Two Distinct Cell-binding Domains in Laminin Can Independently Promote Nonneuronal Cell Adhesion and Spreading

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Abstract. Two subfragments of laminin, E8, a major part of the long arm, and E1–4, the three short arms, promote cell adhesion and spreading. Three distinct types of adhesive behavior are seen in short term (1 h) assays, typified by (*a*) secondary murine fibroblasts, adherent only on fibronectin; (*b*) secondary murine myoblasts, adherent on fibronectin, laminin, and the E8 fragment; and (*c*) Rugli human glioblastoma cells, adherent on fibronectin, laminin, E8, and E1–4.

E8-specific polyclonal antibodies block myoblast adhesion to E8 and to laminin with identical concentration dependence; Rugli binding to E8 but not to laminin is also totally blocked by these antibodies.

Heating of E8 and laminin to ~60°C abolishes cell attachment-promoting activity for myoblasts. Adhesion of Rugli cells to E8 is also lost, but on laminin the attachment-promoting activity remains constant. This is due to an increase in the activity of E1–4 fragment as it is heated.

Thus, major sites for initial cell adhesion to and spreading on laminin lie within the E8 and E1–4 fragments, but not all cells binding to laminin will bind to both fragments. These data may tentatively be explained by the existence of more than one type of receptor for laminin at the cell surface; one is needed for each fragment.

EXTRACELLULAR matrix components can modulate cell differentiation (Hauschka and Konigsberg, 1966; Mauchamp et al., 1979; Sugrue and Hay, 1981; Hay, 1984; Kleinman et al., 1985), cell locomotion (Newgreen and Thiery, 1980; Greenburg and Hay, 1982; Bronner-Fraser, 1984; Goodman and Newgreen, 1985), and cell–cell interaction (Nitkin et al., 1984). The components of the matrix that produce such effects, their cellular receptors, and the molecular events that follow matrix–receptor interaction are incompletely understood.

Laminin is a major protein of the basement membrane, an extracellular matrix that enfolds epithelial, endothelial, muscle, nerve, and fat cells. It appears to act as an adaptor between cell surfaces, collagen type IV matrices, and heparan sulphate proteoglycans. In vitro it promotes the adhesion, spreading, and locomotion of many cell types (Kleinman et al., 1985; von der Mark and Kühl, 1985; Timpl and Dziadek, 1986). Laminin is a cross-shaped multifunctional molecule (mol wt ~900,000) consisting of two nonidentical B chains (mol wt ~220,000) and a heavier A chain (mol wt ~440,000; see Timpl and Dziadek, 1986) and it possesses a domain structure within which the functions are dispersed. However, the exact role and location of the various domains is still in dispute.

The molecular mechanisms by which cells interact with laminin substrates are of great interest. Accordingly, laminin and the cell surface moieties to which it binds have become the subject of much attention (reviewed in von der Mark and Kühl, 1985). One approach to identifying matrix receptors (decisive in the case of fibronectin; Pierschbacher et al., 1982; Pytela et al., 1985) has involved making small proteolytic fragments of a matrix component and studying how cells respond to them. In this way the functional domains that are necessary and sufficient for a specific cellular response may be identified and their cellular targets defined.

A number of proteolytic fragments of laminin have been described (Ott et al., 1982; Rao et al., 1982; Terranova et al., 1983; Timpl et al., 1983; Paulsson et al., 1985; Edgar et al., 1984), specific antibodies prepared against them (Edgar et al., 1984; Paulsson et al., 1985), and some biological functions defined. E1–4, the three short arms (related to α3 of Terranova et al., 1983), is reportedly involved in binding to collagen IV and in promoting the attachment of MCF-7 tumor epithelium-derived cells (Terranova et al., 1983). The E1, P1, and C1 fragments containing short arms but lacking terminal globular domains retain attachment-promoting, but not collagen-binding activity (Terranova et al., 1983). E3, a globular domain from the long arm, holds a major heparin-binding site (Ott et al., 1982). E4 and perhaps also E5 and E6 stem from the ends of the short arms. E5 and E6 (and E1) possess cell attachment sites for hepatocytes, but only E5 stimulates cell spreading (Timpl et al., 1983). E8, the lower 35 nm of the long arm, will stimulate neurite survival and outgrowth (Edgar et al., 1984) and will trigger tyrosine hydroxylase activity in chromaffin cells (Acheson et al., 1986). E9 and 25K are probably from the α-helical regions of E8 and have no known function (Paulsson et al., 1985).

We have been interested in the specific response of skeletal

myocytes (Kuhl et al., 1986) and certain tumor cells (Goodman and Newgreen, 1985) to laminin and we have been searching for the minimal functional domains to which its various biological activities can be ascribed and the corresponding cell surface receptors.

In this study we present evidence that many nonneuronal cells can use a domain in the E8 fragment, and that some can also use a second domain of laminin for cell attachment.

Materials and Methods

Preparation, Purification, and Characterization of Laminin Subfragments

Laminin isolated from mouse Engelbreth-Holm-Swarm tumors (Timpl et al., 1979) was stored at 4°C in the presence of protease inhibitor cocktail (phenylmethylsulfonyl fluoride (PMSF) and *p*-chloromercuribenzoate, each 10 μg/ml). Elastase digestion of laminin in 0.2 M bicarbonate (Ott et al., 1982) was done for 16 h at 37°C using an enzyme/substrate protein ratio of 1:100 and was stopped by addition of PMSF. The digestion products were separated on an Agarose 1.5-M column (4 × 140 cm; Bio-Rad Laboratories, Richmond, CA), typically loading 50 mg of digest in each run. The E1–4 and E8 fragments were further purified after dialysis against 50 mM sodium bicarbonate by passage over heparin-Sepharose (Pharmacia, Uppsala, Sweden) preequilibrated in the same buffer. E8 binds while E1–4 flows through. E8 was eluted with 0.5 M sodium chloride. E3 and E4 were used without further purification.

