

Functionally Distinct Laminin Receptors Mediate Cell Adhesion and Spreading: The Requirement for Surface Galactosyltransferase in Cell Spreading

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Abstract. The molecular mechanisms underlying cell attachment and subsequent cell spreading on laminin are shown to be distinct from one another. Cell spreading is dependent upon the binding of cell surface galactosyltransferase (GalTase) to laminin oligosaccharides, while initial cell attachment to laminin occurs independent of GalTase activity. Anti-GalTase IgG, as well as the GalTase modifier protein, α -lactalbumin, both block GalTase activity and inhibited B16-F10 melanoma cell spreading on laminin, but not initial attachment. On the other hand, the addition of UDP galactose, which increases the catalytic turnover of GalTase, slightly increased cell spreading. None of these reagents had any effect on cell spreading on fibronectin. When GalTase substrates within laminin were either blocked by affinity-purified GalTase or eliminated by prior galactosylation, cell attachment appeared normal, but subsequent cell spread-

ing was totally inhibited. The laminin substrate for GalTase was identified as N-linked oligosaccharides primarily on the A chain, and to a lesser extent on B chains. That N-linked oligosaccharides are necessary for cell spreading was shown by the inability of cells to spread on laminin surfaces pretreated with N-glycanase, even though cell attachment was normal. Cell surface GalTase was distinguished from other reported laminin binding proteins, most notably the 68-kD receptor, since they were differentially eluted from laminin affinity columns. These data show that surface GalTase does not participate during initial cell adhesion to laminin, but mediates subsequent cell spreading by binding to its appropriate N-linked oligosaccharide substrate. These results also emphasize that some of laminin's biological properties can be attributed to its oligosaccharide residues.

THE mechanisms by which cells interact with the basement membrane are of interest due to their significance in a variety of morphogenetic and pathological processes. Laminin is one of the principal components of the basement membrane (Kleinman et al., 1982; Von der Mark and Kuhl, 1985), and multiple cell surface laminin binding proteins have been reported (Rao et al., 1983; Horwitz et al., 1985; Smalheiser and Schwartz, 1987). The best characterized of these is a 68-kD receptor identified by laminin-affinity chromatography (Malinoff and Wicha, 1983; Lesot et al., 1983; Rao et al., 1983), which has been cloned and sequenced (Wewer et al., 1986), and shown to recognize the YIGSR peptide sequence found within the B₁ subunit of laminin (Graf et al., 1987). There is evidence to suggest that members of the integrin receptor family may also function as laminin receptors whereby they may mediate neurite outgrowth on laminin (Tomaselli et al., 1987).

Recently, another cell surface laminin receptor has been identified as the enzyme galactosyltransferase (GalTase),¹ which recognizes specific oligosaccharide residues within laminin (Runyan et al., 1986). Cell surface GalTase participates during a number of cell-cell and cell-matrix interactions including fertilization (Shur and Hall, 1982b; Lopez et al., 1985), morula compaction (Bayna et al., 1988), mesenchymal cell migration (Shur 1982b, Runyan et al., 1986), and intercellular adhesion (Shur, 1982a). In each case, the enzyme acts as a receptor for extracellular lactosaminoglycan-type oligosaccharides, which presumably terminate in N-acetylglucosamine (GlcNAc) residues (Shur and Hall, 1982a; Shur, 1982a). Similar lactosaminoglycan oligosaccharide chains have been found on laminin (Arumugham et al., 1986).

Reagents that block putative cell surface receptors for laminin have pleiotropic effects on cell interactions with

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1. *Abbreviations used in this paper:* α -LA, α -lactalbumin; GalTase, galactosyltransferase.

laminin, inhibiting initial cell attachment to laminin, as well as subsequent process formation and chemotaxis (Graf et al., 1987; Tomaselli et al., 1987). Therefore, the precise role of different laminin receptors in mediating different cell-laminin interactions is unclear. However, in this study, we have been able to define the function of GalTase during cell interactions with laminin by using reagents that perturb cell surface GalTase activity or by eliminating GalTase substrates within laminin. Results establish that GalTase specifically mediates cell spreading on, but not initial cell adhesion to, laminin by binding appropriate N-linked oligosaccharides. Thus, a specific molecular mechanism is provided for cell spreading on laminin, which is distinct from mechanisms that mediate initial attachment to laminin.

Materials and Methods

Reagents

Laminin was obtained either from Bethesda Research Laboratories (Gaithersburg, MD) or was extracted from EHS tumor material by the method of Timpl et al. (1982). Unless specified, all other reagents were obtained from Sigma Chemical Co. (St. Louis, MO). *N*- and *O*-glycanase were obtained from Genzyme Corp. (Boston, MA). Anti-GalTase, and the corresponding preimmune IgG, were prepared from rabbits immunized with affinity-purified bovine milk GalTase as described previously (Lopez et al., 1985; Bayna et al., 1988). Affigel 10 and all electrophoresis chemicals were obtained from Bio-Rad Laboratories (Richmond, CA). UDP[³H]Gal (11.4 Ci/mmol) was obtained from New England Nuclear (Boston, MA).

