Hepatocyte Adhesion to Carbohydrate-Derivatized Surfaces II. Regulation of Cytoskeletal Organization and Cell Morphology

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Abstract. Rat hepatic lectins mediate adhesion of isolated rat hepatocytes to synthetic surfaces derivatized with galactosides. Initial weak adhesion is followed by rapid adhesion strengthening. After hepatocytes contact galactose-derivatized gels, the hepatic lectins move rapidly into an inaccessible patch at the adhesive surface (Weisz, O. A., and R. L. Schnaar. 1991. *J. Cell Biol.* 115:485–493). Hepatic lectin patching, which occurs both at 37°C and 4°C, is not responsible for adhesion strengthening, which does not occur at 4°C. Of various cytoskeletal and metabolic perturbants tested, only a combination of hyperosmotic medium, colchicine, and cytochalasin caused a marked (72%) reduction of adhesion strengthening (without reducing weak cell adhesion). Clathrin and actin were readily detected in the adhesive patch by immunofluorescence microscopy.

Rat hepatocytes also adhered avidly to surfaces derivatized with asialofetuin, a high-affinity ligand for the rat hepatic lectins. However, hepatic lectin molecules did not migrate into a patch on the asialofetuin-derivatized surface, suggesting that hepatic lectin-asialofetuin binding may have resulted in the rapid formation of a ring of essentially irreversibly adherent receptors that prevented diffusion of additional lectin molecules into the contact site. The cells were unable to increase their adhesive contact area by flattening onto the derivatized surface. Treatment of cells with cytochalasin, however, did result in an increase in the size of the contact area. Cells adhering to surfaces derivatized with an adhesion-promoting peptide (containing an arg-gly-asp sequence) had larger contact areas than those adhering to galactoside-derivatized surfaces. A model is proposed in which carbohydrate-mediated adhesion causes specific reorganization of cytoskeletal components, leading to strengthened adhesion and a characteristic spherical cell morphology.

RAT hepatocytes adhere specifically to synthetic galactose-(Gal)[1]-derivatized polyacrylamide surfaces via the rat hepatic lectins (RHLs). During adhesion to Gal-derivatized gels, RHLs rapidly concentrate in a patch at the adhesion site (34). We characterized two steps involved in adhesion of rat hepatocytes to Gal-derivatized surfaces: initial weak adhesion followed by rapid temperature-dependent strengthening (9). The observation that RHLs migrate to the adhesive site on Gal-derivatized gels suggested that strengthening might be explained by an increase in the number of binding sites in the adhesion patch. However, at 4°C no strengthening was observed (9), even though there was complete movement of receptors to the Gal-derivatized surface (34). Conversely, cells incubated on Gal-derivatized gels at 23°C adhered as strongly as cells incubated at 37°C, although they had only 64% as many surface receptors. These data suggest that adhesion strengthening is not directly related to the number of receptors recruited to the adhesive site. To test whether components of the submembranous cytoskeleton are responsible for adhesion strengthening, we examined the effects of cytoskeletal and metabolic perturbants on adhesive strengthening, as well as on the expression and topography of the hepatic lectin. As a complementary approach, cytoskeletal components were localized on adherent membrane patches by immunohistochemistry. These results, in many ways, confirm and extend previous observations of cytoskeletal involvement in cell adhesion (8).

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; ASF, asialofetuin; Gal, galactose; GlcNAc, N-acetylglucosamine; RGD, arginyl-glycyl-aspartic acid; RHL, rat hepatic lectin.

Materials and Methods

General

Hepatocytes were isolated by in situ collagenase perfusion of rat liver essentially as described previously (22, 31). Polyacrylamide gel disks were derivatized with aminohexyl galactoside or aminohexyl N-acetylglucosaminide using the N-succinimidyl ester of acrylaminohexanoic acid (3). Weak and strong cell adhesion to derivatized gels were quantitated using an assay which applied a defined centrifugal force to remove nonadherent cells (9, 34). The rat hepatic lectins were quantitated on hepatocytes in suspension or adherent to derivatized gels using the synthetic, high-affinity ligand ^{125}I-Gal$_{39}$BSA (GalBSA) as reported previously (34). Asialofetuin (ASF) and asialoagalactofetuin were prepared as described (12) and deriva-

Table I. Effect of Temperature, Cytoskeletal Perturbants, and Metabolic Inhibitors on Hepatocyte Adhesion to Gal-derivatized Gels and on Cell Surface RHL Expression

Perturbant	Incubation temperature	Cell adhesion: Strong adhesion (250–500 *g*)	Cell adhesion: Weak adhesion (1 *g*)	^{125}I-GalBSA bound: Surface binding sites
	°C	(% of 37°C control)		(% of control)
None	4	−1 ± 1*	114 ± 12	
None	37	100	100	100
HO	37	65* ± 5	102 ± 2	52 ± 3
CO	37	73* ± 5	106 ± 4	48 ± 1
Cyto D	37	73 ± 9	128 ± 6	143 ± 13
HO/CY	37	71 ± 5	ND	62 ± 1
CY/CO	37	66 ± 2	ND	63 ± 2
HO/CO	37	62 ± 9	ND	15 ± 3
HO/CY/CO	37	28* ± 5	112 ± 3	16 ± 1
Monensin	37	63* ± 7	103 ± 2	56 ± 2
Vanadate	37	84* ± 4	109 ± 6	51 ± 1
Rot/Oligo	37	62* ± 7	103 ± 3	64 ± 3

Cells were preincubated with or without the indicated perturbants (singly or in concert) as described in Materials and Methods, then placed on Gal-derivatized gels for 45 min at the indicated temperature (*HO*, hyperosmotic medium; *CO*, colchicine; *CY*, cytochalasin *D*; *Rot*, rotenone; *Oligo*, oligomycin). At the end of the incubation, cells were subjected to a detachment force of 250–500 *g* (strong adhesion) or 1 *g* (weak adhesion) and cell adhesion was quantitated. Specific adhesion at 37°C, the percentage of adherent untreated cells minus background adhesion (measured in the presence of 25 mM GalNAc), was normalized to 100%. Asterisks denote strong adhesion values which were significantly different from control ($p < 0.05$) using statistical paired analysis. Strong specific adhesion averaged 55% with a background of 12% ($n = 22$). Weak specific adhesion averaged 70% with a background of 21% ($n = 4$). Cell surface RHL concentration on unattached cells was determined by binding ^{125}I-GalBSA as described in Materials and Methods and normalized to binding sites on control (untreated) cells. Surface ^{125}I-GalBSA binding sites on control cells averaged 5.3×10^5 sites/cell ($n = 8$). Values are the means ± SEM for triplicate or quadruplicate determinations from 1–8 separate experiments.

tized on activated polyacrylamide surfaces (25) using AEMAS as linker (30). Preparation of gels coderivatized with a synthetic nonapeptide containing the arg-gly-asp (RGD) adhesion sequence and either aminohexyl galactoside or aminohexyl N-acetylglucosaminide was as described (5).