Pepsin digestion of purified E1–4 and E8 in 0.5 M acetic acid was for 24 h at 4°C using an enzyme/substrate protein ratio of 1:100, and was stopped by addition of the inhibitor cocktail and dialysis against 0.2 M NH_4HCO_3. The pepsin digest of E1–4, containing P1 fragments, was sometimes further purified by HPLC over columns (TSK-SW3000 and TSK-SW2000; Beckman Instruments, Inc., Fullerton, CA).

Attachment Assays

Preparation of Substrates. Proteins were diluted (usually to 10 μg/ml) in 145 mM NaCl, 5 mM KCl, 7 mM $Na_2HPO_4 \cdot 2H_2O$, 1.5 mM KH_2PO_4, pH 7.2 (PBS), and 0.3 ml of the solution allowed to adsorb overnight at 4°C onto 24-well tissue culture plates (Costar; Cambridge, MA). After washing (twice in 1 ml PBS), remaining protein binding sites on the plate were saturated with filtered, heat-treated (1 h at 70°C) BSA solutions (Serva, Heidelberg, Federal Republic of Germany) 0.5% in PBS, for 2 h at 37°C and the plates stored (<48 h at 4°C) in BSA/PBS.

Standard Adhesion Assay. Cell monolayers were washed with PBS and harvested with 0.1% trypsin (Biochrom KG, Berlin, Federal Republic of Germany), 0.025% EDTA in PBS (trypsin/EDTA, 2 min at 37°C, TR126 required 5 min). The reaction was stopped by addition of fibronectin-depleted 10% FCS in DME (FCS−/DME) (Dessau et al., 1978) and the cells pelleted (1,000 *g* for 5 min), resuspended in 0.5% BSA in DME (BSA/DME), and allowed to recover at 37°C for 15 min. Typically, 5 × 10^4 cells in 0.2 ml were plated into a final volume of 0.5 ml of DME/BSA on 10 μg/ml protein-coated Costar wells.

After 60 min at 37°C (unless otherwise noted in text) the supernatant was aspirated and nonadherent cells washed away (twice in 1 ml PBS). Adherent cells were removed with trypsin/EDTA (0.5 ml) and PBS washing (twice in 0.5 ml), added to DME/FCS (0.5 ml) and counted in a cell counter (Coulter Electronics, Luton, Bedfordshire, England).

The number of cells binding to wells coated with BSA alone (nonspecific attachment) was 1–2% of the maximum specific binding. These nonspecific values were routinely subtracted from the specific cell counts. Assays were performed several times and in triplicate or quadruplicate. The variation between wells rarely exceeded 5–10% of the mean. Attachment assays according to Landegren (1984) gave results identical to those of the cell counter.

For microscale screening the assay was performed on Terasaki plates (Falcon Labware, Oxnard, CA). 3 × 10^3 cells (in 2 μl) were plated in 10 μl of DME/BSA on coated wells. After 60 min at 37°C, the plates were washed with PBS and fixed (3% paraformaldehyde in PBS for 30 min at 20°C). Cell attachment and spreading were scored on a four-point scale ranging from extensive attachment and spreading (++) to none (−).

Preparation of Cells. TR126, Hep-2, and HeLa (human carcinoma-derived); A549 and Stromer (human malignant melanoma); HT1080 (human fibrosarcoma); Rugli (human glioblastoma); Madin-Darby bovine kidney (MDBK, bovine epithelial) established cell lines; and secondary mouse fibroblasts (passage 3–5), were grown to semiconfluency on 75 cm^2 plates in DME/10% FCS, 0.58 mg/ml L-glutamine, 50 μg/ml sodium ascorbate, 100 U/ml penicillin, and 100 U/ml streptomycin (media, sera, and antibiotics from Biochrom KG) in a 7.5% CO_2/92.5% air atmosphere. Rugli were the generous gift of Professor B. Odermatt, University Hospital, Zurich, Switzerland. Other established cell lines have been described in detail elsewhere (Lane et al., 1982; Rupniak et al., 1982; Goodman and Newgreen, 1985).

Secondary murine skeletal muscle myoblasts (>90% fusogenic) and fibroblasts (<1% fusogenic) were prepared essentially as described (Kuhl et al., 1982, 1986) by adsorbing collagenase/dispase-dispersed 3-d postnatal thigh muscles onto tissue culture plastic for 15 min (fibroblasts). However, the supernatant was then adsorbed onto laminin-coated BSA-saturated plastic for 30 min (myoblasts). After growth to ~80% confluency (3–4 d) in DME containing 1% chick embryo extract and 20% horse serum, cultures were passaged with 0.1 mg/ml dispase (*B. polymyxa* neutral protease; Boehringer Mannheim, Mannheim, Federal Republic of Germany) and replated onto laminin substrates. This procedure gives a highly enriched fusion-competent myoblast population (Kuhl et al., 1986). At 70–90% confluency (2–3 d in proliferative phase, <5% fusion) the cultures were passaged using trypsin/EDTA for adhesion assays.