Cells

B16-F10 cells were a gift from Dr. Eve Briles (M. D. Anderson Cancer Center). Cells were passaged in DME (Gibco Laboratories, Grand Island, NY) containing 12% heat inactivated (56°C, 30 min) FCS (HyClone, Logan, UT). Cells were collected by trituration after being rinsed in Ca⁺⁺ and Mg⁺⁺ free Tyrode's salt solution (Gibco Laboratories) and incubated in 2 mM EDTA in Ca⁺⁺ and Mg⁺⁺ free Tyrode's for 10 min. Dissociated cells were pelleted by centrifugation, resuspended in DME, and aliquots added to the appropriate buffers.

Cell Spreading Assays

Cells were resuspended in DME containing ITS supplement (insulin, 5 µg/ml; transferrin, 5 µg/ml; and selenium, 5 ng/ml; Collaborative Research, Inc., Waltham, MA). Cells (5,000 cells in a final volume of 500 µl) were added to wells of 48-well tissue culture plates that had been previously coated with 10–15 µg of laminin for at least 2 h and washed before use. During a 5-h assay, cells attached and spread only on laminin or fibronectin-coated surfaces; i.e., cells did not attach and spread on uncoated or BSA (2 mg/ml) coated surfaces. For some experiments, the laminin was incubated overnight with various enzymes as indicated before addition of cells. Alternatively, various reagents including α-lactalbumin, lysozyme, bovine milk GalTase, sugar nucleotides, and antibodies were added to the wells at the indicated concentration. At various intervals after the cells were plated, the dishes were removed from the incubator and photographed using Hoffman modulation optics (Hoffman Modulation Optics, Inc., NY), which produces a three-dimensional view of the cellular morphology. Cells were considered adherent if they were firmly anchored to the substrate (i.e., resistant to agitation) and considered spread if they deviated from a round morphology and were producing cytoplasmic extensions. All data were scored independently at Iowa (R. B. Runyan) and at Houston (B. D. Shur) and results for each assay were within 10% of one another. Significance of the data was calculated using *t* test relative to control incubations.

Enzyme Assays

Cell surface GalTase activity on B16-F10 melanoma cells was assayed as described (Shur, 1982b). Briefly, aliquots of intact cells in Medium B (Shur,

1982a) were incubated with 100 µM UDPGal, 1 µCi UDP[³H]Gal, 10 mM MnCl₂, and 30 mM GlcNAc. After a 60-min incubation at 37°C, 10 µl of ice-cold 0.2 M EDTA was added to each tube and 50 µl of incubation mixture was spotted on paper (type 3MM; Whatman Inc., Clifton, NJ). The dried paper was subjected to high voltage borate electrophoresis to remove unused UDPGal and any breakdown products, and the incorporated radioactivity remaining at the origins representing the extracellular galactosylated product was determined by liquid scintillation spectroscopy.

GalTase activity was immunoprecipitated from solubilized plasma membranes collected from B16-F10 cells as described below. A 50-µl aliquot of solubilized membrane protein was incubated with 30 µg anti-GalTase IgG for 1 h at 4°C. 100 µl of protein-A Sepharose (Sigma Chemical Co.) in a 1:1 slurry with solubilization buffer was added and incubated overnight at 4°C to bind IgG. The protein A-Sepharose, with adsorbed IgG, was pelleted by centrifugation, and the residual GalTase activity in the supernatant was determined by high voltage borate electrophoresis as described (Shur, 1982a). Control incubations were treated in parallel with 30 µg normal rabbit IgG or with buffer.

Identification of Enzyme Substrate

8.4 × 10⁵ B16-F10 melanoma cells and 34 µg of bovine milk GalTase were individually incubated with 10 µCi UDP[³H]Gal, 1 mM MnCl₂, and 123 µg of purified laminin in Medium B for 3 h at 37°C. Control reactions without GalTase were incubated in parallel. The mixtures were precipitated by the addition of an equal volume of cold (–20°C) acetone, dried, resuspended in electrophoresis sample buffer, and separated by 4% SDS-PAGE (Laemmli, 1970). Gels were prepared for fluorography as described (Bonner and Laskey, 1974) except that glacial acetic acid rather than dimethyl sulfoxide was used as solvent. Purified laminin was used as the standard on adjacent lanes and identified by Coomassie Blue staining. After fluorography, the areas corresponding to A and B chains of laminin were excised from the gel and the radioactivity determined by liquid scintillation spectroscopy.

To examine the nature of the substrate oligosaccharide chains found on laminin, a similar incubation of laminin and UDP[³H]Gal was performed, after which aliquots of [³H]galactosylated laminin were acetone precipitated, washed three times with acetone, and the dry pellets were resuspended in 0.2 ml of the appropriate buffer (0.2 M Na phosphate, pH 8.6 for *N*-glycanase; 0.02 M Tris maleate, pH 6.0 for neuraminidase/*O*-glycanase). Neuraminidase (5.7 U; Boehringer-Mannheim Biochemicals, Indianapolis, IN) was added to galactosylated laminin aliquots suspended in 0.02 M Tris maleate, pH 6.0, to remove sialic acids that may sterically hinder *O*-glycanase digestion. After a 4 h incubation at 37°C, 24 U *N*-glycanase (Genzyme Corp.) or 40 mU *O*-glycanase (Genzyme Corp.) were added to the appropriate tubes and incubated another 14 h at 37°C. The number of enzyme units used were recommended by the distributor (Genzyme Corp.). The reaction was terminated by acetone precipitation, the pellets were washed with acetone, and the labeled glycoproteins resolved by SDS-PAGE fluorography as above.