Cell Treatment with Cytoskeletal Perturbants

Stocks were prepared as follows (chemicals obtained from Sigma Chemical Co., St. Louis, MO unless indicated). Cytochalasin D (1 mM), monensin (1 mM), and colchicine (0.8 mM) were prepared in DMSO; rotenone (0.4 mg/ml) was prepared in 100% ethanol; and oligomycin was prepared (2 mg/ml) in 75% ethanol. Stocks were stored at −20°C. Sodium vanadate, ortho (Fischer Scientific, Fair Lawn, NJ) was prepared fresh as a 100-mM stock in 100 mM Hepes acid, and the pH (of the supplemented medium) adjusted to 7.4. Hyperosmotic medium (500 mOsm) was prepared by adding a concentrated sucrose solution to twofold concentrated Hepes-buffered DME (DMEM-H; [17]).

Freshly isolated rat hepatocytes were preincubated in rotation culture for 60 min at 37°C, then centrifuged and resuspended in control medium or in medium containing cytochalasin B (10 μM final concentration), cytochalasin D (1 μM), monensin (25 μM), colchicine (4 μM), vanadate (5 mM), or rotenone (2 μM) and oligomycin (2 μg/ml). Cells treated with hyperosmotic medium were incubated in DMEM-Hepes for 50 min in rotation culture at 37°C, then centrifuged, resuspended in hyperosmotic medium, and returned to the rotating shaker for an additional 10 min before use (17, 24). Cells treated with the other agents were incubated in rotation culture for 60 min at 37°C, then centrifuged and resuspended in fresh medium containing the desired agent(s).

Immunofluorescence Microscopy

Affinity-purified polyclonal α-RHL antibody (19) was generously provided by Dr. Ann Hubbard (The Johns Hopkins School of Medicine). Anticlathrin antibody (monoclonal IgM raised against bovine brain clathrin) was from Boehringer Mannheim Corp. (Indianapolis, IN). Indirect immunofluorescence microscopy on intact cells was performed as described (34). For detection of actin, cells were fixed with 2% glutaraldehyde, permeabilized (7) with 0.005% digitonin in Dulbecco's PBS (6) for 15 min, then rinsed three times with PBS and incubated for 15 min with rhodamine phalloidin. The gels were then washed three times with PBS, the solution was removed, and 15 mg/ml Dabco (Aldrich Chemical Co., Milwaukee, WI) in PBS:glycerol (3:1) was added. After transferring to a microscope slide, the gels were overlaid with a drop of glycerol and a coverslip. Cells were viewed using a Zeiss Axioplan Universal Microscope. Morphometric analyses were performed on photomicrographs using the computer program SigmaScan (Jandel Scientific, Sausalito, CA).

Results

Effect of Cytoskeletal Perturbants on the Strengthening of Hepatocyte Adhesion to Gal-derivatized Surfaces

Hepatocytes were incubated on Gal-derivatized gels at 37°C or at 4°C for 45 min, then subjected to weak (1 *g*) or strong (250–500 *g*) detachment forces before quantitating adherent cells. As reported previously (9), cells incubated at 37°C adhered strongly to Gal-derivatized gels, while those incubated at low temperature adhered only weakly (Table I). To determine whether cytoskeletal elements were required for adhesive strengthening, cells were treated with various perturbants or combinations of perturbants, before and during incubation on the derivatized surfaces. Single perturbants caused modest but significant reductions in strong cell adhesion. Treatment with pairs of perturbants produced a similar decrease in strengthening, while combined treatment with the three direct structural perturbants (hyperosmotic medium, cytochalasin D, and colchicine) caused a marked reduction in strong adhesion. In contrast, none of the treatments (including the combination of three perturbants) affected weak adhesion to Gal-derivatized gels, suggesting that they did not compromise cell viability or RHL binding to the derivatized surface (Table I).

Several of the perturbants have been reported to modulate surface RHL expression (11, 13, 18). However, the surface expression of RHLs (measured as ^{125}I-GalBSA binding sites) did not correlate with adhesive strengthening (Table I). Cells

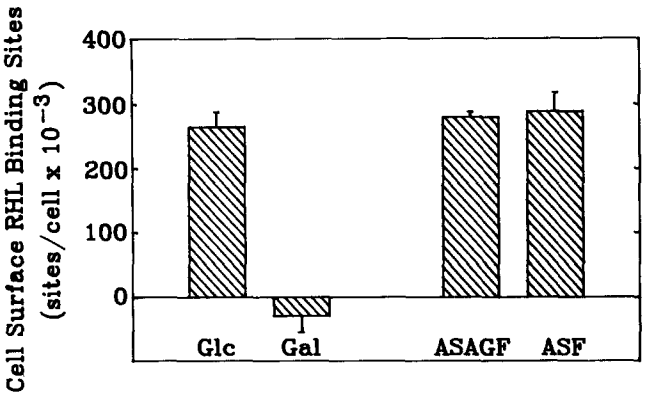

Figure 1. Adhesion to ASF-derivatized gels does not decrease accessible surface RHLs. Cells were incubated on gels derivatized with the indicated ligands for 45 min at 37°C, then rapidly chilled, and ^{125}I-GalBSA (12.5 nM) was added to measure accessible cell surface binding sites (34). ASAGF, asialoagalacto-fetuin.

treated with a combination of hyperosmotic medium and colchicine reduced RHL surface expression by 85%, yet strong adhesion was only modestly reduced (by 38%). Conversely, cells treated with cytochalasin D expressed 43% more surface ^{125}I-GalBSA binding sites than control cells, but had decreased strengthening.

To test whether the perturbants affected the ability of surface RHLs to accumulate as a patch at the site of cell adhesion to Gal-derivatized gels, cells were pretreated with the agents, then incubated on gels for 45 min at 37°C, rapidly chilled, and the RHLs remaining accessible were probed with ^{125}I-GalBSA (34). In all cases, >90% of the preexisting cell surface receptors became inaccessible to ^{125}I-GalBSA binding when cells were incubated on Gal-derivatized gels, suggesting that the rapid lateral diffusion of surface receptors to the adhesive surface is neither energy nor cytoskeleton dependent (data not shown).

Hepatocyte Adhesion to Asialofetuin-derivatized Gels

The observation that adhesion strengthening was not directly related to the number of lectin molecules in contact with the adhesive surface was confirmed by an unexpected observation made using gels derivatized with asialofetuin. ASF is a glycoprotein with branched galactose-terminated glycosylation sites optimally spaced for high-affinity binding to RHLs (33). Derivatization of polyacrylamide gel surfaces was designed to generate a dense monolayer of immobilized ASF (25). Calculations based on immobilization of ^{125}I-ASF (data not shown) suggest a density of $>2 \times 10^4$ glycoprotein molecules/μm^2 at the gel surface.

Hepatocytes adhered with equal strength to ASF-derivatized and Gal-derivatized gels, with 48 and 46% of input cells resisting a detachment force of 250 *g*, respectively. The areas of the adhesion sites (detected after immunohistochemical staining, see below) were also remarkably similar, both being uniform and circular with a mean contact area of 140 μm^2 for ASF-adherent cells and 150 μm^2 for Gal-adherent cells. However, when accessible surface RHLs were measured on the adherent cells the results were distinctly different. Whereas RHLs on hepatocytes adhering to Gal-derivatized gels had migrated into an adhesive patch (34) such that no ^{125}I-GalBSA binding sites remained accessible, the number of available RHLs on cells adhering to ASF-derivatized gels was not measurably reduced (Fig. 1), even though ample glycoprotein was available for binding all surface RHLs.

