecular weight inhibitor GalNAc (221 D, 25 mM final concentration) or $Gal_{39}BSA$ (~75,000 D, 12.5 nM final concentration) was added, each at >30-fold their respective IC_{50}'s, and the incubation was continued for an additional 1 h at 4°C. Cell adhesion to the gels was then determined as described in Materials and Methods. Cells adhered avidly and specifically to Gal-derivatized gels. Adhesion was blocked or reversed by addition of 25 mM GalNAc (background adhesion was ~20% of input cells, appropriate for the low detachment force used [11]). In contrast, GalBSA was unable to reverse adhesion to the Gal-derivatized gels subsequent to cell adhesion (Fig. 2), although it is fully capable of blocking adhesion if added at 0 min (data not shown). This suggested that the macromolecular ligand was excluded from the adhesion site, while the smaller GalNAc diffused into the adhesion site and effectively competed for RHL binding.

Adhesion-dependent Disappearance of Accessible Cell Surface RHLs

Cells were incubated for 60 min at 4°C on Gal derivatized gels, to which they adhere, or on control Glc-derivatized gels. Subsequent determination of ^{125}I-GalBSA binding on adherent and control cells (see Materials and Methods) revealed that Gal-adherent hepatocytes had only 9% as many accessible RHLs as cells incubated on control gels (Fig. 3 *A*). In contrast, hepatocytes that were incubated with ^{125}I-GalBSA for 60 min before incubation on derivatized gels (for an additional 60 min at 4°C), and then washed in an identical fashion, demonstrated a high level of accessible cell surface RHLs (Fig. 3 *B*). We interpret these results to indicate movement of nearly all of the RHLs to the adhesive Gal-derivatized surface within 60 min at 4°C. This conclusion is

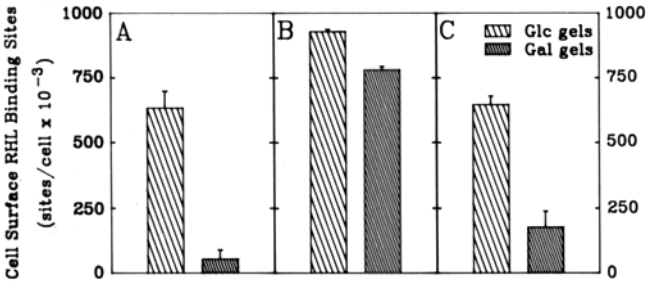

Figure 3. Disappearance of accessible RHLs upon adhesion of rat hepatocytes to Gal-derivatized surfaces. (*A*) Hepatocytes were incubated on Gal- or Glc-derivatized gels for 60 min at 4°C before addition of ^{125}I-GalBSA for an additional 60 min at 4°C. (*B*) Hepatocytes were incubated with ^{125}I-GalBSA (12.5 nM) in suspension at 4°C for 1 h with gentle agitation before placement on Gal- or Glc-derivatized gels for an additional 1 h at 4°C. (*C*) Hepatocytes were placed on Gal- or Glc-derivatized gels in the presence of ^{125}I-GalBSA for 1 h at 4°C. After each of the above incubations, cells were washed, and radioligand binding was determined as described in Fig. 1 and in the text.

dependent on the observations that ligand internalization does not occur at 4°C, and that the off rate of ^{125}I-GalBSA bound to the RHL is negligible within the time course of our experiments (6, 31). The rapid kinetics of RHL disappearance is indicated in Fig. 3 *C*, where ^{125}I-GalBSA was added to the hepatocytes concomitant with their placement on the derivatized gels. Cells incubated on Gal-derivatized surfaces had only 27% as much ligand bound as cells incubated on Glc-derivatized surfaces. This "competition" experiment suggests that the movement of RHLs to the adhesive surface was more rapid than their association with the radioligand. Rapid receptor diffusion to the adhesive surface occurred under all conditions tested including after pretreatment of the cells with a variety of metabolic or cytoskeletal perturbants (cytochalasin B [10 μM], monensin [25 μM], colchicine [4 μM], vanadate [5 mM], rotenone [2 μM] and oligomycin [2 μg/ml], or hyperosmotic medium [500 mOsm with sucrose], data not shown).

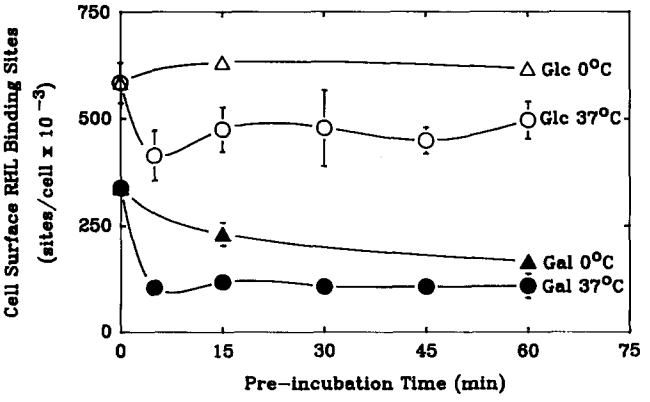

Figure 4. Kinetics of disappearance of accessible RHLs upon adhesion to Gal-derivatized surfaces. Cells were incubated on Gal- or Glc-derivatized gels at 37°C or 0°C for the indicated times. The chambers were then rapidly chilled to 0°C (if necessary), ^{125}I-GalBSA (12.5 nM) was added to an additional 60 min, and specific radioligand binding was quantitated as described in Fig. 1 and in Materials and Methods.