Affinity Chromatography of GalTase on Laminin

Purified laminin was coupled to Affigel 10 (Bio-Rad Laboratories) according to the manufacturer's instructions. The final concentration of laminin coupled to the matrix was 0.75 mg/ml. 4.06 × 10⁵ B16-F10 cells were harvested and suspended in a buffer containing 0.1 M NaCl, 25 mM Hepes, pH 7.2, and protease inhibitor cocktail (Bayna et al., 1988), and homogenized with a Polytron (Brinkmann Instruments Co., Westbury, NY). The homogenate was subjected to low speed centrifugation (800 g, 10 min) to remove nuclei and intact cells and the membranes pelleted from the supernatant at 33,000 g for 1 h. The membrane pellet was resuspended in 0.25 M sucrose, 15% Percoll, and 1 mM TEA, pH 7.2, plus protease inhibitor cocktail, layered over a 2.5-ml cushion of 2.5 M sucrose plus protease inhibitor cocktail, covered with another 20 ml of Percoll/TEA/sucrose and centrifuged at 40,000 g for 20 min. The top 5 ml portion of the gradient containing the plasma membrane layer was diluted into 13.5 ml of Medium B, pH 7.2, and centrifuged at 100,000 g for 3 h. The resulting membrane pellet was solubilized in 0.1% Triton X-100, 0.1 M NaCl, 10 mM Tris, pH 7.4, plus protease inhibitor cocktail, and applied to the laminin affinity column. The column was washed with additional buffer until protein in the effluent was undetectable by Bradford assay (Bradford, 1976), and eluted with a linear gradient of 0.1–1.0 M NaCl. Fractions of the column were assayed for GalTase activity as described above, and proteins resolved by SDS-PAGE.

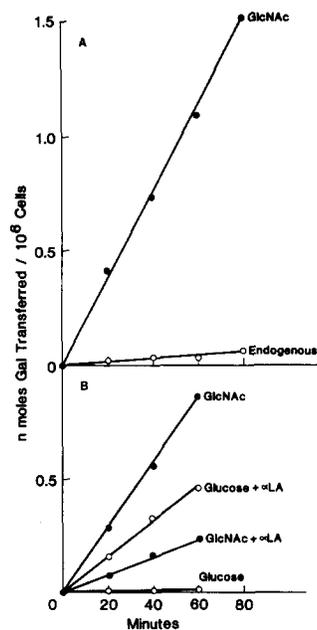


Figure 1. Intact B16-F10 melanoma cells have abundant surface GalTase activity. (A) Activity towards soluble exogenous substrates (●) is >25 times higher than activity towards cell-bound endogenous substrates (○). (B) α-LA lessens the activity of surface GalTase on B16-F10 melanoma cells for its conventional substrate, GlcNAc (●), and increases it for glucose (○), a substrate of normally low affinity.

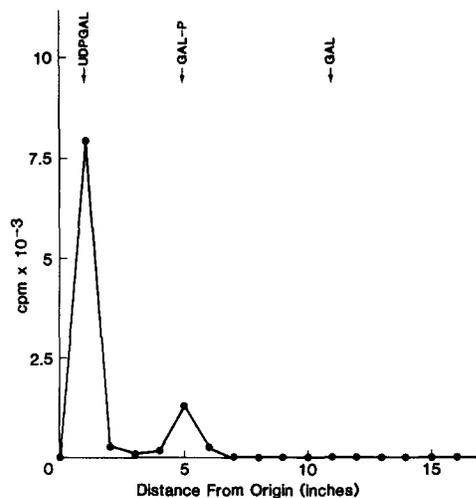


Figure 2. Hydrolysis of UDPGal during B16-F10 melanoma surface GalTase assays. At the end of the incubation, there is no detectable pool of free, radiolabeled galactose.

Results

Surface GalTase on B16-F10 Melanoma Cells

Surface GalTase activity on B16-F10 cells was linear with time of incubation (Fig. 1 A) and cell number (not shown). GalTase activity towards exogenous substrates was more than an order of magnitude higher than cell-bound endogenous substrates, demonstrating that during the course of the assay, surface GalTase is free to interact with extracellular glycoconjugates (Fig. 1). Assays were conducted under optimized conditions previously developed to detect surface GalTase activity (Shur, 1982a). That this activity was not due to hydrolysis, uptake, and intracellular use of labeled galactose is shown by the lack of free [³H]galactose in the incubation mixture (Fig. 2). Furthermore, as will be shown later, B16-F10 plasma membranes, isolated by subcellular fractionation, had abundant GalTase activity.