Two possible explanations were apparent to explain the lack of disappearance of ^{125}I-GalBSA binding sites on cells adherent to ASF-derivatized gels. The first was that receptors had actually "patched" on ASF-derivatized gels, but that the ^{125}I-GalBSA probe penetrated the adhesive patch and bound to the clustered receptors. However, a concentration of GalBSA (25 nM) far above its K_D (0.5 nM) did not reverse strong adhesion to ASF-derivatized gels (data not shown), suggesting that the macromolecular ligand did not penetrate the adhesion patch. A second possibility was that RHLs on the hepatocyte surface bound the immobilized ASF with such high affinity that they became essentially irreversibly bound and formed a barrier that excluded diffusion of additional cell surface RHLs into the adhesive patch. Since the area of the adhesive patch is only ~7% of the total cell surface (34), >90% of the RHLs would be expected to remain accessible.

The presence of RHLs outside of the adhesion site on ASF-adherent cells was confirmed using a probe for RHLs that could not penetrate the "patch," desialylated erythrocytes. PBS-washed sheep erythrocytes were incubated with 0.2 mU/ml of *Vibrio cholera* neuraminidase (Calbiochem, San Diego, CA) for 2 h at 37°C, then washed with PBS and suspended in DMEM-H. Desialylated erythrocytes bound avidly to hepatocytes via a GalNAc-inhibitable mechanism, but were much too large to penetrate the adhesion site. Hepatocytes were incubated on Gal- or ASF-derivatized gels at 37°C, chilled, neuraminidase-treated erythrocytes were added, the incubation was continued at 0°C for 30 min, and the adherent cells were examined microscopically. While Gal-adherent hepatocytes did not bind erythrocytes (due to the patching of the RHLs), all of the hepatocytes adhering to ASF-derivatized gels were extensively decorated with the desialylated erythrocytes (Fig. 2).

The observation that hepatocytes adhering to ASF-derivatized gels had most of their RHLs available on the cell surface outside of the adhesion site, yet did not flatten to bring more of the lectin molecules in contact with the derivatized surface led us to hypothesize that carbohydrate-mediated adhesion generated a cytoskeletal structure that imposed restrictions on cell shape. This led to several testable questions. (*a*) Are cytoskeletal elements localized at the site of adhesion? (*b*) Do cytoskeletal perturbants modify the morphology of the adherent cells or the adhesive membrane patch? (*c*) Are topographical restrictions on cell shape specific for RHL-mediated adhesion?

RHL Distribution on Adherent Hepatocytes

Indirect immunofluorescence labeling with α-RHL antibody was used to determine lectin distribution on strongly adherent hepatocytes (weakly adherent cells did not withstand the labeling procedure). Cells adhering to ASF-derivatized gels had more fluorescence outside the adhesive site than cells on Gal-derivatized gels (Fig. 3). This fluorescence appeared to be uniformly distributed around the plasma membrane and surrounded an area of exclusion that was very similar in size to the area delineated by (and including) the brightly stained ring on cells adhering to Gal-derivatized gels. Membrane patches left behind by cells sheared from ASF-derivatized

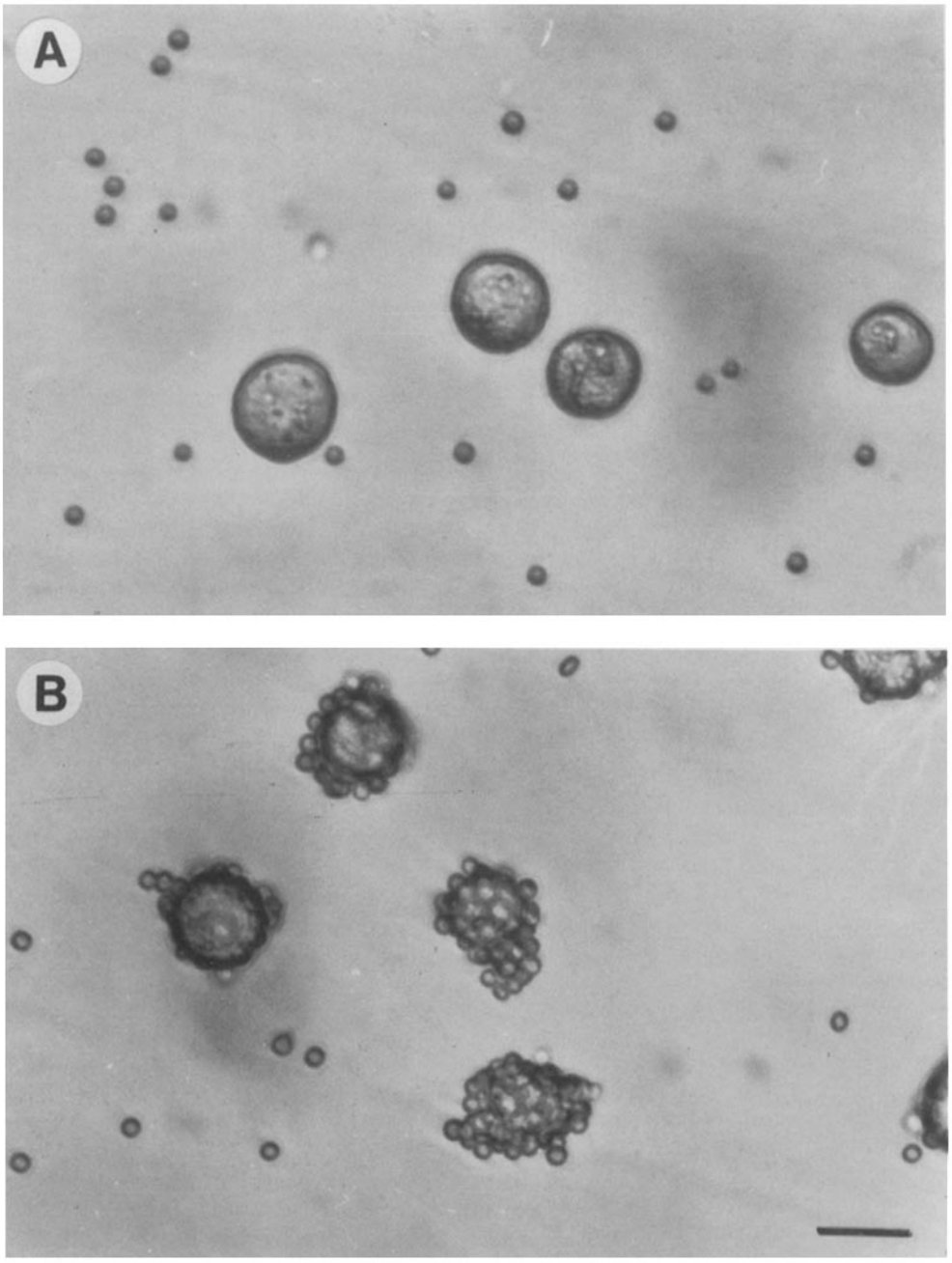

Figure 2. Neuraminidase-treated erythrocyte adhesion to hepatocytes incubated on ASF- and Gal-derivatized gels. Hepatocytes (40,000/well) were incubated on Gal- (*A*) or ASF- (*B*) derivatized gels for 60 min at 37°C, chilled, and 100-fold excess of neuraminidase-treated erythrocytes added to each well. The wells were centrifuged for 3 min at 186 *g* to bring the erythrocytes into contact with the adherent hepatocytes, incubated at 0°C for 30 min, then immersed in ice-cold PBS and kept inverted for 10 min to remove nonadherent cells (hepatocytes and erythrocytes). Phase microscopy photomicrographs are presented. Bar, 25 μm.

gels more often displayed a bright ring surrounding an area of exclusion, in contrast to the patches left behind by cells on Gal-derivatized gels which were more often uniformly labeled (data not shown).