Kinetics of Adhesion

Substrates and cells were prepared as for the standard adhesion assay, the wells prefilled with 0.3 ml of DME/BSA, and 0.2 ml cell suspension added at predetermined time points. The attachment reactions were stopped simultaneously.

Concentration Dependence of Attachment

The proteins were serially diluted with PBS, and 0.3 ml allowed to adsorb onto Costar wells overnight at 4°C before BSA saturating, washing, and standard attachment assay (see above). The quantity of protein adsorbing onto the substrate was measured by using radiolabeled probes (see below).

Antibody Blocking Experiments

Anti-E8 antibodies, the generous gift of Dr. R. Timpl, Martinsried, Federal Republic of Germany (the specificity of the antibodies has been previously described by Paulsson et al., 1985) or control preimmune rabbit sera were complement inactivated at 56°C for 20 min, serially diluted with DME/BSA, and incubated with protein-coated Costar wells in a total volume of 0.3 ml. After 1 h at 37°C, 0.2 ml of cell suspension was added for a standard attachment assay. The results are plotted taking the binding on each substrate in the presence of 1:50 diluted preimmune sera as 100%.

Thermal Inactivation Studies

Stock solutions of 200 μg/ml laminin, fibronectin, E1–4, or E8 in PBS were diluted to 10 μg/ml in PBS, stored on ice, and heated by transfer for 10 min to a thermostatically controlled heating block before being returned to the ice bath. The heated proteins were used to coat Costar wells for a 45-min attachment assay. Controls showed that the apparent concentration of protein in the coating solutions (Bradford, 1976) was unaltered (±10%), and the size of the component protein chains on SDS-PAGE unchanged by the heat treatment.

Binding Capacity of the Substrates

10–15 ng of ^{125}I-protein (~4,000 Bq) were added to increasing amounts of serially diluted nonradioactive protein (see above) and adsorbed onto Costar wells overnight at 4°C, then washed and incubated with BSA/PBS exactly as in the standard adhesion assay. After three further PBS washes the bound protein was hydrolyzed from the plate by incubation with 3 M NaOH (0.5 ml, 4 h at 80°C), the wells washed (twice in 1 ml PBS), and the total radioactivity counted. Over 99% of the adsorbed radioactivity was removed by this procedure. The number of moles of protein adsorbed was calculated assuming mol wts of 900,000 for laminin (Timpl et al., 1979), 440,000 for E1–4, 250,000 for E8 (Deutzmann, R., R. Timpl, and M. Paulsson, unpublished observations), and 480,000 for fibronectin.

Miscellaneous Methods

Human serum plasma fibronectin was generously provided by Dr. H. Richter, Martinsried, Federal Republic of Germany. E8, E1–4, and laminin

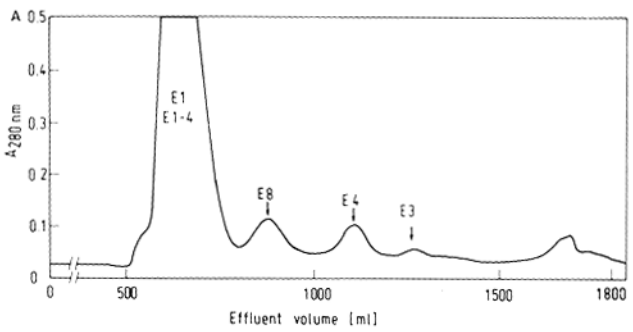
were iodinated to specific activities of 9.5×10^6, 5.0×10^6, and 6.1×10^6 cpm/μg, respectively, by a coupled glucose oxidase/lactoperoxidase technique (Hubbard and Cohn, 1975). Protein concentrations were determined by the method of Bradford (1976) (Bio-Rad Laboratories) using BSA as standard. SDS-PAGE was performed according to Laemmli (1970) in 3–25% gradient gels. For rotary shadowing transmission electron microscopy (Kühn et al., 1981), the laminin fragments were dissolved in 0.2 M bicarbonate, pH 7.9 (20–50 μg/ml). After addition of an equal volume of glycerol, they were sprayed onto mica disks and rotary shadowed.

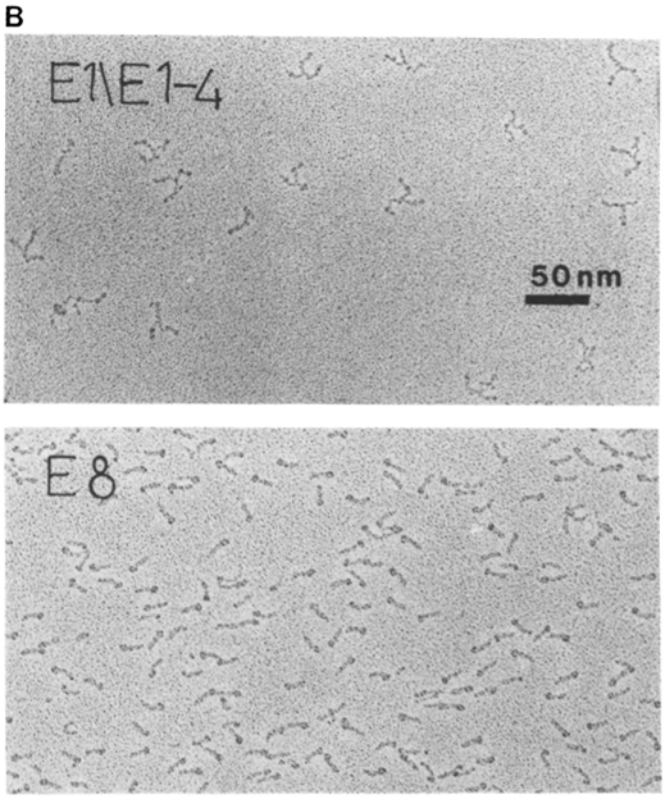
Competitive ELISA for E8 was performed by coating 96-well polystyrol plates (Nunc, Roskilde, Denmark) with 1 μg/ml E8 in PBS, saturating with BSA/PBS containing 0.02% Tween 20, and incubating with anti-E8 antiserum in the presence of serial dilutions of competing proteins for 1 h at 37°C. After washing with BSA/PBS/Tween, bound antibody was detected with horseradish peroxidase–conjugated goat anti-rabbit IgG (1:5000 dilution; Bio-Rad Laboratories) using tetramethylbenzamidine (Miles Laboratories, Naperville, IL) as substrate.