Reagents that Inhibit Surface GalTase Inhibit Cell Spreading on, but Not Cell Attachment to, Laminin

During a conventional 4–5-h assay, cells would attach and spread only on laminin or fibronectin-coated surfaces (Fig. 3). (In this, as well as all subsequent photographs, a fine granularity on the laminin surface is evident by Hoffman modulation optics, which is undetectable by phase contrast microscopy.) Cells did not attach and spread on either uncoated or BSA-coated surfaces. Furthermore, pretreating (or blocking) the laminin or fibronectin surface with BSA (2 mg/ml) before addition of cells had no effect on the apparent rate or extent of cell attachment or spreading.

α-Lactalbumin. α-Lactalbumin (α-LA) is a 14.2-kD protein that binds to GalTase and modifies its substrate specificity away from its conventional substrate, GlcNAc, and toward glucose, a substrate of normally low affinity (Morrison and Ebner, 1971). The ability of α-LA to modify the sub-

strate specificity of B16-F10 cell surface GalTase is shown in Fig. 1 B. In the presence of α-LA, cells attached normally to laminin, but failed to spread and migrate (Fig 3). The inhibition of cell spreading by α-LA was dose dependent (data not shown) at concentrations similar to those used previously in other systems (Shur et al., 1982b; Runyan et al., 1986).

As control, the structurally similar protein lysozyme, as well as BSA, had no effect on either cell attachment or spreading under identical conditions (Fig. 3). Since previous studies (Runyan et al., 1986) showed that bovine plasma fibronectin is not a substrate for GalTase, the attachment and spreading of melanoma cells on fibronectin were examined in the presence of α-LA (Fig. 3). Cell adhesion and spreading were normal on fibronectin in the presence of α-LA, which eliminated any possibility that α-LA had nonspecific metabolic effects. Furthermore, virtually all the cells recovered from the effects of α-LA if cultured overnight in control medium.

Anti-GalTase IgG. The effects of anti-GalTase IgG on cell attachment and spreading were examined. Anti-GalTase IgG, raised against affinity-purified bovine milk GalTase, recognizes murine GalTase as judged by immunoprecipitation of enzyme activity (Lopez et al., 1985; Bayna et al., 1988), by inhibition of enzyme activity (Lopez et al., 1985; Bayna et al., 1988), by immunoprecipitation of a single metabolically labeled membrane protein (Bayna et al., 1988), and by indirect immunofluorescence (Lopez et al., 1985; Bayna et al., 1988; Eckstein and Shur, manuscript submitted for publication). That anti-GalTase IgG also recognizes B16-F10 cell GalTase was shown by the ability of the antibody to immunoprecipitate enzyme activity as described in Materials and Methods. Anti-GalTase IgG immunoprecipitated 74% (944 cpm/1-h assay) of the GalTase activity solubilized from plasma membranes isolated from B16-F10 cells, whereas under identical conditions, preimmune rabbit IgG removed 1% (13 cpm/1-h assay) of the GalTase activity. Immunoprecipitation of GalTase activity was dose dependent relative to the

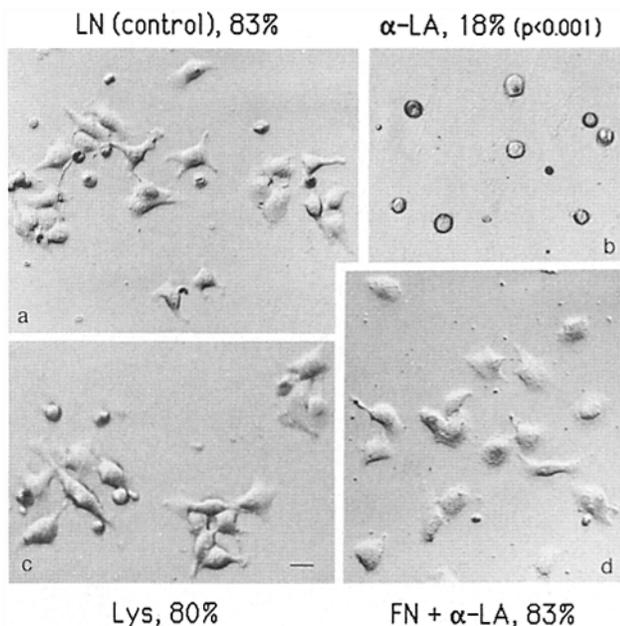


Figure 3. Cell spreading on laminin, but not cell adhesion, is inhibited by the GalTase substrate modifier protein, α -LA. (a) Cells on laminin (control; 310 cells scored); (b) 5 mg/ml α -LA inhibits cell spreading, but not cell adhesion (437 cells scored); (c) 5 mg/ml of the structurally similar protein, lysozyme, has no effect (350 cells scored); (d) 5 mg/ml α -LA has no effect on cell spreading on fibronectin (360 cells scored). All photographs were taken after 4 h of culture. The percentages of cells spread are shown. This experiment was repeated three times with similar results. In all photographs, a fine granularity on the laminin surface is evident by Hoffman modulation optics. Bar, 10 μ m.

IgG. Only 74% of the GalTase activity was immunoprecipitated, since under the conditions used the IgG-binding capacity of the protein A-Sepharose was limiting. This experiment was repeated four times with similar results.