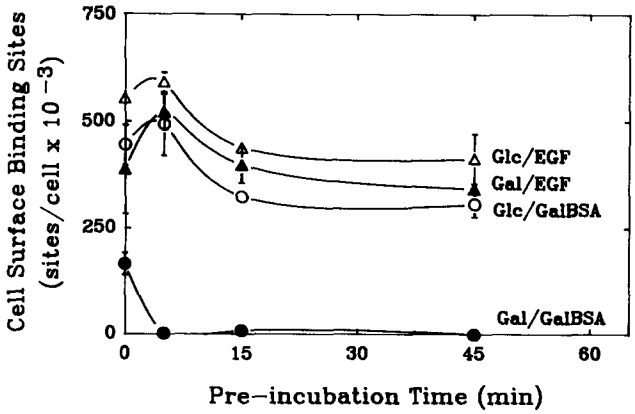

Figure 5. Adhesion to galactose-derivatized gels does not decrease surface EGF receptors. Cells were incubated on Gal- or Glc-derivatized gels at 37°C for the indicated times. The chambers were chilled to 0°C and ^{125}I-EGF (25 nM) or ^{125}GalBSA (12.5 nM) added for 60 min to quantitate accessible surface binding sites. Nonspecific binding of ^{125}I-EGF was determined in the presence of 50-fold excess of unlabeled EGF (Amgen, Thousand Oaks, CA).

Kinetics of Disappearance of Accessible Cell Surface RHLs

Cells were incubated on Gal- or Glc-derivatized gels for up to 60 min at 0°C or 37°C, then chilled (to 0°C) and ^{125}I-GalBSA was added for an additional 60 min at 4°C. Specific ligand binding was quantitated as described in Materials and Methods. The decrease in surface binding to cells incubated on Gal-derivatized gels was complete within 5 min of incubation at 37°C (Fig. 4). This adhesion-dependent decrease was slower, although equally extensive on cells incubated solely at 0–4°C, suggesting that receptors were not internalized but instead redistributed on the cell surface. When the cells were allowed to adhere at 18°C, the disappearance of surface sites showed intermediate kinetics (data not shown). The decrease in RHL binding at 0 min preincubation was due to receptor redistribution during the incubation with radioligand at 4°C (compare Fig. 3 *C*).

Adhesion to Gal-derivatized Gels Does Not Affect Cell Surface EGF Receptors

If the decrease in ^{125}I-GalBSA binding to hepatocytes incubated on Gal-derivatized gels was due to the movement of RHLs to the Gal-derivatized surface, the binding of unrelated hepatocyte surface receptors should be unaffected. This was the case for EGF receptors. Cells were incubated on carbohydrate-derivatized surfaces for up to 60 min at 37°C, then chilled to 0°C, ^{125}I-EGF (25 nM) or ^{125}I-GalBSA (12.5 nM) added for 60 min at 4°C, and binding was quantitated as described in Materials and Methods. While the RHLs on hepatocytes incubated on Gal-derivatized gels completely disappeared, EGF receptors were unaffected compared to control cells incubated on Glc-derivatized gels (Fig. 5).

Immunofluorescence Labeling of Adherent Hepatocytes

The surface distribution of RHLs on adherent rat hepatocytes was examined by indirect immunofluorescence microscopy. Hepatocytes were incubated on Gal-derivatized gels