Results

Two Distinct Subfragments of Laminin Promote Cell Adhesion as Substrates

To locate the functional domains of laminin responsible for cell attachment and spreading, we prepared laminin fragments using limited elastase digestion (Ott et al., 1982; Timpl et al., 1983; Paulsson et al., 1985) and separated them using column chromatography (Fig. 1). The fragment E8, comprising a major part of the long arm, was purified from contaminating E1–4 by chromatography on heparin-Sepharose. The fragments were screened as substrates for cell adhesion and spreading, and the results are shown in Table I. Only two fragments, E1/E1–4 and E8, showed significant adhesion- and spreading-promoting activity. The fragments were essentially homogenous, as assessed by the discrete peaks seen in column chromatography (Fig. 1 *A*), unique morphology in rotary shadowing electron microscopy (Fig. 1 *B*), their behavior in SDS-PAGE (Fig. 1 *C*), and by competitive ELISA (Fig. 5 *B*). In agreement with earlier studies (Ott et al., 1982) we found that in the E1/E1–4 preparations some fragments lacked one or more arm-terminal globular domains, as seen in the double band in SDS-PAGE. We will describe this mixture as E1–4, the major component, throughout the text. In E8 the truncated laminin A and B chains are not covalently linked and separate under SDS-PAGE to give an ~140-kD and an ~80-kD band (Deutzmann, R., manuscript in preparation). Pepsin treatment of E1–4 produces a P1 fragment of ~250 kD that runs anomalously slowly (Fig. 1 *C*, lane *2*). By contrast, pepsin digests E8 to low molecular mass material (Fig. 1 *C*; lane *4*).





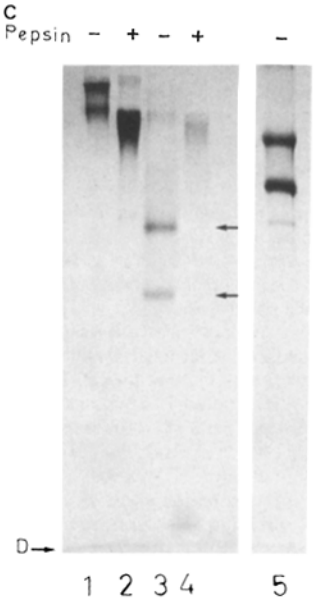


Figure 1. Preparation of laminin subfragments. Laminin was treated with elastase and chromatographed on an agarose 1.5-M column (*A*). Two fractions, purified using heparin–Sepharose, are shown as rotary-shadowing electron micrographs in *B*; these molecules promoted significant cell adhesion (see Materials and Methods for details). (*C*) SDS-PAGE of E1–4 (lanes *1* and *2*), E8 (lanes *3* and *4*) (nonreducing conditions), and laminin (lane *5*) (reducing conditions), before (lanes *1*, *3*, and *5*) and after (lanes *2* and *4*) treatment with pepsin. 25 μg/ml protein per track. The truncated laminin A and B chains in E8 (*arrows*) are degraded by pepsin. Note absence of E8 chains in E1–4 (lane *1*) and P1 (lane *2*).

Table I. Cell Attachment and Spreading to Elastase Digestion Products of Laminin

	Fragment No.																	
	Laminin		E1-4		E3		E4		E8		25K		Small peptides‡		BSA		Fibronectin	
Cell type*	A§	S	A	S	A	S	A	S	A	S	A	S	A	S	A	S	A	S
Mb	++	++	−	−	−	−	−	−	++	++	−	−	−	−	−	−	++	++
Fb	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	++	++
HeLa	++	++	+	++	±	−	−	−	++	++	−	−	−	−	±	−	++	++
Hep-2	++	++	±	−	−	−	−	−	++	++	−	−	−	−	−	−	++	++
TR126	+	+	±	±	−	−	−	−	+	+	−	−	−	−	−	−	++	++
MDBK	+	+	−	−	−	−	−	−	+	+	±	−	−	−	±	−	++	++
A549	+	±	−	−	−	−	−	−	±	+	−	−	−	−	−	−	++	++
Stro.	++	++	−	−	−	−	−	−	+	++	−	−	−	−	−	−	++	++
Rugli	++	++	+	++	±	−	−	−	++	++	−	−	−	−	−	−	++	++
HT1080	+	++	−	−	−	−	−	−	+	+	−	−	−	−	−	−	++	++

* Mb, primary mouse skeletal muscle myoblasts; Fb, primary mouse fibroblasts; other cell lines as described in Materials and Methods.
‡ Complex of low (<20,000) mol-wt material in digest.
§ A, Adhesion; S, spreading, scored on the following subjective scale: ++, adhesion or spreading comparable to that on laminin; +, adhesion or spreading apparent but weaker than on laminin; ±, some adhesion or spreading visible; −, no adhesion or spreading.
See Materials and Methods for details of the technique.