As shown in Fig. 4, anti-GalTase IgG, but not normal IgG, inhibited cell spreading on laminin after a 4-h incubation. By 20 h, there was no significant difference between anti-GalTase IgG-treated cells and controls (data not shown), indicating that either turnover of GalTase and/or IgG occurred or that additional spreading mechanisms were recruited. The specificity of anti-GalTase IgG treatment was shown by its inability to influence cell spreading on fibronectin (Fig. 4). As before, this substrate specificity eliminated considerations of nonspecific metabolic effects of the antibody.

Increasing Surface GalTase Activity Promotes Cell Spreading on Laminin

GalTase requires two substrates for catalytic activity, an oligosaccharide chain terminating in the appropriate residue and the galactose donor, UDPGal. GalTase is thought to function in cell interactions by binding to its complementary oligosaccharide substrate on adjacent cells and/or in the extracellular matrix, creating an inactive, and stable, enzyme-substrate complex. The addition of UDPGal completes the catalytic reaction. If GalTase mediates cell spreading on laminin, it may not necessarily be dependent upon the availability of UDPGal in situ; nevertheless GalTase function may

be tested by the addition of exogenous UDPGal, which forces galactosylation and increases the turnover of the enzyme-substrate complex. The addition of UDPGal produced a slight increase in B16-F10 cell spreading on laminin, while the inappropriate sugar nucleotide, UDPglucose, had no effect (no addition: 39% spread; UDPglu: 36%, $P > 0.4$; UDPGal: 54%, $P < 0.005$; Fig. 5). The effect of UDPGal was subtle, and evident only if assayed soon after plating cells ($\sim 1\frac{1}{2}$ h), at which time control cells had not yet fully extended their processes (Fig. 5). After 4 h of incubation cells were fully spread, and consequently UDPGal no longer had the opportunity to increase the rate of cell spreading.

Blocking or Consuming GalTase Substrates in Laminin Selectively Inhibits Cell Spreading

Soluble GalTase. If surface GalTase recognition of laminin oligosaccharides is involved in cell spreading, then blocking or modifying GalTase substrates within laminin should inhibit subsequent cell spreading. Accordingly, laminin-coated surfaces were incubated with soluble GalTase purified from bovine milk to apparent homogeneity by α -LA affinity chromatography (Lopez et al., 1985). Unbound GalTase was removed by washing, and B16-F10 melanoma cells were added. As shown in Fig. 6, cells were able to attach normally but were unable to spread on laminin when its oligosaccharide substrates were blocked by soluble GalTase.

Galactosylation. A further, more specific modification of the laminin substrate was produced by incubating the laminin surface with affinity-purified GalTase and UDPGal to consume GalTase substrates by covalent glycosylation. After incubation, the laminin surface was washed to remove remaining GalTase and UDPGal. As shown in Table I, preincubation of laminin surfaces with purified GalTase and UDPGal eliminated >98% of the GalTase substrates, as assayed by a second incubation with purified GalTase and UDP 3 H-Gal. When cells were added to these galactosylated laminin surfaces, their attachment appeared normal, but subsequent cell spreading was totally inhibited (Fig. 6) presumably due to the absence of binding sites (i.e., substrates) for surface GalTase. As control, cells were added to laminin surfaces preincubated with boiled GalTase and UDPGal followed by rinsing; cells attached and spread normally (not shown).

Oligosaccharide Linkage of GalTase Substrates in Laminin

Aliquots of laminin were glycosylated with UDP 3 H-Gal and a source of GalTase, and the linkage of the oligosaccharide substrates was determined by appropriate enzyme digestions. The products were resolved by SDS-PAGE fluorography and the radiolabeled bands corresponding to the A and B chains of laminin (identified by Coomassie Blue-stained laminin standards) were excised and the incorporated radioactivity quantitated by liquid scintillation spectroscopy. We were unable to resolve the B₁ and B₂ chains from one another, and consequently, they were excised together. Experiments using either intact B16-F10 melanoma cells or affinity-purified bovine milk GalTase produced similar results.

Both A and B classes of laminin chains were galactosylated, although the A chain had about three times the specific activity of the B chains (Table II). Similar results were found when an aliquot of EHS matrix (an extract from Englebreth