Effect of Cytoskeletal Perturbants on RHL Topography

When cells were treated with hyperosmotic medium or colchicine and incubated on Gal-derivatized gels at 37°C, there were no marked differences in RHL patch morphology, except that the diameter of the RHL "halo" around the adhesive patch was slightly smaller than on control cells (Fig. 4). This may be explained by the presence of fewer surface ^{125}I-GalBSA binding sites. In contrast, cytochalasin D treatment of cells caused dramatic changes in both cell morphology and RHL patch topography. Cells were irregularly shaped and had membrane blebs, but remained viable (Fig. 4 *D*). The areas of exclusion outlined by antibody staining of cytochalasin-treated cells were larger and irregularly shaped compared to those on control cells. Antibody appeared to be less accessible to the region adjacent to the adhesive patch on cytochalasin-treated cells compared to control cells, since the RHL "halo" around this area was narrower than on control cells. Whereas control cells had one circular adhesive patch, individual cytochalasin-treated cells often appeared to have small satellite patches, resulting in a wide range of patch sizes and shapes. This was more evident on adherent membrane patches left behind by sheared cells (data not shown).

The adhesive patches on control and cytochalasin-treated

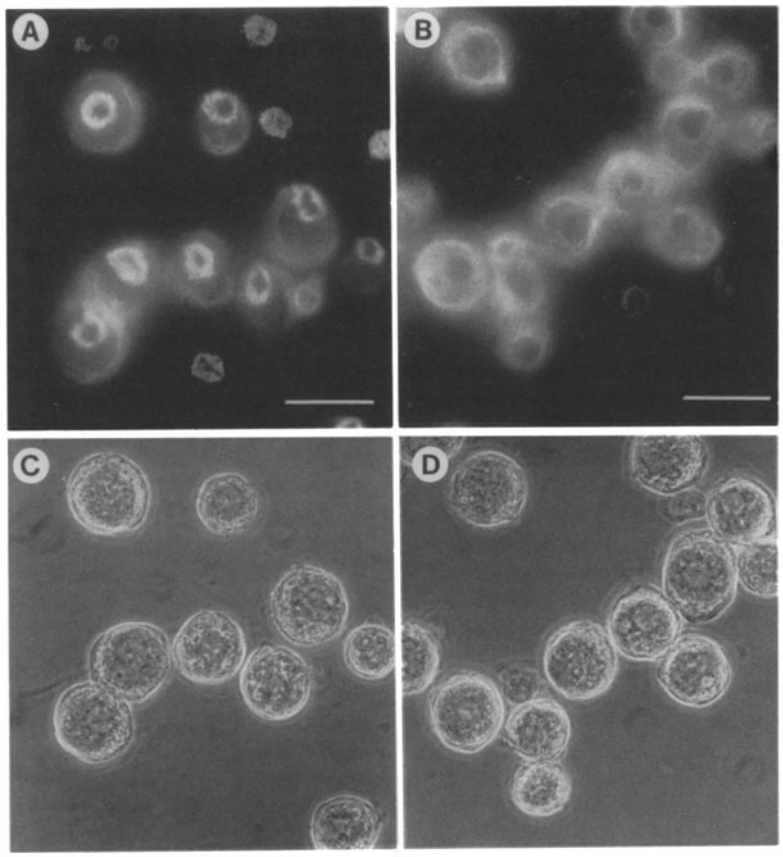

Figure 3. RHL distribution on cells adhering to Gal- or ASF-derivatized surfaces. Cells were incubated on Gal- or ASF-derivatized gels for 45 min at 37°C, then fixed and processed for indirect immunofluorescence labeling using α-RHL antibody (34). (*A*) Gal-derivatized gel, fluorescence micrograph focused at the adhesive surface; (*B*) ASF-derivatized gel, fluorescence micrograph focused at the adhesive surface; (*C* and *D*) corresponding phase-contrast micrographs of cells depicted in *A* and *B*, respectively, focused at the level of the cell nucleus. Bars, 25 μm.

cells were subjected to morphometric analysis. α-RHL-stained patches on control cells were compared with "major" patches (i.e., not associated with satellite patches) on cytochalasin-treated cells. Both the area and perimeter of patches on cytochalasin-treated cells were significantly larger than those on control cells (Fig. 5). In addition, the range of patch sizes and shapes on cytochalasin-treated cells was much greater than those on untreated cells, as evidenced by increased standard deviations. Similar results were obtained using cytochalasin-treated cells on ASF-derivatized gels (data not shown).

Localization of Clathrin and Actin on Adherent Membrane Patches

Anticlathrin antibodies and rhodamine-phalloidin were used to examine the distribution of clathrin and actin on membrane patches adhering to Gal-derivatized gels (initial studies using permeabilized whole cells revealed intense general staining which obscured the adhesion site). Anticlathrin antibody bound in a distinct punctate pattern to adherent membrane patches (Fig. 6), and often stained an annular structure (as opposed to a patch) on adherent membranes left by cells sheared from ASF-derivatized surfaces (data not shown). Rhodamine phalloidin staining revealed a highly structured radial array of actin cables extending from within the patch on Gal-adherent cells (Fig. 6). In a few cases, these radial arrays appeared to emanate from a focal point at the center of the patch.

Adhesive Site Morphology of Peptide-adherent Cells

The characteristic reproducible morphology of the adhesive site of Gal- and ASF-adherent cells led us to compare it with a different mode of cell adhesion based on the interaction of cell surface fibronectin receptors (10) with an immobilized RGD-containing peptide. Cell morphology and RHL distribution on cells adhering to gels copolymerized with RGD-peptide and either aminohexyl Gal or aminohexyl GlcNAc (control) were compared. Cells adhered to both surfaces and remained adherent during fixation and subsequent immunofluorescence labeling. The morphology of the adherent cells and adhesive patches was distinctly different on RGD-peptide-derivatized surfaces compared to carbohydrate-derivatized surfaces (Fig. 7). Cells were more flattened and adhesive patches were larger and more irregular in shape (similar to those on cytochalasin-treated cells on Gal-derivatized gels). Morphometric quantitation revealed that the mean area of the adhesive patches on cells adherent to RGD-containing gels (210 μm^2) was significantly greater than the same cells adhering to Gal-derivatized surfaces (150 μm^2) and was similar to that of cytochalasin-treated cells adhering to Gal-derivatized gels (250 μm^2). RHLs on hepatocytes adhering to gels copolymerized with Gal and the RGD peptide became completely inaccessible to ^{125}I-GalBSA binding (data not shown). Furthermore, adherent membrane patches left by sheared cells stained heavily with α-RHL antibody, suggesting that the presence of immobilized adhesion peptide did not interfere with RHL diffusion into the adhesion