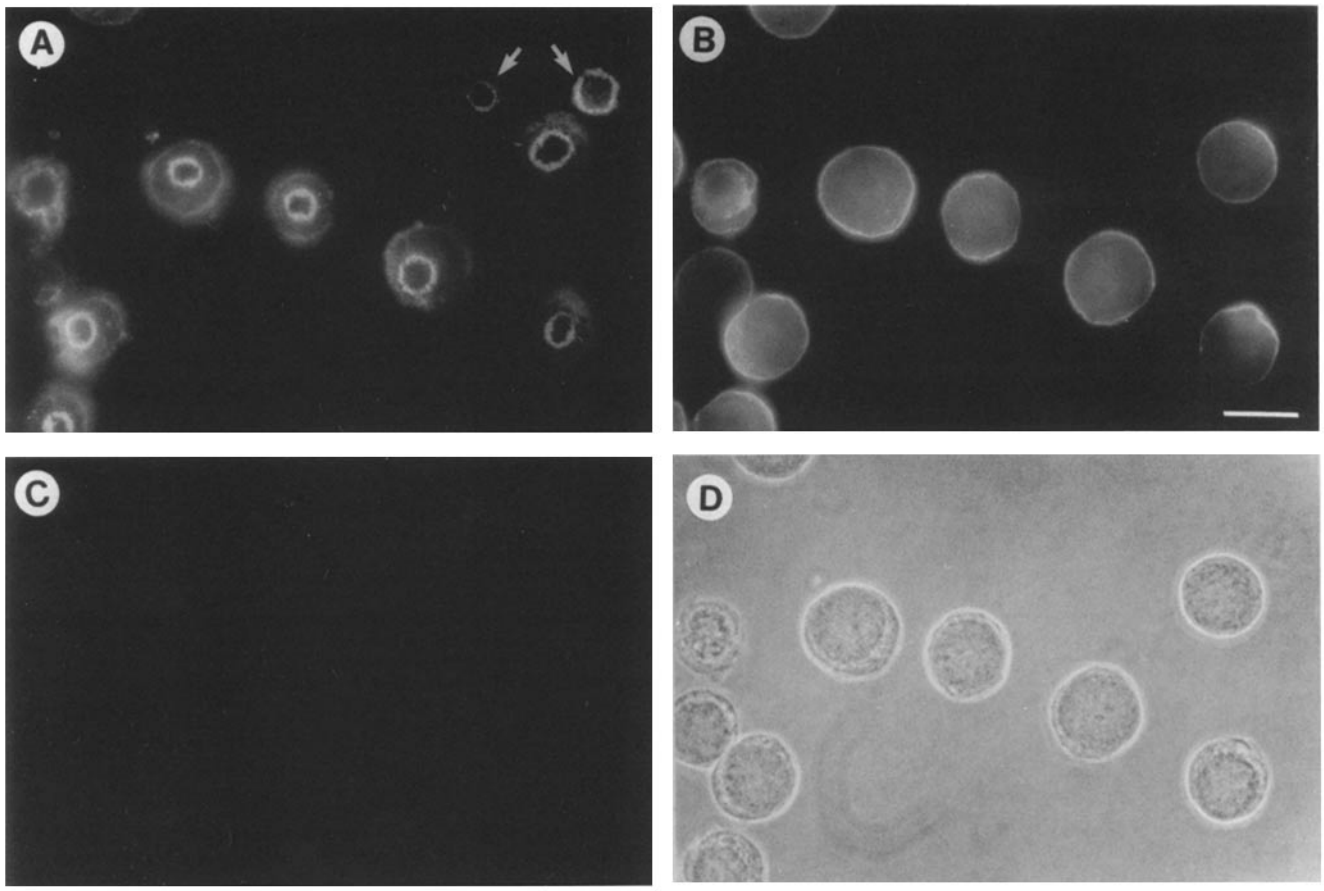

Figure 6. Immunohistochemical labeling of RHL reveals a structured ring at the site of adhesion to Gal-derivatized gels. Cells were incubated on Gal-derivatized gels for 1 h at 37°C, fixed, and subjected to indirect immunofluorescence staining using a polyclonal antibody directed against the RHL as described in Materials and Methods. (*A*) Fluorescence at the level of the derivatized surface; (*B*) Fluorescence at level of cell nucleus; (*C*) Nonspecific fluorescence (second antibody only); (*D*) Phase-contrast image at level of cell nucleus. Fluorescent structures associated with residual membrane patches are marked with arrows. Bar, 25 μm.

for 60 min at 37°C, fixed, and labeled with a polyclonal antibody directed against RHLs followed by FITC-conjugated second antibody. By phase microscopy most of the cells appeared intact, round, and refractile. When examined by immunofluorescence microscopy, the plasma membranes of the cells appeared uniformly lightly stained, except for the area surrounding the site of adhesion, where a brightly stained structured ring surrounding a circular area of exclusion was consistently visible (Fig. 6). Presumably, the area of exclusion contained receptors that were inaccessible to α-RHL antibody, surrounded by a limited ring of accessible epitopes. In addition, numerous brightly stained rings and patches were visible on areas of the gel surface that did not contain cells (Fig. 6 *A*, *arrows*). Some of these corresponded to membrane fragments when viewed by phase microscopy. We interpret these structures as adherent membrane patches left behind by cells sheared during fixation and staining. Cells stained with antibodies specific for RHL-1 or RHL-2/3 had indistinguishable staining patterns, suggesting that all RHL subtypes move to the Gal-derivatized surface during patching (data not shown).

Confocal microscopy was used to determine the three-dimensional distribution of antibody labeling of intact rat hepatocytes adhering to Gal-derivatized gels. After 1 h at 37°C, adherent cells were processed for indirect immunofluorescence using either α-RHL or α-EGF-receptor antibodies. The number of accessible RHLs remaining was quantitated on companion gels. Greater than 80% of the RHLs of these hepatocytes became inaccessible to ^{125}I-GalBSA (data not shown). Individual rat hepatocytes labeled with α-RHL antibody were optically sectioned by confocal microscopy to confirm that the structured ring surrounding the excluded area was indeed at the level of the gel surface (Fig. 7 *A*). In contrast, staining of adherent cells with a polyclonal antibody directed against the EGF receptor showed a uniform surface distribution (Fig. 7 *B*).