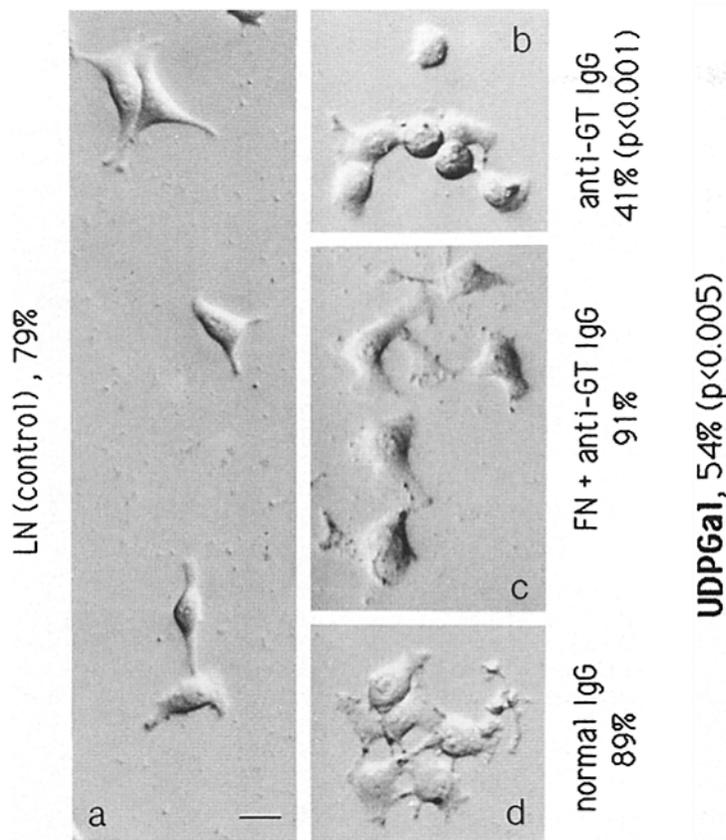


Figure 4. Cell spreading on laminin, but not cell adhesion, is inhibited by anti-GalTase IgG. (a) Control cells on laminin (185 cells scored); (b) cells on laminin in the presence of 120 μ g/ml anti-GalTase IgG (108 cells scored); (c) 120 μ g/ml anti-GalTase IgG has no effect on cell spreading on fibronectin (123 cells scored); (d) cell spreading on laminin in the presence of 120 μ g/ml normal rabbit IgG (101 cells scored). All photographs were taken after 4 h of culture. The percentage of cells spread are shown. This experiment was repeated five times with similar results. Bar, 10 μ m.

Holm Swarm sarcoma) was substituted for purified laminin (data not shown). Purified laminin was galactosylated as above and digested with either *N*-glycanase or with neuraminidase followed by *O*-glycanase to hydrolyze the corresponding oligosaccharide linkage. *N*-glycanase released most of the [3 H]galactose from the A chain and 50% of the label on B chains (Table II). Neuraminidase/*O*-glycanase digestion did not release any galactose label.

These results suggest that surface GalTase mediates cell spreading by recognizing and binding to one or more N-linked oligosaccharides in laminin. This was confirmed by the inability of laminin surfaces pretreated with *N*-glycanase and washed, to support cell spreading, even though cell attachment appeared normal (Fig. 6).

Differential Elution of Surface GalTase and Other Putative Laminin Receptors from Laminin Affinity Columns

Since cell surface GalTase functions as a laminin receptor on B16-F10 melanoma cells, we determined whether GalTase could be distinguished from other laminin binding proteins,

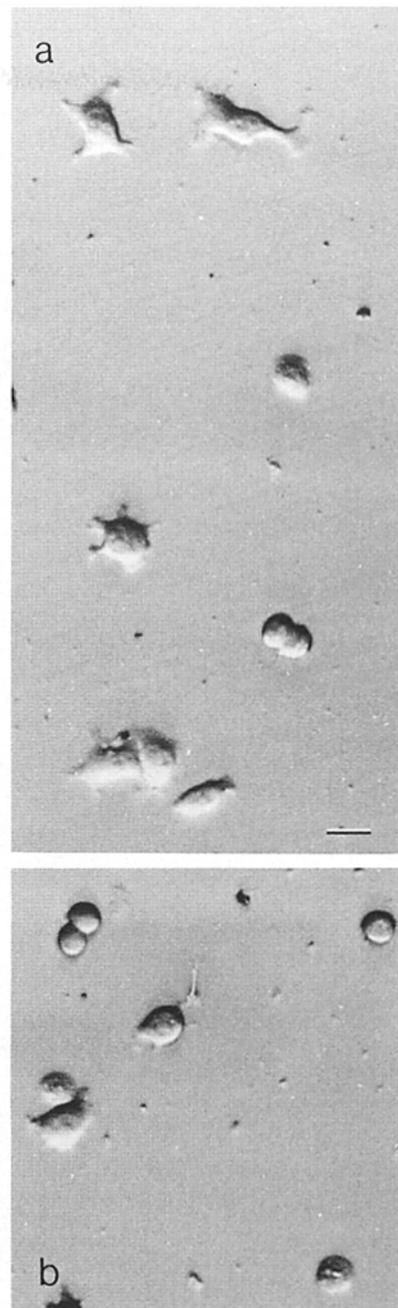


Figure 5. Cell spreading on laminin is accelerated by UDPGal. (a) UDPGal increases cell spreading (304 cells scored) relative to controls containing the inappropriate sugar nucleotide UDPGlu (b; 290 cells scored). The presence of UDPGlu has no significant effect on cell spreading relative to no sugar nucleotide addition (92% of control, $P > 0.4$). All photographs were taken after 1½ h of culture. After cells are fully spread (i.e., by 4 h of incubation), UDPGal no longer facilitates cell spreading. The percentages of cells spread in the presence of 20 mM sugar nucleotide are shown; 5 mM sugar nucleotide produced similar results. This experiment was repeated six times with similar results. Bar, 10 μ m.

most notably the 68-kD receptor, by laminin-affinity chromatography. Previously published protocols for laminin receptor chromatography were modified (omission of EDTA) to preserve GalTase activity and a solubilized plasma mem-

binding proteins, are present on B16-F10 cells as determined by affinity chromatography. In this report, we show that an independent laminin receptor, cell surface GalTase, recognizes N-linked oligosaccharides primarily on the A chain. By perturbing GalTase binding to its complementary laminin substrate, the function of GalTase in cell spreading was defined and distinguished from cell attachment.