All cell types that attached to a laminin substrate also attached to the E8 fragment and most of these showed adhesion and spreading on E8 comparable to that seen on laminin (secondary murine myoblasts, TR126, Hela and Hep-2 human and MDBK bovine epithelial, A549 and Stromer human melanoma, Rugli human glioblastoma, and HT1080 human fibrosarcoma established cell lines; Tab. I, Fig. 2). Only Rugli and Hela attached to E1-4. Fragments E4 and 25K were inactive, while E3 showed only slight activity to Rugli and Hela. MDBK and HeLa also showed very weak adhesion on BSA. In the 30-min screen, only murine fibroblasts showed no inclination to attach to laminin, while A549 at-

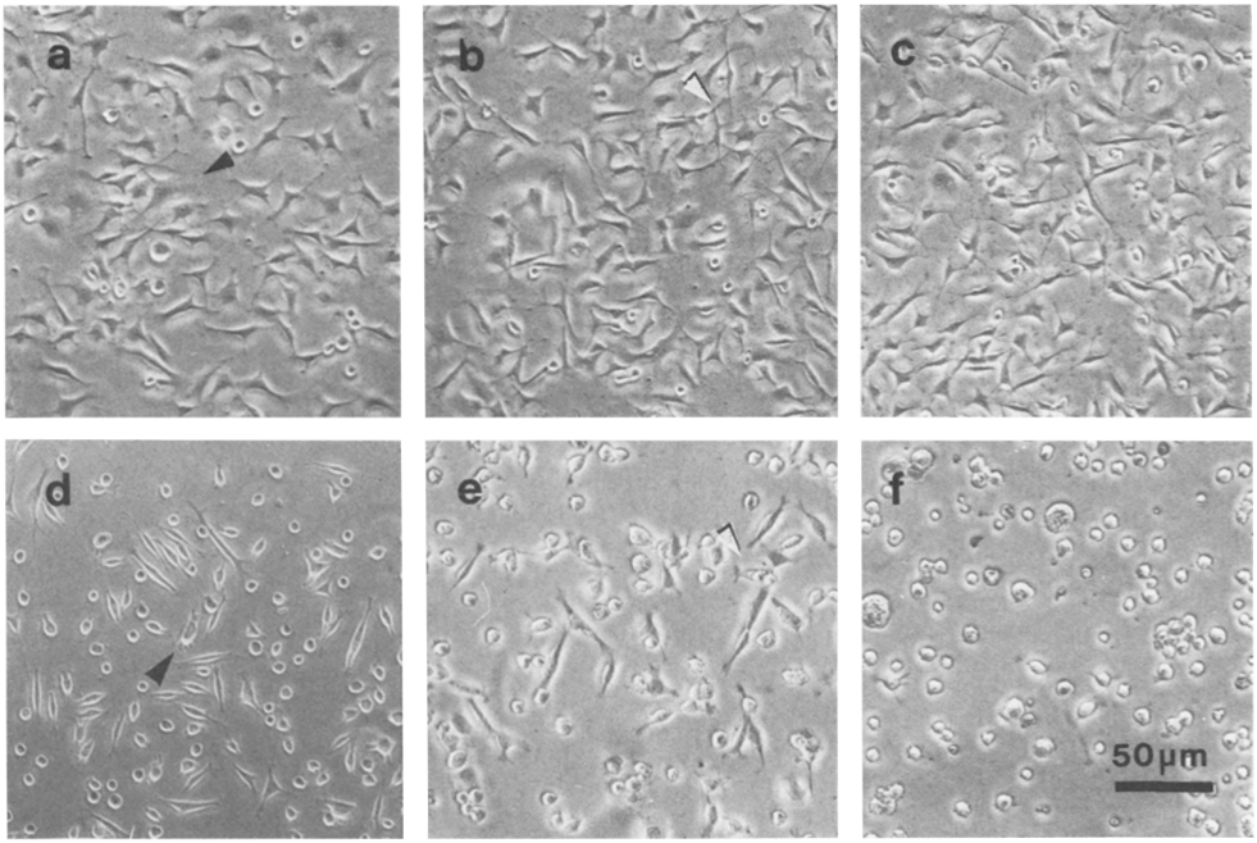


Figure 2. The adhesion and spreading of cells to laminin and its fragments. Rugli glioblastoma cells (*a–c*) and secondary myoblasts (*d–f*), were allowed to attach for 75 min to laminin (*a* and *d*), E8 (*b* and *e*), and E1–4 (*c* and *f*) and photographed without removal of unbound cells. Note, on laminin, Rugli and myoblasts (*a* and *d*) sending out broad ruffling lamellae (*black arrowheads*), note on E8, angular and spindle formed cells (*b* and *e*; *white arrowheads*), and note E1–4 where Rugli spreads (*c*).

tached and spread only poorly. The appearance of myoblasts and Rugli cells on laminin, E8, and El–4 before removal of nonadherent cells is shown in Fig. 2.