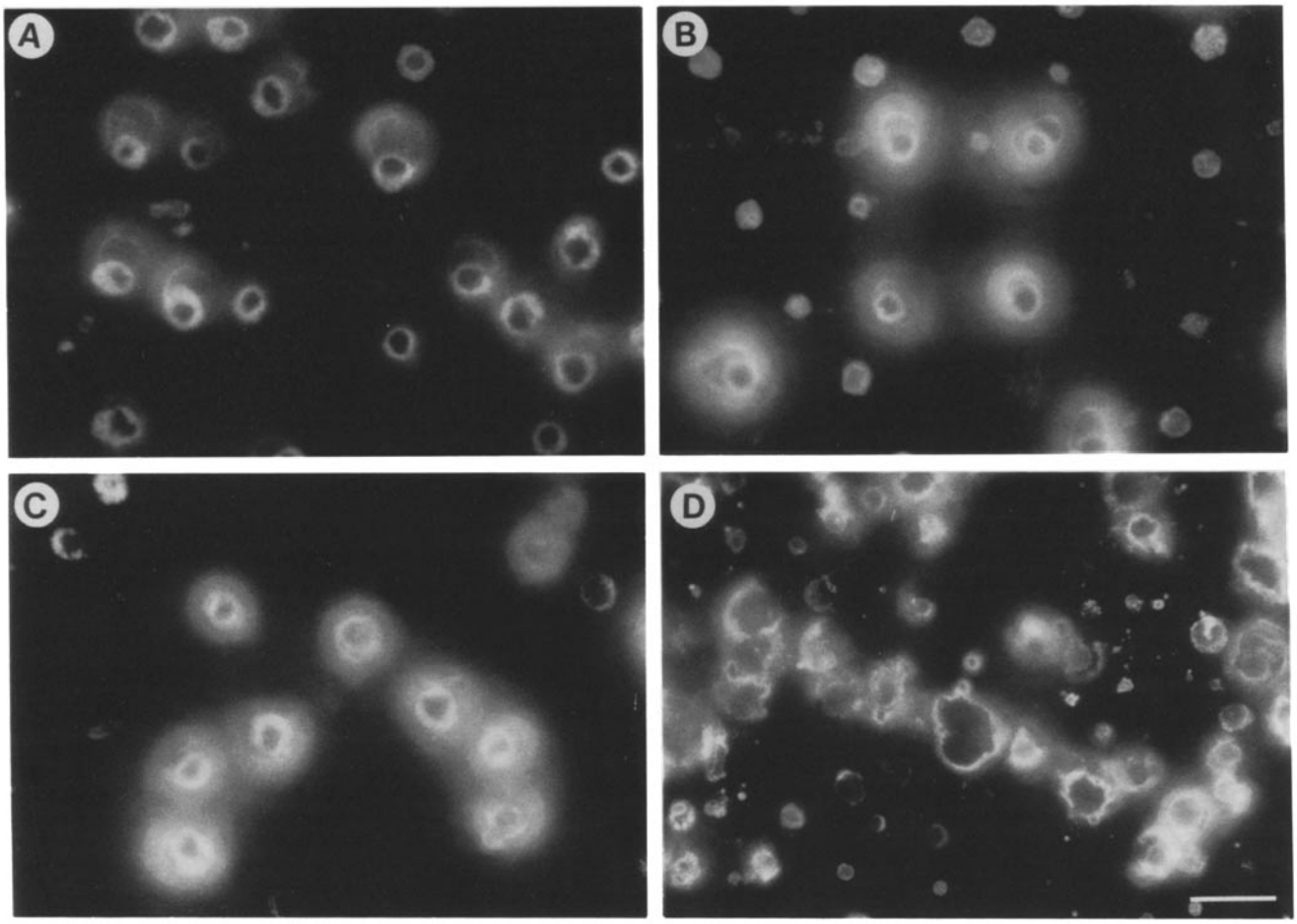

Figure 4. RHL distribution on Gal-adherent hepatocytes treated with cytoskeletal perturbants. Cells were pretreated with cytoskeletal perturbants, then incubated on Gal-derivatized gels for 60 min at 37°C, and processed for indirect immunofluorescence using α-RHL antibody as described in Materials and Methods. Fluorescence photomicrographs focused at the level of the adhesive surface are presented for control cells (*A*) and cells subsequently treated at 37°C with hyperosmotic medium for 10 min (*B*); colchicine for 60 min (*C*); or cytochalasin D for 60 min (*D*) before addition to derivatized gels as described in the text. Bar, 25 μm.

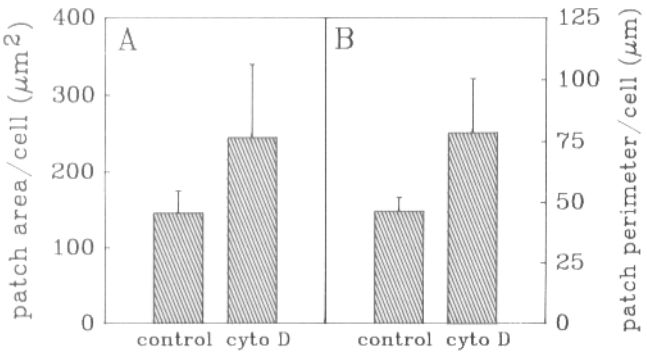

Figure 5. Morphometric analysis of the adhesive patches of control and cytochalasin-treated cells adhering to Gal-derivatized gels. Photomicrographs of control and cytochalasin D–treated cells after adhesion to Gal-derivatized gels for 60 min at 37°C and stained with α-RHL antibody (see text) were enlarged for morphometric analysis. The contact area (*A*), and adhesive site periphery (*B*) values are presented (mean ± SD; control, $n = 107$; cytochalasin D-treated, $n = 45$).

patch (Fig. 7 *A*, *inset*). Surface ^{125}I-GalBSA binding was only slightly attenuated on cells adhering to gels derivatized both with RGD and GlcNAc (control sugar), and indirect immunofluorescence labeling of these cells revealed diffuse α-RHL staining over the entire plasma membrane and surrounding an area of exclusion at the RGD-mediated adhesion site (Fig. 7).

Discussion

Cytoskeletal Involvement in Strong Adhesion to Galactose-derivatized Surfaces

Several observations demonstrated that strong adhesion to Gal-derivatized surfaces was not dependent upon nor caused by RHLs migrating to the adhesive site. (*a*) Although RHLs diffused into an adhesive patch on cells incubated on Gal-derivatized gels at 4°C (34), only weak adhesion was detected at this temperature. (*b*) Strong adhesion measured on cells incubated on Gal-derivatized gels at 25°C was identical