Cell surface GalTase was assayed under optimal conditions with respect to required substrates and cofactors in which there was no detectable contribution by intracellular GalTase activity. Surface GalTase on B16-F10 melanoma cells is available to bind extracellular glycoside substrates, and as shown previously, laminin can serve as substrate for surface GalTase (Runyan et al., 1986). The nature and function of GalTase interaction with laminin was examined in this study by specifically perturbing the surface GalTase and/or its laminin substrate.

Two reagents that inhibit GalTase recognition of glycoside substrates inhibited cell spreading. α -LA and anti-GalTase IgG both prevented cell process formation on laminin-coated substrates. Since neither α -LA nor anti-GalTase IgG prevented cell attachment, attachment and spreading on laminin can be distinguished at the mechanistic level. That neither α -LA nor anti-GalTase IgG influenced cell attachment or spreading on fibronectin demonstrates specificity of GalTase action and eliminates from consideration nonspecific metabolic effects of these reagents.

In the presence of the appropriate sugar nucleotide, UDPGal, cells spread at a slightly faster rate. We do not know why such high concentrations of UDPGal were required to produce this effect, but relative to UDPglucose controls, the results were statistically significant. While this does not establish that the enzyme actually consumes an endogenously supplied sugar donor during spreading, the results are consistent with a role for GalTase during spreading, and suggest that the turnover or release of cell attachments is rate limiting.

GalTase substrates within laminin were modified to inhibit binding by cell surface GalTase. First, soluble, affinity-purified GalTase was added as a competitive receptor to block the appropriate laminin oligosaccharides. Second, GalTase substrates were consumed by earlier galactosylation with purified GalTase and UDPGal. Finally, the N-linked oligosaccharide substrates for GalTase were enzymatically removed. All modifications strongly inhibited cell spreading, though cell attachment appeared normal.

In general, reagents that perturbed GalTase on B16-F10 melanoma cells (i.e., α -LA, anti-GalTase IgG, UDPGal) produced short-lived effects, since cells recovered from these reagents after prolonged incubation (i.e., 24 h). On the other hand, covalent modification of the substrate by galactosylation appeared irreversible, as cells were unable to spread even after a 24-h incubation. This implies that laminin galactosylation markedly influences the cell's synthetic activity, since the cells would be expected to secrete new extracellular matrix capable of supporting spreading.

The ability to discriminate, at the mechanistic level, between cell attachment and subsequent cell spreading on laminin is noteworthy in light of recent studies suggesting that laminin contains two distinct cell-binding domains recognized by distinct cell surface receptors (Goodman et al., 1987; Aumailley et al., 1987). The short arms of laminin

Table II. Galactosylation of Laminin Oligosaccharides

Laminin chain	Enzyme pretreatment	cpm [3 H]galactose incorporated	Percent of control
A	Buffer (control)	48,739	—
	N-glycanase	12,574	26
	Neuraminidase/O-glycanase	52,394	107
B	Buffer (control)	16,766	—
	N-glycanase	8,819	53
	Neuraminidase/O-glycanase	19,274	115

Laminin was galactosylated with UDP[3 H]Gal and affinity-purified β 1,4 GalTase from bovine milk as described. Aliquots of the galactosylated product were pretreated with the specified glycosidase and resolved by SDS-PAGE. The galactosylated products were identified by fluorography, excised from the gel, and the radioactivity was determined by liquid scintillation spectroscopy. This experiment was performed three times with similar results.

(fragment 1) appear to bind to the 68-kD receptor via the YIGSR amino acid sequence. The laminin long arm (fragment 8) also binds to high affinity surface receptors whose identity is unknown. Likely candidates for surface receptors that recognize the long arm (fragment 8) are GalTase as well as members of the integrin family, both of which have been implicated in cell process spreading on laminin (Edgar et al., 1984; Tomaselli et al., 1987). However, antibodies against integrin-like receptors block initial cell attachment as well as neurite formation on laminin (Tomaselli et al., 1987), unlike anti-GalTase antibodies, which selectively inhibit cell spreading. Furthermore, fragment 8 binds to surface receptors with an affinity higher than that expected for integrin-mediated binding (Aumailley et al., 1987). In this regard, the high-affinity binding of GalTase to laminin columns suggests that GalTase may be one of the long arm receptors. It is possible that neurite extension may be analogous, at the mechanistic level, to filopodial extension in nonneuronal cells such that the neurite promoting site on the laminin long arm (Chiu et al., 1986; Edgar et al., 1984; Engvall et al., 1986; Tomaselli et al., 1987) is, in fact, an N-linked oligosaccharide domain recognized by GalTase on neurite growth cones. It is of interest to note that neurite outgrowth on laminin is inhibited by α -LA in quail neural tube cultures (Runyan et al., 1986).