On laminin, Rugli (Fig. 2 *a*) and myoblasts (Fig. 2 *d*) rapidly extended ruffling lamellae and flattened. TR126 were poorly spread, and fibroblasts hardly at all (not shown). On E8, Rugli was angular and spindly with fewer cells expressing lamellae. Myoblasts were also more elongated on E8 than on laminin (Fig. 2, *d* and *e*). Only Rugli spread on El–4 and showed a similar morphology as on E8 (Fig. 2 *b* and *c*). On fibronectin each cell type spread rapidly and sent out broad ruffling lamellae (not shown).

Concentration Dependence and Kinetic of Cell Adhesion to El–4 and E8

The attachment to laminin and its fragments of secondary mouse myoblasts and fibroblasts, and of Rugli were studied in detail, and three distinct types of behavior were seen. After 1 h, both myoblasts and Rugli attached well on laminin; but fibroblasts were extremely poorly attached (Figs. 3 and 4; Table II). On E8, both Rugli and myoblasts attached with a concentration dependence similar to that on laminin (Fig. 3). On El–4, Rugli attached but required some two orders of magnitude more protein as substrate to produce the same cell attachment as E8. However, myoblasts did not attach on El–4 (at the highest coating concentration tested, 30 μg/ml). Fibroblasts showed meager attachment to laminin and fragments. All the cells tested could adhere to fibronectin-coated substrates, Rugli and fibroblasts requiring lower concentrations for maximal adhesion than did myoblasts (Figs. 3 and 4, Table II).

The kinetics of adhesion confirmed the concentration data (Fig. 4; Table II). Rugli attached about one-half and one-fifth as fast to E8 and to El–4 as they did to laminin. Myoblasts attached at almost the same rate to laminin as to E8, a quarter as fast as Rugli attached to laminin. They did not attach to El–4. Fibroblasts showed very slow attachment to laminin. These data are summarized and expressed in terms of the molar concentration of the substrates used in Table II. By 24 h the plating efficiency of Rugli onto laminin, fibronectin, E8, and El–4 was >80%.

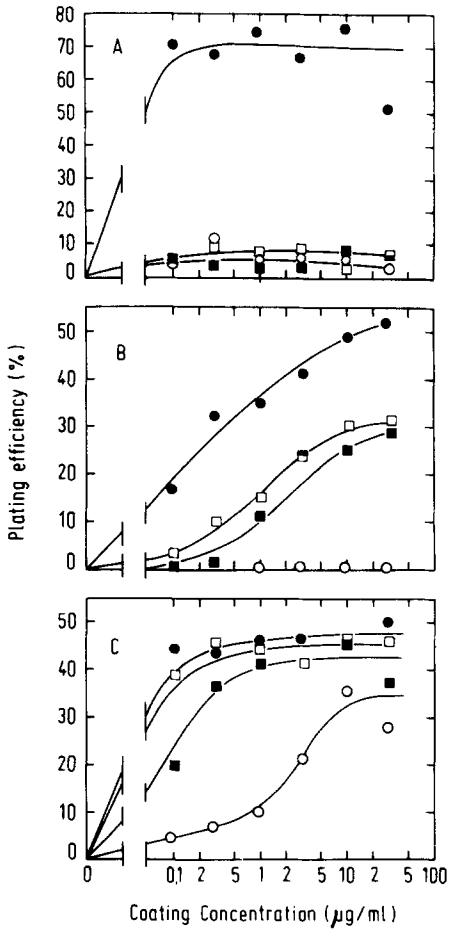


Figure 3. The effect of concentration of substrate molecules on the binding of cells to the substrate. Plastic substrates were coated with protein solutions at the concentrations shown and (*A*) mouse fibroblasts, (*B*) mouse myoblasts, and (*C*) human Rugli glioblastoma cells were allowed to attach as described in Materials and Methods. (*Solid squares*) Laminin; (*open squares*) E8; (*open circles*) El–4; (*solid circles*) fibronectin (see Materials and Methods for details).

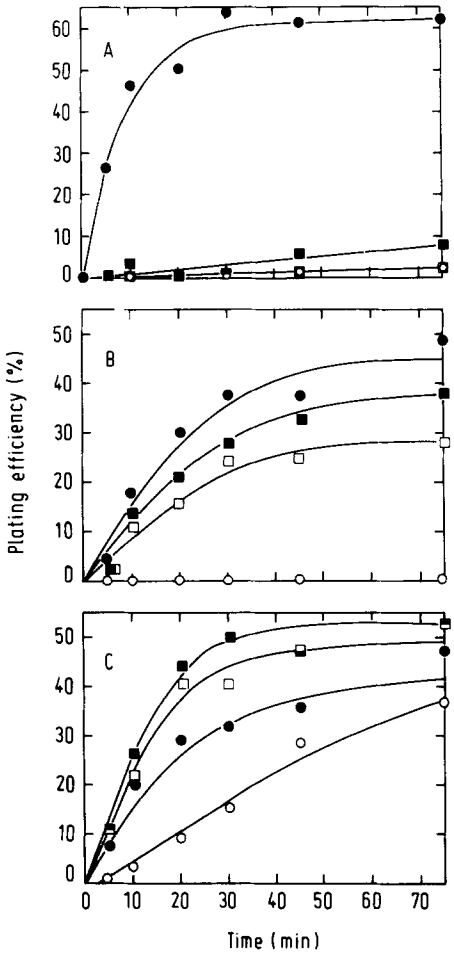


Figure 4. The kinetics of cell attachment to various substrates. The kinetics of cell adhesion to 10 μg/ml protein-coated plastic substrates were measured as described in Materials and Methods. Key and cell types as described in the legend to Fig. 3.