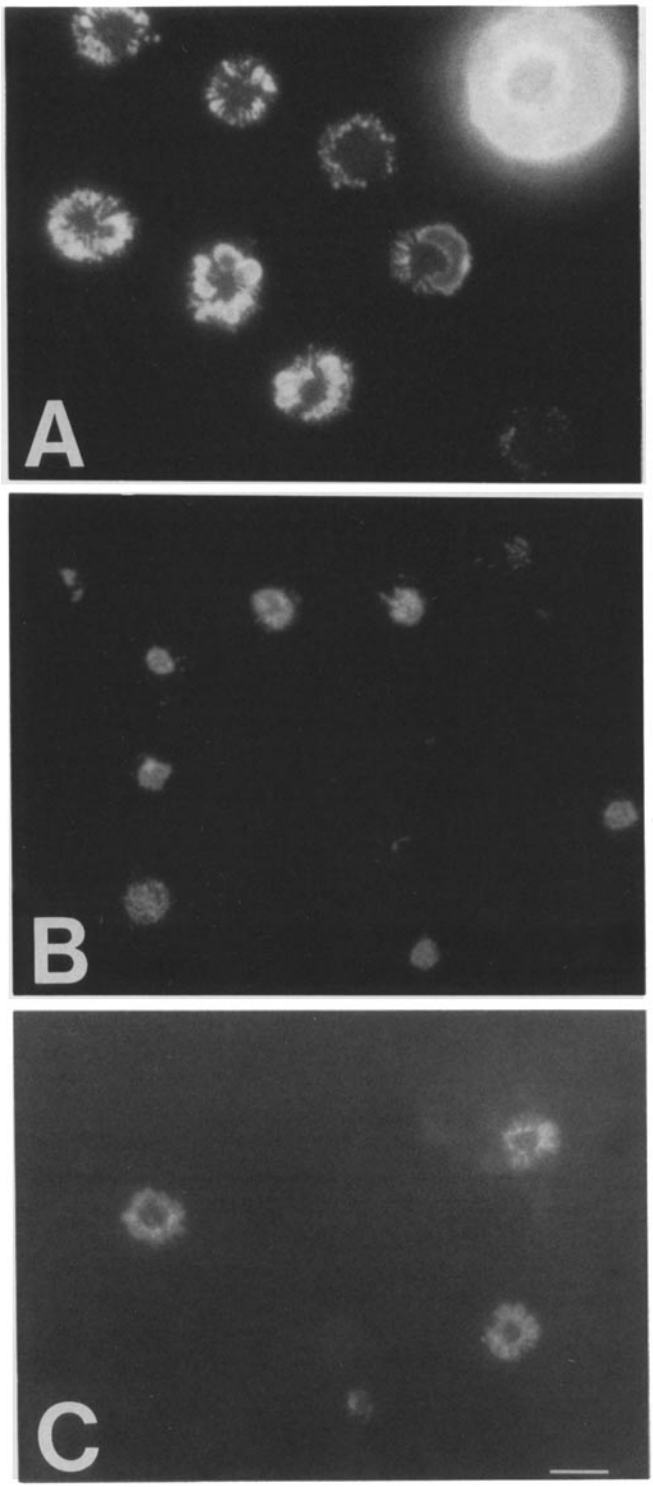

Figure 6. Distribution of clathrin and actin on adherent membrane patches. Cells were incubated on Gal-derivatized gels for 1 h at 37°C. The medium above the gels was vigorously triturated to shear adherent cells from the derivatized surface, and the gels were immediately fixed as described in Materials and Methods and separate gels were labeled with α-RHL antibody (*A*), α-clathrin antibody (*B*), or with rhodamine-conjugated phalloidin (*C*). An adherent cell remains in *A* for comparison. Bar, 10 μm.

to that determined for cells incubated at 37°C, even though cells incubated at the lower temperature had a third fewer surface ^{125}I-GalBSA binding sites (data not shown). (*c*) Hyperosmotic medium in conjunction with colchicine reduced cell surface ^{125}I-GalBSA binding by 85%, but had only a modest effect on strong adhesion. (*d*) Nearly all of the RHLs remained accessible on cells incubated on ASF-derivatized surfaces (patching was blocked), yet the cells adhered strongly. Together, these findings led us to implicate cytoskeletal elements as key components in the establishment and maintenance of strong adhesion to carbohydrate-derivatized surfaces.

Inhibition studies suggest that strong hepatocyte adhesion to Gal-derivatized surfaces involves concerted actions of clathrin, microtubules, and actin. While treatment with individual agents that depolymerize cytoskeletal components had only modest effects on strong cell adhesion, strong (but not weak) adhesion to Gal-derivatized surfaces was markedly compromised when cells were treated under conditions in which clathrin arrays, actin filaments, and microtubules were all perturbed. These data, combined with the observation that clathrin and actin are found on carbohydrate-adhesive membrane patches, suggest that RHLs at the site of adhesion form the focus of a cytoskeleton/membrane association. Microtubules may be indirectly required for organization of a stable cytoskeletal structure at the adhesion site without being directly involved, since strong adhesion was measured after chilling the cells to a temperature (<4°C) at which microtubules would depolymerize.

Clathrin has been found at the focal adhesion sites of cells adhering to various substrata. Large sheets of clathrin lattices have been observed on the ventral surfaces of adherent HeLa cells (16) and on fibroblasts cultured in the absence of serum (35). In addition, an adhesion structure containing clathrin-coated sheets was described in normal rat kidney cells (21). Time-lapse interference reflection microscopy of those cells suggested that the clathrin in their patches was static. Clathrin lattices may represent a distinct mode of cell adhesion. This adhesion may involve clathrin stabilization of aggregations of substratum-bound receptors, some of which have been thought to be involved primarily in endocytosis. The substratum-bound receptors may control recruitment of clathrin to the plasma membrane (15), and could directly influence cytoskeletal organization at these sites.

Relationship of RHL Redistribution and Adhesion Strengthening to "Capping"

Cell surface molecules on various cell types patch and redistribute into a "cap" over the Golgi region of the cell upon exposure to multivalent antibodies or binding proteins. This capping process may be accompanied by endocytosis of the resulting clusters into coated vesicles (28). The capping process is energy dependent and requires intact actin filaments. Interestingly, actin filaments may associate directly with clathrin coats at the cap site. In addition, microtubules radiating from microtubule organizing centers near the nucleus appear to concentrate underneath the cap (1), although microtubule-depolymerizing agents do not block capping. RHL migration to the adhesive site on Gal-derivatized gels (34) differs from "capping" in several respects. Capping does not occur at low temperatures but proceeds rapidly at 37°C.