RHL Patching Does Not Block Endocytosis

RHL surface expression and recycling on carbohydrate-adherent cells were determined by quantitating ^{125}I-GalBSA endocytosis. As in Fig. 3 *A*, a sharp reduction (81%) in accessible receptors was detected when the cells were incubated on Gal-derivatized gels at 4°C (Fig. 8, 0 min). Surprisingly, upon warming to 37°C, the rate and extent of endocytosis by cells on Gal-derivatized gels was ~50% of that by cells on control (Glc-derivatized) gels (Fig. 8). Endocytosis by the adherent cells was relatively rapid, even though the steady-state number of accessible binding sites remained low during the 90-min incubation at 37°C (Fig. 8,

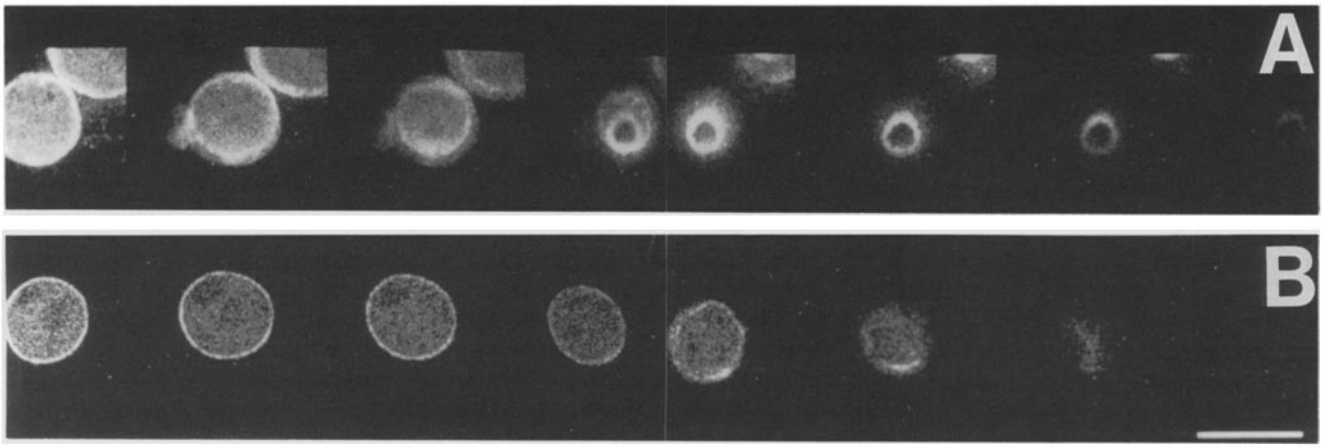

Figure 7. Serial optical sections of adherent cells labeled with anti-RHL and anti-EGF-receptor antibodies. Cells were incubated on Gal-derivatized gels for 1 h at 37°C, then fixed and processed for indirect immunofluorescence staining using α-RHL antibody (*A*) or α-EGF-receptor antibody (*B*) as described in Materials and Methods. The confocal effect was used to generate a series of 0.65-μm sections from the nuclear level (*left*) to the derivatized surface (*right*). Bar, 25 μm.

triangles). Under identical conditions, endocytosis of ^{125}I-EGF was unaffected by adhesion to Gal-derivatized gels (data not shown).

Quantitation of RHLs in the Adhesive Patch

The observation that cells depleted of most of their accessible cell surface RHLs endocytosed appreciable levels of ^{125}I-GalBSA suggested that most of the uptake was mediated by receptors recruited to the cell surface from intracellular pools. If newly exocytosed receptors could continually become incorporated into the adhesive patch in the absence of ligand, all receptors might eventually become associated with the patch on the derivatized surface. To test this possibility, we quantitated the RHLs in the patch after recovery of the cells from adhesive surfaces. Initial experiments demonstrated that adherent cells were difficult to recover intact after adhesion to Gal-derivatized gels at 37°C. This was the case even after the cells were incubated in CMF-PBS supplemented with EDTA (data not shown). Therefore, gels were derivatized with aminohexyl glycosides using the reversible (disulfide-containing) acrylic immobilization linker, AEMAS (26). Cells (2×10^5 cells in 0.5 ml DMEM-H)

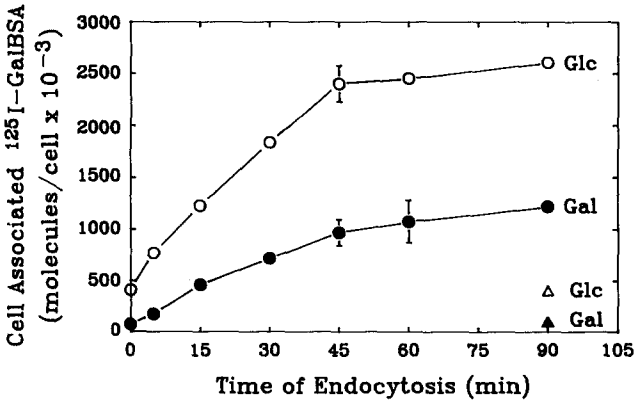

Figure 8. Kinetics of ^{125}I-GalBSA endocytosis after RHL patching. Cells were preincubated on Gal- or Glc-derivatized gels for 60 min at 37°C before chilling and addition of ^{125}I-GalBSA (12.5 nM). Endocytosis was initiated by returning the chambers to 37°C for the indicated times. The triangles represent cell surface binding sites quantitated at 4°C after a 90-min preincubation at 37°C in the absence of ligand. Similar results were obtained in two other experiments.