GalTase is not uniformly distributed on the cell surface but rather is preferentially localized on cell lamellipodia and filopodia as assayed by indirect immunofluorescence, consistent with its proposed role in cell spreading and migration (Eckstein and Shur, manuscript submitted for publication; Shur, 1982b). Furthermore, GalTase colocalizes with actin-containing microfilaments, suggesting that its distribution may be directed by a cytoskeletal association. Studies are now in progress to define the ultrastructural localization of GalTase, its cytoskeletal association, and its precise oligosaccharide substrates in laminin.

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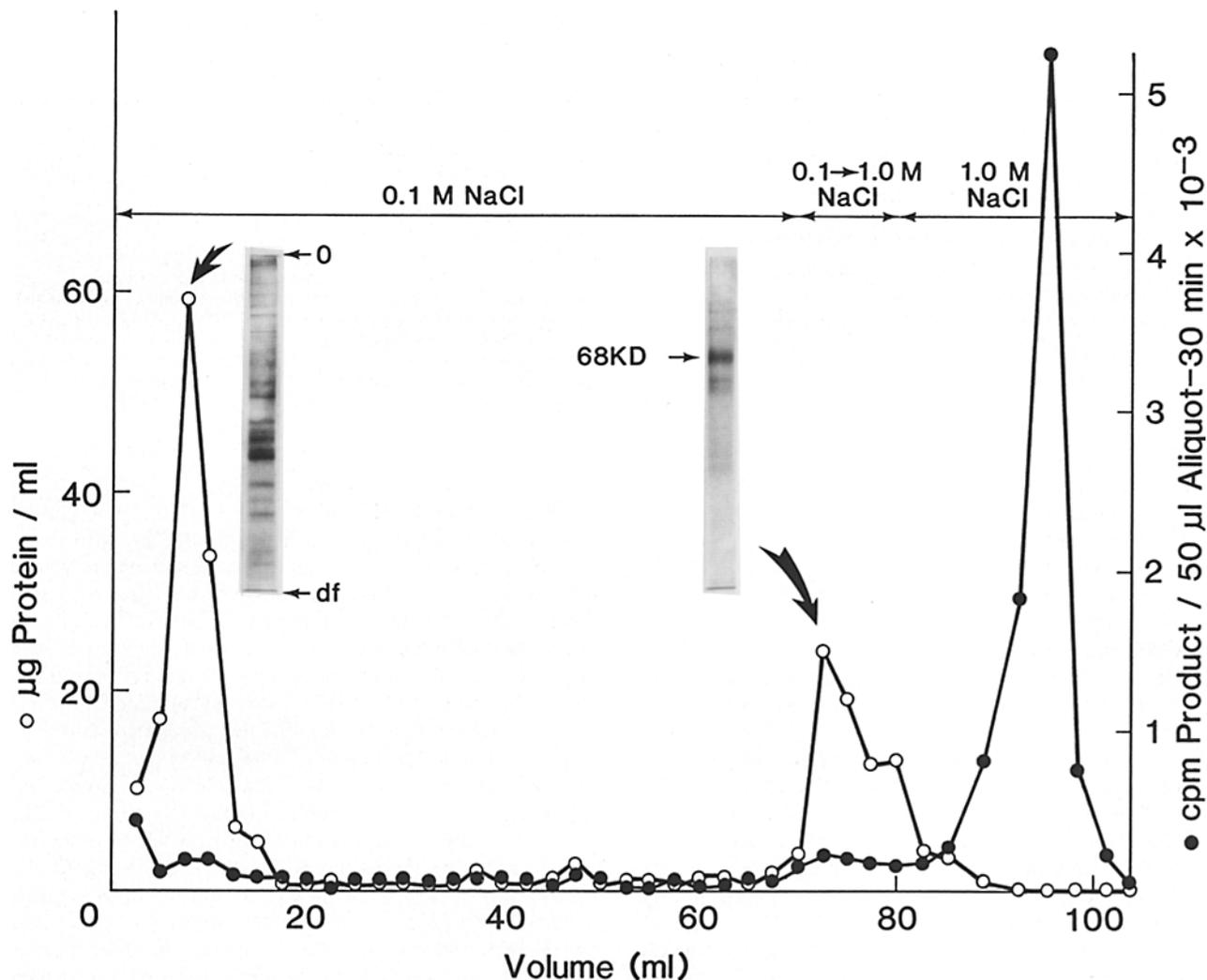


Figure 7. Differential elution of distinct laminin binding proteins from laminin affinity columns. Detergent-solubilized B16-F10 plasma membranes were applied to a laminin affinity column as described in Materials and Methods. After washing away unbound protein, a salt gradient eluted the 68-kD receptor, as well as other laminin-binding proteins, at 0.2–0.3 M NaCl. GalTase eluted at 1.0 M NaCl as assayed by enzymatic activity. The eluted protein was resolved by silver stained SDS-PAGE, and representative fractions are illustrated. GalTase has an apparent molecular mass of 60 kD by immunoblot analysis.

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