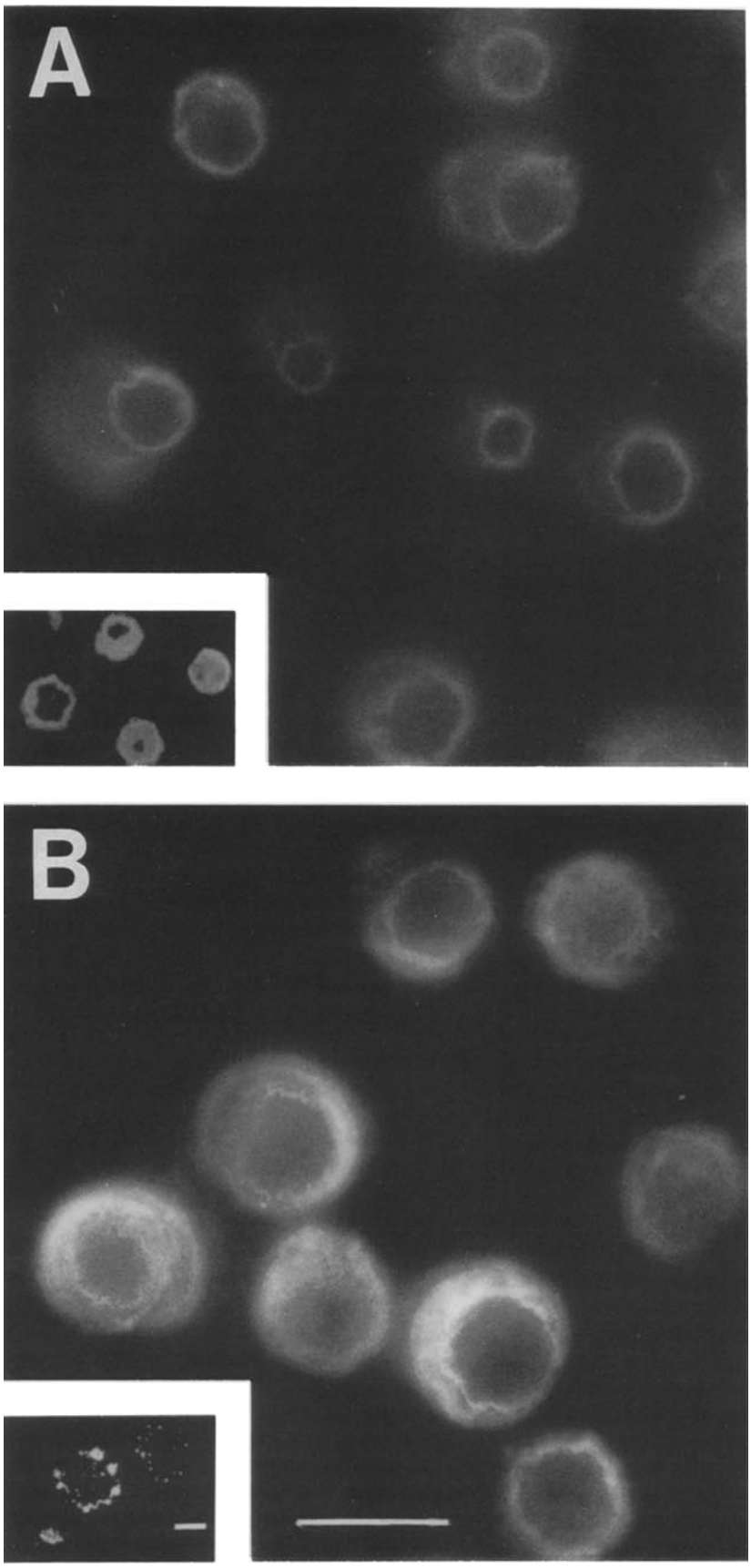

Figure 7. RHL distribution on cells adhering to RGD/Gal- and RGD/GlcNAc-derivatized gels. Cells were incubated for 60 min at 37°C on polyacrylamide gels coderivatized with an RGD-containing nonapeptide (4) and either Gal or GlcNAc. The cells were then fixed and processed for indirect immunofluorescence staining using α-RHL antibody as described in Materials and Methods. (*A*) Cells incubated on RGD/Gal-derivatized gels; (*B*) cells incubated on RGD/GlcNAc-derivatized gels. (*insets*) Membrane patches left behind by cells sheared from RGD/Gal- (*A*) and RGD-GlcNAc-derivatized gels (*B*). Bars: 25 μm; (*insets*) 10 μm.

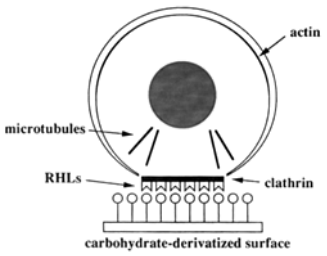

Figure 8. Model for RHL-mediated adhesion to Gal-derivatized surfaces. Hepatocyte contact with Gal-derivatized surfaces results in rapid accumulation of receptors in a patch at the site of adhesion. Temperature-dependent strengthening occurs as clathrin, actin, and microtubules organize to form a structured array at the adhesive site. The resultant structure lends turgidity to the hepatocyte, and limits the cell–cell contact area while stabilizing the hepatocyte against detachment.

In contrast, disappearance of surface RHLs after cell adhesion to Gal-derivatized gels occurs rapidly even at 0°C. Furthermore, agents that perturb actin formation blocked antibody-induced capping and coated vesicle formation, while they had no effect on RHL redistribution.

While RHL redistribution and capping are mechanistically separable, the requirements for adhesion strengthening on carbohydrate-derivatized gels resemble those described for the capping process. Strengthening and capping are both inhibited by low temperature and metabolic poisons, and appear to involve receptor associations with clathrin and actin. However, the mechanism for capping does not adequately describe the cellular changes associated with adhesion strengthening. A particularly striking difference between the two processes is their sensitivity to actin-disrupting agents. Whereas treatment of hepatocytes with cytochalasin B or D does not markedly inhibit the cells' abilities to establish strong adhesive contacts to Gal-derivatized surfaces, lymphocyte capping is completely abolished by similar treatment (28).

A Model for Carbohydrate-mediated Hepatocyte Adhesion

One model for carbohydrate-mediated cell adhesion that is consistent with our data is presented in Fig. 8. After initial contact, hepatocytes begin to flatten, forming a circular adhesive contact region between the plasma membrane and the derivatized surface. The growing area of contact is limited, however, by the submembranous cytoskeleton which resists cell deformation. In this model, the actin cortex which lines the cytoplasmic face of the plasma membrane maintains the spherical "turgid beach ball" shape of rat hepatocytes and restricts flattening, even when appropriate cell surface receptors for substratum binding sites are available on the membrane adjacent to the adhesive patch, such as occurs on ASF-derivatized gels. The effects of combined treatment with cytoskeleton-depolymerizing agents lead us to hypothesize that actin, clathrin, and microtubules interact specifically to generate a stable structure at the adhesion site. When the constraining actin cortex is perturbed by cytochalasin, the resistance to deformation is lessened, and the cell flattens further.

At the adhesive site, the cooperative association of a large number of substratum-bound receptors with a rigid submembranous cytoskeleton might require the simultaneous breaking of many receptor–substratum bonds in order to reverse cell adhesion (26). When the cytoskeletal structure is not formed (at 4°C) or is perturbed (by a combination of cytoskeletal perturbants), a weak force may be able to "peel" the cell progressively from the edge of its attachment site, much like Velco strips can be readily separated if peeled from the edge.

Peptide-mediated Hepatocyte Adhesion

The contact sites of cells adhering to surfaces containing an integrin-related RGD-adhesion peptide were larger than those of cells adhering to Gal- or ASF-derivatized gels, and were similar to the contact sites of cytochalasin-treated cells adhering to Gal-derivatized gels. Since carbohydrate-mediated cell adhesion results in a more restricted contact area, this suggests that the cytoskeletal organization of adherent cells (and the resulting cell morphology) is determined by the molecular interactions occurring at the substratum.

Long term adhesion of hepatocytes to various surfaces, including Gal-derivatized gels, results in cell "spreading" (2, 20, 23, 27, 29). Cells adhering to type IV collagen eventually generate an adhesive patch having a peripheral "adhesion annulus" rich in clathrin-coated structures and microfilaments (20), although the microfilaments do not appear to be directly attached to the adhesion plaque. The observation that cell spreading on Gal-derivatized surfaces is enhanced by serum (23) suggests that spreading involves extracellular matrix components (such as fibronectin) either added exogenously or produced by the hepatocytes themselves (32). This would be consistent with our data demonstrating short-term "spreading" of hepatocytes on RGD-derivatized surfaces, even in the presence of immobilized galactosides.