Table II. The Binding of Cells to Fibronectin and Laminin-derived Substrates

Cell type*	$t_{1/2}$‡				$C_{1/2}$§				$A_{1/2}$‖			
	LN	E1-4	E8	FN	LN	E1-4	E8	FN	LN	E1-4	E8	FN
	min	*min*	*min*	*min*	*nM*	*nM*	*nM*	*nM*	*fmol/cm²*	*fmol/cm²*	*fmol/cm²*	*fmol/cm²*
Fb	~360	~3,000	~3,000	6		–	–	<0.1	–	–	–	ND
Mb	30	>6,000	38	16	17	–	18	0.4	8	–	50	ND
Rugli	9	45	18	11	0.14	8.9	<0.2	<0.1	4	63	<1	ND
TR126	76	~350	~600	17	~70	–	–	0.71	100	–	–	ND

* Fb, Mouse fibroblasts; Mb, mouse myoblasts; Rugli, human glioblastoma; TR126, human carcinoma.
‡ Time required for half maximal cell attachment to plastic coated with 10 μg/ml laminin (LN), E1-4, E8, or fibronectin (FN), and BSA blocked (cf. Fig. 4) assuming that the binding curves will attain the highest value reached in the experiment. A linear extrapolation has been made where approximate values are stated.
§ Protein concentrations in coating solution required to produce half maximal cell attachment at 1 h (cf. Figs. 3 and 5). Same assumptions as in ‡.
‖ Quantities of protein adsorbed onto the substrate when maximal cell attachment at 1 h is observed (cf. Figs. 3 and 5). Same assumptions as in ‡.
¶ Where a – is shown, the increase in cell adhesion with increasing protein concentration was too low to be measurable.

Cell Attachment to E1-4 and E8 Cannot Be Explained by Differential Adsorption or Contamination Artefacts

Under the conditions of the kinetic attachment assay ~60 ng/cm² (~70 fmol) of radioiodinated laminin, ~45 ng/cm² (~100 fmol) of E1-4, and ~20 ng/cm² (~70 fmol) of E8 were strongly adsorbed onto Costar wells (i.e., comparable amounts; Fig. 5 *A*). When E8 or E1-4 were treated with pepsin and then used as substrates, the attachment-promoting activity of E8 was reduced to the BSA background levels both for Rugli and for myoblasts, while the activity of E1-4 remained essentially unaltered (Table III). SDS gels of E8 and E1-4 before and after pepsin digestion (Fig. 1 *C*) showed that the A and B chains of E8 were not visible in the E1-4 preparation, and were totally removed from E8 by pepsin treatment. E1-4 was reduced in mass by ~150 kD by pepsin giving a P1 fragment. When the pepsin digest of E1-4 was further purified by HPLC, the cell attachment-promoting activity ran with the P1 fragment (Table III). Competitive ELISA measurements showed that the quantities of E8 present in 50 μg/ml E1-4 and P1 were below 0.2 μg/ml and below 0.01 μg/ml, respectively (Fig. 5 *B*).

E8-specific Antibodies Block Myoblast but Not Rugli Adhesion to Laminin

The attachment-promoting activity of E8 and laminin for myoblasts and E8 for Rugli was abolished by preincubation of the substrate with similar concentrations of E8-specific antiserum (half-maximal inhibitions ~1:3000 dilution for Rugli, ~1:4000 for myoblasts; Fig. 6, cf. Fig. 3 and Table II). But, while anti-E8 antibody could totally abolish myoblast adhesion to laminin, Rugli adhesion was still ~70% of maximum even at high concentrations of antibody (1:50 dilution). Normal rabbit serum had no effect on cell adhesion. Neither sera affected cell attachment onto fibronectin or E1-4 substrates at 1:50 dilution (not shown). These data support the notion that Rugli recognizes two domains in laminin, one in E8 and one in E1-4, while myoblasts only recognize the domain in E8.

The Thermal Stability of Laminin and Its Fragments also Reveals Independent Receptors for E1-4 and E8

E8 and laminin both undergo thermal transitions at around 60°C (Ott et al., 1982; Paulsson et al., 1985). We found that at this temperature the ability of heated laminin and E8 to support myoblast attachment and spreading activity was suddenly lost. Similarly, the ability of E8 to support Rugli adhesion disappears at ~60°C. In marked contrast, Rugli attaches only 20% less well to laminin that has been heated to 95°C for 10 min (Fig. 7, *A* and *B*). When the effect of heat on the adhesion-promoting activity of E1-4 was examined, the reason for this curious result became clear. The activity of E1-4 increased with temperature, undergoing a sharp rise between 40 and 50°C and a gradual increase thereafter. After