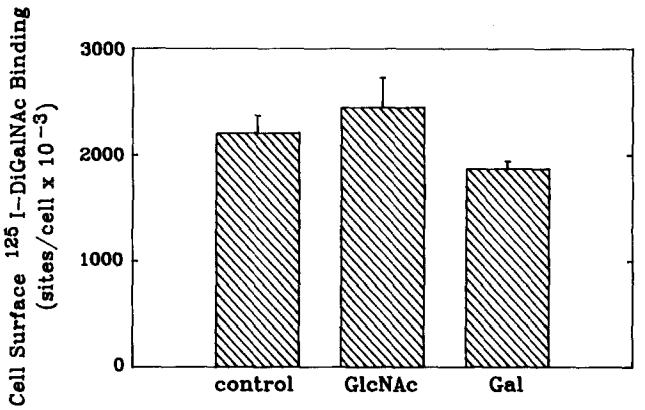

Figure 9. Quantitation of RHLs on cells recovered after adhesion to carbohydrate-derivatized gels. Cells (2×10^5 in 0.5 ml DMEM-H) were incubated for 1 h at 37°C on gels derivatized with aminohexyl β-Gal or β-GlcNAc using the disulfide-containing acrylic linker AEMAS, then rapidly chilled on ice. Cells were recovered from gels as follows. CMF-HBS, pH 7.6, containing 10 mM DTT and 10 mM EDTA (0.5 ml/well) was added and the cells were incubated for 60 min at 0°C. The cells were removed by agitation, transferred to a test tube, collected by centrifugation, washed once in CMF-HBS containing 5 mM EDTA, centrifuged through Percoll (15), resuspended in 0.5 ml of DMEM-H, and surface ^{125}I-DiGalNAc binding sites measured in suspension at 4°C as follows. An aliquot (25 μl) of the radioligand (0.55 μM) was added and the suspension gently agitated for 60 min at 4°C. A portion of the suspension (400 μl) was added to 9 ml of ice-cold PBS, the cells collected by centrifugation and resuspended in 1 ml of 0.5% Triton X-100 in 0.1 M sodium phosphate buffer, pH 7. Radioactivity and cell recovery were determined as described in Materials and Methods. Control cells were treated identically except that they were incubated at 37°C in rotation culture for 1 h instead of on gels before addition of DTT/EDTA solution.

were incubated at 37°C for 60 min on gels derivatized with Gal or GlcNAc using AEMAs as linker. After chilling to 0°C, cells were recovered from gels by addition of 10 mM DTT and 10 mM EDTA in 0.5 ml CMF-HBS, pH 7.6. After 60 min at 0°C, cells were removed by mild trituration, collected by centrifugation, then washed with 5 mM EDTA in CMF-HBS. Viable cells were recovered by centrifugation through Percoll as described in Materials and Methods and resuspended in ice-cold DMEM-H. This procedure resulted in recovery of >50% of the cells originally added to the gels. Control cells were incubated in rotation culture for 60 min at 37°C, chilled, treated with the same DTT/EDTA solution, washed, and ligand binding was determined in parallel with recovered adherent cells. Since cells recovered from Gal-derivatized gels would be likely to have closely clustered RHLs, steric factors might be expected to reduce binding of the macromolecular ligand, ^{125}I-GalBSA. Therefore, in these experiments RHLs were quantitated using an engineered high-affinity (4 nM) low molecular weight ligand referred to as Di-GalNAc (16). Cells recovered from Gal-derivatized or control (GlcNAc-derivatized) surfaces, and control cells were incubated with ^{125}I-Di-GalNAc at 4°C for 60 min, then washed by centrifugation in PBS and bound ligand quantitated. Cells preincubated on Gal-derivatized gels had comparable (84%) surface receptors to control cells (in suspension) or to those incubated on GlcNAc-derivatized surfaces (Fig. 9). The observation that surface receptor number did not increase due to cell adhesion to Gal-derivatized gels suggests kinetic or physical restraints on the size or receptor density of the adhesive patch.

Discussion

Adhesion-dependent Surface RHL Redistribution

The rapid disappearance of accessible RHLs on the surface of hepatocytes adherent to Gal-derivatized gels under all conditions tested (including 0°C and in the presence of metabolic, endocytic, or cytoskeletal perturbants) suggests that movement of lectins to the derivatized surface proceeds via unrestricted lateral diffusion in the plane of the membrane followed by kinetic "trapping" at the highly multivalent derivatized surface. The rate of receptor disappearance (Figs. 4 and 5) can be used to estimate the minimum lateral diffusion coefficient (D_L) of RHLs at 0°C and 37°C. The diffusion of randomly distributed RHLs to the adhesive patch, where they become irreversibly bound (within the time frame of our experiments), can be treated mathematically as a "diffusion to capture" problem (2), a formal mathematical treatment of which is given in the Appendix (with isolated hepatocytes treated as spheres, a reasonable approximation). Given (*a*) an average hepatocyte radius of 12.5 μm; (*b*) an average radius of the adhesive patch of 6.8 μm (35); and (*c*) an average time to capture (time to half-maximal disappearance of receptors) of <50 min at 0°C and <5 min at 37°C, we calculate the D_L to be $\geq 9 \times 10^{-10}$ cm^2/s at 0°C and $\geq 9 \times 10^{-9}$ cm^2/s at 37°C. These values are comparable to the most readily diffusible proteins, and approach the D_L for the lipids in the bilayer (14). We conclude that RHLs diffuse too readily to be associated with the submembraneous cytoskeleton during their movement to the adhesive surface. Upon migration to that surface, however, the RHLs rapidly associate with cytoskeletal components as evidenced by indirect immunofluorescence and by inhibition of adhesion strengthening by cytoskeletal perturbants (35).