Our data suggest that initial recognition of Gal-derivatized surfaces by hepatocytes leads to the regulation of cytoskeletal components, resulting in a specific mode of attachment that differs from that induced by integrins. Furthermore, the affinity of the cell surface receptor for its substratum-bound ligand (Gal vs. ASF) dictates the topography of the receptor, although cell morphology is not affected. Through these mechanisms, qualitative or quantitative modulation of cell surface receptors and ligands may direct cytoskeletal structure, cell morphology, and the availability of molecular components for subsequent cell adhesions.

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References

1. Albertini, D. F., and J. I. Clark. 1975. Membrane-microtubule interactions: Concanavalin A capping induced redistribution of cytoplasmic microtubules and colchicine binding proteins. *Proc. Natl. Acad. Sci. USA.* 72:4976–4980.
2. Bissell, D. M., S. C. Stamatoglou, M. V. Nermut, and R. C. Hughes. 1986. Interactions of rat hepatocytes with type IV collagen, fibronectin and laminin matrices. Distinct matrix-controlled modes of attachment and spreading. *Eur. J. Cell Biol.* 40:72–78.
3. 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.
4. Brandley, B. K., and R. L. Schnaar. 1988. Covalent attachment of an Arg-Gly-Asp sequence peptide to derivatizable polyacrylamide surfaces: support of fibroblast adhesion and long-term growth. *Anal. Biochem.*

172:270–278.
5. Brandley, B. K., J. H. Shaper, and R. L. Schnaar. 1990. Tumor cell haptotaxis on immobilized N-acetylglucosamine gradients. *Dev. Biol.* 140:161–171.
6. Dulbecco, R., and M. Vogt. 1954. Plaque formation and isolation of pure lines with poliomyelitis viruses. *J. Exp. Med.* 99:167–182.
7. Fiskum, G., S. W. Craig, G. L. Decker, and A. L. Lehninger. 1980. The cytoskeleton of digitonin-treated rat hepatocytes. *Proc. Natl. Acad. Sci. USA.* 77:3430–3434.
8. Geiger, B. 1989. Cytoskeleton-associated cell contacts. *Curr. Opin. Cell Biol.* 1:103–109.
9. 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.
10. Johansson, S. 1985. Demonstration of high affinity fibronectin receptors on rat hepatocytes in suspension. *J. Biol. Chem.* 260:1557–1561.
11. Kaiser, J., R. J. Stockert, and A. W. Wolkoff. 1988. Effect of monensin on receptor recycling during continuous endocytosis of asialoorosomucoid. *Exp. Cell Res.* 174:472–480.
12. Kim, Y. S., J. Perdomo, and J. Nordberg. 1971. Glycoprotein biosynthesis in small intestinal mucosa I. A study of glycosyltransferases in microsomal subfractions. *J. Biol. Chem.* 246:5466–5476.
13. Kolset, S. O., H. Tolleshaug, and T. Berg. 1979. The effects of colchicine and cytochalasin B on uptake and degradation of asialo-glycoproteins in isolated rat hepatocytes. *Exp. Cell Res.* 122:159–167.
14. Lee, Y. C., C. P. Stowell, and M. J. Krantz. 1976. 2-Imino-2-methoxyethyl 1-thioglycosides: New reagents for attaching sugars to proteins. *Biochemistry.* 15:3956–3963.
15. Mahaffey, D. T., M. S. Moore, F. M. Brodsky, and R. G. W. Anderson. 1989. Coat proteins isolated from clathrin coated vesicles can assemble into coated pits. *J. Cell Biol.* 108:1615–1624.
16. Maupin, P., and T. D. Pollard. 1983. Improved preservation and staining of HeLa cell actin filaments, clathrin-coated membranes, and other cytoplasmic structures by tannic acid-glutaraldehyde-saponin fixation. *J. Cell Biol.* 96:51–62.
17. McAbee, D. D., J. A. Oka, and P. H. Weigel. 1989. Loss of surface galactosyl receptor activity on isolated rat hepatocytes induced by monensin or chloroquine requires receptor internalization via a clathrin coated pit pathway. *Biochem. Biophys. Res. Commun.* 161:261–266.
18. McAbee, D. D. B. L. Clarke, J. A. Oka, and P. H. Weigel. 1990. The surface activity of the same subpopulation of galactosyl receptors on isolated rat hepatocytes is modulated by colchicine, monensin, ATP depletion, and chloroquine. *J. Biol. Chem.* 265:629–635.
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. Nermut, M. V., L. D. Williams, and S. C. Stamatoglou. 1986. Ultrastructure of ventral membranes of rat hepatocytes spread on type IV collagen. *Eur. J. Cell Biol.* 42:35–44.
21. Nicol, A., and M. V. Nermut. 1987. A new type of substratum adhesion structure in NRK cells revealed by correlated interference reflection and electron microscopy. *Eur. J. Cell Biol.* 43:348–357.
22. 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.
23. 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.
24. Oka, J. A., M. D. Christiensen, and P. H. Weigel. 1989. Hyperosmolarity inhibits galactosyl receptor-mediated but not fluid phase endocytosis in isolated rat hepatocytes. *J. Biol. Chem.* 264:12016–12024.
25. 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.
26. Rees, D. A., Lloyd, C. W., and D. Thom. 1977. Control of grip and stick in cell adhesion through lateral relationships of membrane glycoproteins. *Nature (Lond.).* 267:124–128.
27. Rubin, K., S. Johansson, M. Hook, and B. Obrink. 1981. Substrate adhesion of rat hepatocytes. On the role of fibronectin in cell spreading. *Exp. Cell Res.* 135:127–135.
28. Salisbury, J. L., J. S. Condeelis, and P. Satir. 1980. Role of coated vesicles, microfilaments, and calmodulin in receptor-mediated endocytosis by cultured B lymphoblastoid cells. *J. Cell Biol.* 87:132–141.
29. Sawada, N., A. Tomomura, C. A. Sattler, G. L. Sattler, H. K. Kleinman, and H. C. Pitot. 1987. Effects of extracellular matrix components on the growth and differentiation of cultured rat hepatocytes. *In Vitro Cell. Dev. Biol.* 23:267–273.
30. 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.
31. Seglen, P. O. 1976. Preparation of isolated rat liver cells. *Methods Cell Biol.* 13:29–83.
32. Stamatoglou, S. C., R. C. Hughes, and U. Lindahl. 1987. Rat hepatocytes in serum-free culture elaborate an extensive extracellular matrix containing fibrin and fibronectin. *J. Cell Biol.* 105:2417–2425.
33. Townsend, R. R., M. R. Hardy, T. C. Wong, and Y. C. Lee. 1986. Binding of N-linked bovine fetuin glycopeptides to isolated rabbit hepatocytes: Gal/GalNAc hepatic lectin discrimination between Galβ(1,4)GlcNAc and Galβ(1,3)GlcNAc in a triantennary structure. *Biochemistry.* 25:5716–5725.
34. Weisz, O. A., and R. L. Schnaar. 1991. Hepatocyte adhesion to carbohydrate-derivatized surfaces: I. Surface topography of the rat hepatic lectin. *J. Cell Biol.* 115:485–493.
35. Woods, A., C. G. Smith, D. A. Rees, and G. Wilson. 1983. Stages in specialization of fibroblast adhesion and deposition of extracellular matrix. *Eur. J. Cell Biol.* 32:108–116.