Indirect immunofluorescence labeling using α-RHL antibody on cells adhering to Gal-derivatized surfaces revealed a ring-like structure of characteristic size and shape surrounding an area of exclusion (Fig. 6). These ringlike structures delineate the periphery of a "patch" of receptors largely inaccessible to antibody or ^{125}I-GalBSA on intact cells. Residual membrane patches apparently left behind by sheared cells were also labeled. In many cases the residual membrane patches were uniformly labeled, suggesting that antibody had greater access to this "patch" once the cells were removed. The remainder of the plasma membrane on intact cells was only lightly stained, suggesting that most surface RHLs were sequestered in the "patch." In contrast, α-EGF-receptor antibody staining of adherent cells was uniform, suggesting that EGF receptor remained randomly distributed in the plasma membrane of adherent cells. The mean area bounded by the outer edge of α-RHL antibody staining of adherent hepatocytes was 146 μm^2, representing ~7.5% of the surface area of a 25-μm diameter spherical hepatocyte. Assuming the area occupied by an RHL molecule (molecular mass is ~50 kD) is 50 nm^2, the patch could hold up to 2.8×10^6 ideally packed RHLs. Up to 1.3 million high-affinity cell surface binding sites were revealed by binding of the low molecular weight ligand ^{125}I-Di-GalNAc. This suggests that the adhesive patch is relatively saturated with RHLs. In the only previous study published on this subject, intact adherent cells were removed from Gal-derivatized surfaces with EGTA, then labeled with FITC-conjugated ASOR (32). The average patch size reported was only 4 μm^2, corresponding to 0.2% of the cell surface. This much smaller area may have been due to shearing of the cells from the adhesive surface or by diffusion of receptors out of the patch during cell recovery.

Endocytosis of ^{125}I-GalBSA by Adherent Cells

Adherent cells with few accessible surface ^{125}I-GalBSA binding sites still endocytosed radioligand at ~50% of the rate of nonadherent cells (Fig. 8). Since surface binding sites (measured at 4°C) remained inaccessible on cells that had been preincubated for 90 min at 37°C, we conclude that intracellular pools of RHL, which are known to recycle rapidly to the cell surface (29), most likely mediated the observed ligand uptake. These data do not rule out an equilibrium between the intracellular pool and receptors in the patch.

RHLs in the Adhesive Patch

The significant rate of endocytosis on adherent cells suggested that at steady state, many of the cell's RHLs were not associated with the adhesive patch. To investigate this directly, we measured surface ligand binding on cells recovered (at 4°C) after adhesion to Gal-derivatized gels for 60 min at 37°C, during which time net migration of RHLs to the surface might be expected to occur. Surprisingly, we found no net increase in surface RHLs due to hepatocyte adhesion to the Gal-derivatized surfaces. This implied that the number of receptors associated with the adhesive patch was limited either physically or kinetically. Data reported in the accompanying paper demonstrate the generation of a

submembraneous cytoskeletal organization which apparently limits the size of the adhesive patch.

Appendix

The following solution for diffusion to capture on the surface of a sphere was proposed by Dr. Howard Berg (Harvard University) (2) and solved by Dr. Alfredo Torruella (University of Puerto Rico) with contributions from Dr. Dennis Noe (The Johns Hopkins School of Medicine), to whom we are indebted. Analytical expressions have been reported (5, 30) for diffusion to capture which agree closely with our solution, especially for a small adhesion patch.

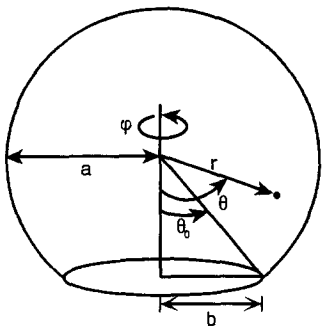

Figure A1.

A spherical hepatocyte of radius a is adherent to a surface via a circular contact area (adhesion patch) of radius b (see Fig. A1). A receptor on the surface of the hepatocyte at a position defined by ray r with length a and coordinates (θ,ϕ) diffuses randomly until it contacts the adhesion patch ($\theta = \theta_0$), at which time it is irreversibly captured. By arguments set forth in Berg and Purcell (2), Equation A1 defines the relationship between W and D_L, where W is the mean of all the observed times-to-capture for a large number of receptors, D_L is the lateral diffusion coefficient, and ∇^2 is the Laplacian in spherical coordinates.

$$\nabla^2 W = -\frac{1}{D_L} \tag{A1}$$

For the geometry outlined in Fig. A1, the Equation A2 applies:

$$\left(\frac{1}{\sin\theta}\right)\frac{\partial}{\partial\theta}\left(\sin\theta\frac{\partial W}{\partial\theta}\right) + \left(\frac{1}{\sin^2\theta}\right)\frac{\partial^2 W}{\partial\varphi^2} = -\frac{a^2}{D_L}. \tag{A2}$$

With the boundary condition $W = 0$ at $\theta = \theta_0$ (e.g., at capture). Since neither the source term nor the boundary condition involves ϕ, $W = W(\phi)$ and Equation A2 reduces to A3

$$\left(\frac{1}{\sin\theta}\right)\frac{\partial}{\partial\theta}\left(\sin\theta\frac{\partial W}{\partial\theta}\right) = -\frac{a^2}{D_L}. \tag{A3}$$

Equation A3 integrates to:

$$W(\theta) = \frac{a^2}{D_L}\int_{\theta_0}^{\theta}\cot\theta d\theta + A\int_{\theta_0}^{\theta}\frac{d\theta}{\sin\theta}, \tag{A4}$$

where A is a constant of integration. Integrating Equation A4 gives Equation A5:

$$W(\theta) = \frac{a^2}{D_L}\ln\left(\frac{\sin\theta}{\sin\theta_0}\right) + A\ln\left(\left(\frac{1-\cos\theta}{\sin\theta}\right)\left(\frac{\sin\theta}{1-\cos\theta_0}\right)\right). \tag{A5}$$

This reduces to Equation A6:

$$W(\theta) = \frac{a^2}{D_L}\ln\left(\frac{\sin\theta}{\sin\theta_0}\right) - A\ln\left(\frac{\sin\theta}{\sin\theta_0}\right) + A\ln\left(\frac{1-\cos\theta}{1-\cos\theta_0}\right). \tag{A6}$$

Since the solution must be finite at $\theta = \pi$, $A = a^2/D_L$ to cancel the singular log (sin θ) terms, giving Equation A7, where $\theta_0 \leqslant \theta \leqslant \pi$:

$$W(\theta) = \frac{a^2}{D_L}\ln\left(\frac{1-\cos\theta}{1-\cos\theta_0}\right). \tag{A7}$$

The average of W over the surface $\theta \geqslant \theta_0$ gives the average time to capture, $\bar{t}_c$.

$$\bar{t}_c = \frac{1}{B}\int_0^{2\pi}\int_{\theta_0}^{\pi} W(\theta)a^2\sin\theta d\theta d\varphi; \tag{A8}$$

$$\bar{t}_c = \left(\frac{2\pi a^2}{B}\int_{\theta_0}^{\pi}[1n(1-\cos\theta) - 1n(1-\cos\theta_0)]\sin\theta d\theta\right)\frac{a^2}{D_L}; \tag{A9}$$

and finally,

$$\bar{t}_c = \left(\frac{2\pi a^2}{B}(21n2 - 2 - 21n(1-\cos\theta_0) + (1-\cos\theta_0))\right)\frac{a^2}{D_L}. \tag{A10}$$

Where B equals the surface area of the cell outside of the patch, $2\pi a^2(1 + \cos\theta_0)$. Since a, b, and $\bar{t}_c$ are experimentally determined, and θ_0 = arcsine (b/a), D_L can be calculated from Equation A10.

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References

1. Ashwell, G., and J. Harford. 1982. Carbohydrate-specific receptors of the liver. *Annu. Rev. Biochem.* 51:531–554.
2. Berg, H. C., and E. M. Purcell. 1977. Physics of chemoreception. *Biophys. J.* 20:193–219.
3. Bischoff, J., S. Libresco, M. A. Shia, and H. F. Lodish. 1988. The H1 and H2 polypeptides associate to form the asialoglycoprotein receptor in human hepatoma cells. *J. Cell Biol.* 106:1067–1074.
4. Brandley, B. K., and R. L. Schnaar. 1985. Phosphorylation of extracellular carbohydrates by intact cells: chicken hepatocytes specifically adhere to and phosphorylate immobilized N-acetylglucosamine. *J. Biol. Chem.* 260:12474–12483.
5. Chao, N. M., S. H. Young, and M. M. Poo. 1981. Localization of cell membrane components by surface diffusion into a "trap." *Biophys. J.* 36: 139–153.
6. Connolly, D. T., R. R. Townsend, K. Kawaguchi, M. K. Hobish, W. R. Bell, and Y. C. Lee. 1983. Binding and endocytosis of glycoproteins and neoglycoproteins by isolated rabbit hepatocytes. *Biochem. J.* 214:421–431.
7. Drickamer, K. 1988. Two distinct classes of carbohydrate-recognition domains in animal lectins. *J. Biol. Chem.* 263:9557–9560.
8. Drickamer, K., J. F. Mamon, G. Binns, and J. O. Leung. 1984. Primary structure of the rat liver asialoglycoprotein receptor. *J. Biol. Chem.* 259:770–778.
9. Dulbecco, R., and M. Vogt. 1954. Plaque formation and isolation of pure lines with poliomyelitis viruses. *J. Exp. Med.* 99:167–182.
10. Dunn, W. A., T. P. Connolly, and A. L. Hubbard. 1986. Receptor-mediated endocytosis of epidermal growth factor by rat hepatocytes: receptor pathway. *J. Cell Biol.* 102:24–36.
11. Guarnaccia, S. P., and R. L. Schnaar. 1982. Hepatocyte adhesion to immobilized carbohydrates. I. Sugar recognition is followed by energy-dependent strengthening. *J. Biol. Chem.* 257:14288–14292.
12. Halberg, D. F., R. E. Wager, D. C. Farrell, J. Hildreth, IV, M. S. Quesenberry, J. A. Loeb, E. C. Holland, and K. Drickamer. 1987. Major and minor forms of the rat liver asialoglycoprotein receptor are independent galactose-binding proteins. *J. Biol. Chem.* 262:9828–9838.
13. Herzig, M. C. S., and P. H. Weigel. 1990. Surface and internal galactosyl receptors are heterooligomers and retain this structure after ligand internalization or receptor modulation. *Biochemistry.* 29:6437–6447.
14. Houslay, M. D., and K. K. Stanley. 1982. Dynamics of biological membranes. John Wiley and Sons, Chichester. 330 pp.
15. Kreamer, B. L., J. L. Staecker, N. Sawada, G. L. Sattler, M. T. S. Hsia, and H. C. Pitot. 1986. Use of a low-speed, iso-density Percoll centrifugation method to increase the viability of isolated rat hepatocyte preparations. *In Vitro Cell. Dev. Biol.* 22:201–211.
16. Lee, R. T., and Y. C. Lee. 1987. Preparation of cluster glycosides of N-acetylgalactosamine that have subnanomolar binding constants towards the mammalian hepatic Gal/GalNAc-specific receptor. *Glycoconjugate J.* 4:317–328.
17. Lee, Y. C., C. P. Stowell, and M. J. Krantz. 1976. 2-Imino-2-methoxyethyl 1-thioglycosides: New reagents for attaching sugars to pro-

teins. *Biochemistry.* 15:3956–3963.
18. McLean, I. W., and P. K. Nakane. 1974. Periodate-lysine-paraformaldehyde fixative. A new fixative for immunoelectron microscopy. *J. Histochem. Cytochem.* 22:1077–1083.
19. Mueller, S. C., and A. L. Hubbard. 1986. Receptor-mediated endocytosis of asialoglycoproteins by rat hepatocytes: receptor-positive and receptor-negative endosomes. *J. Cell Biol.* 102:932–942.
20. Obrink, B., M. S. Kuhlenschmidt, and S. Roseman. 1977. Adhesive specificity of juvenile rat and chicken liver cells and membranes. *Proc. Natl. Acad. Sci. USA.* 74:1077–1081.
21. Oka, J. A., and P. H. Weigel. 1986. Binding and spreading of hepatocytes on synthetic galactose culture surfaces occur as distinct and separable threshold responses. *J. Cell Biol.* 103:1055–1060.
22. Pless, D. D., Y. C. Lee, S. Roseman, and R. L. Schnaar. 1983. Specific cell adhesion to immobilized glycoproteins demonstrated using new reagents for protein and glycoprotein immobilization. *J. Biol. Chem.* 258:2340–2349.
23. Rice, K. G., O. A. Weisz, T. Barthel, R. T. Lee, and Y. C. Lee. 1990. Defined geometry of binding between triantennary glycopeptide and the asialoglycoprotein receptor of rat hepatocytes. *J. Biol. Chem.* 265: 18429–18434.
24. Sawyer, J. T., J. P. Sanford, and D. Doyle. 1988. Identification of a complex of the three forms of the rat liver asialoglycoprotein receptor. *J. Biol. Chem.* 263:10534–10538.
25. 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.
26. Schnaar, R. L., B. G. Langer, and B. K. Brandley. 1985. Reversible covalent immobilization of ligands and proteins on polyacrylamide gels. *Anal. Biochem.* 151:268–281.
27. Schwartz, A. L., A. Marshak-Rothstein, D. Rup, and H. F. Lodish. 1981. Identification and quantification of the rat hepatocyte asialoglycoprotein receptor. *Proc. Natl. Acad. Sci. USA.* 78:3348–3352.
28. Seglen, P. O. 1976. Preparation of isolated rat liver cells. *Methods Cell Biol.* 13:29–83.
29. Spiess, M. 1990. The asialoglycoprotein receptor: a model for endocytic transport receptors. *Biochemistry.* 29:10009–10018.
30. Weaver, D. L. 1983. Diffusion-mediated localization on membrane surfaces. *Biophys. J.* 41:81–86.
31. Weigel, P. 1980. Characterization of the asialoglycoprotein receptor on isolated rat hepatocytes. *J. Biol. Chem.* 255:6111–6120.
32. Weigel, P. H. 1980. Rat hepatocytes bind to synthetic galactoside surfaces via a patch of asialoglycoprotein receptors. *J. Cell Biol.* 87:855–861.
33. 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.
34. 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.
35. Weisz, O. A., and R. L. Schnaar. 1991. Hepatocyte adhesion to carbohydrate-derivatized surfaces. II. Regulation of cytoskeletal organization and cell morphology. *J. Cell Biol.* 115:495–504.