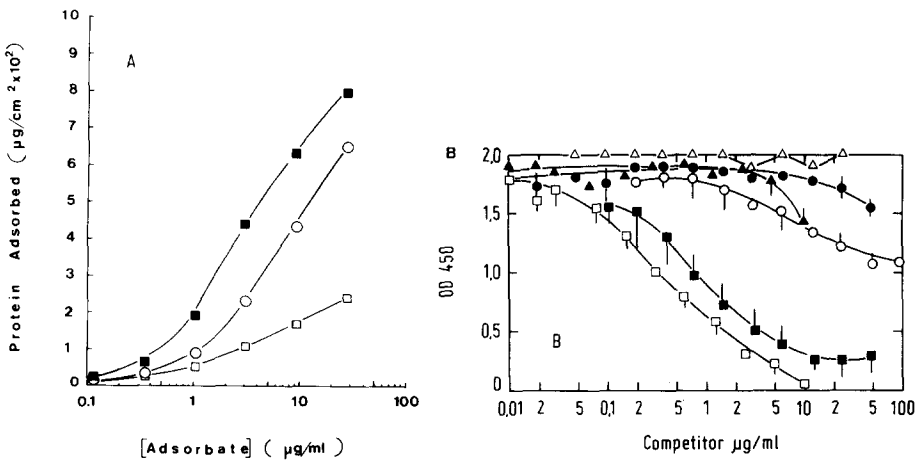


Figure 5. The nature of the adhesive substrate. (A) ^{125}I protein adsorbed onto plastic substrates. Radioiodinated laminin (*solid squares*), E8 (*open circles*), or E1-4 (*open squares*) were serially diluted with carrier protein and adsorbed to Costar wells under the same experimental conditions as used in Figs. 2-4 (see Materials and Methods for details). (B) Competitive ELISA against E8. The ability of E8 (*open squares*), pepsin-treated E8 (*solid triangles*), E1-4 (*open circles*), P1 (*open triangles*), laminin (*solid squares*), and fibronectin (*solid circles*) to compete with anti-E8 antibodies for substrate-bound E8. Uncompeted antibody binding to the plate was detected by horseradish peroxidase second layer antibodies.

Table III. The Binding of Cells to Protease-treated Laminin-derived Substrates

Cell type	Substrate protein	Coating concentration	Plating efficiency on: Untreated protein	Pepsin* -treated protein	Pepsin/HPLC‡ -treated protein
		μg/ml			
Rugli					
	Laminin	10	47.7 ± 5.7	ND	ND
		1	32.0 ± 5.9	ND	ND
		0.1	6.1 ± 4.0	ND	ND
	E8	10	42.5 ± 6.8	3.5 ± 4.8	ND
		1	42.9 ± 8.1	3.0 ± 4.8	ND
		0.1	4.9 ± 3.9	3.5 ± 4.6	ND
	E1-4	10	30.1 ± 5.1	25.0 ± 4.1	20.2 ± 4.0
		1	6.0 ± 3.6	8.8 ± 3.3	5.8 ± 3.5
		0.1	3.5 ± 3.8	4.5 ± 2.9	3.6 ± 2.9
	BSA§	5,000	4.1 ± 3.5	ND	ND
Myoblasts					
	Laminin	10	33.4 ± 7.9	ND	ND
		1	21.0 ± 8.6	ND	ND
		0.1	6.7 ± 6.8	ND	ND
	E8	10	28.6 ± 4.0	6.0 ± 1.6	ND
		1	21.0 ± 3.0	5.9 ± 2.2	ND
		0.1	2.9 ± 1.1	3.0 ± 1.6	ND
	E1-4	10	6.1 ± 8.4	4.5 ± 1.4	ND
		1	5.5 ± 8.3	5.4 ± 3.7	ND
		0.1	5.2 ± 8.0	5.6 ± 1.6	ND
	BSA	5,000	2.8 ± 2.9	ND	ND

The values given are the mean plating efficiency ±SD (n = 4).
* The protein was treated with pepsin before use as substrate.
‡ The pepsin-treated protein was repurified by HPLC before use as substrate.
§ Background cell adhesion.

heating, E1–4 was still not active in supporting fibroblast and myoblast adhesion. Fibronectin showed a gradual decay of activity with heat, half maximal activity being at ~65°C (Fig. 7 *B*). Neither the amount of protein in the coating solutions nor the size of the component peptides on SDS-PAGE was affected by heating (not shown).

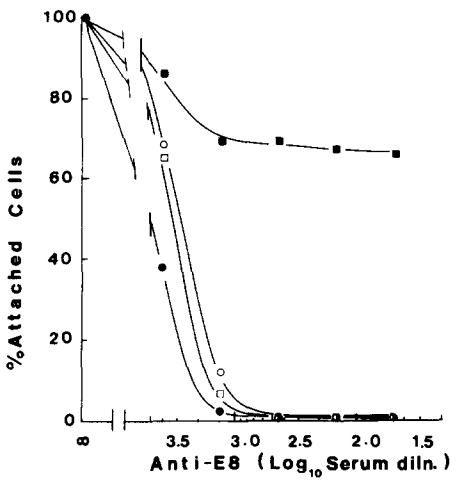


Figure 6. Inhibition of cell attachment on laminin and E8 by anti-E8 antiserum. Laminin (*solid squares, solid circles*) and E8 (*open circles, open squares*) substrates were preincubated with the indicated quantities of anti-E8 antiserum before addition of mouse myoblasts (*solid circles, open circles*), or Rugli cells (*solid squares, open squares*), and attachment assays as described in Materials and Methods. 100% cell attachment represents a plating efficiency of 51 and 40% for myoblasts on laminin and E8, and 70 and 73% for Rugli on laminin and E8, respectively.

Discussion

We have studied the adhesive response of cells to proteolytic fragments of laminin and found that at least one major cell

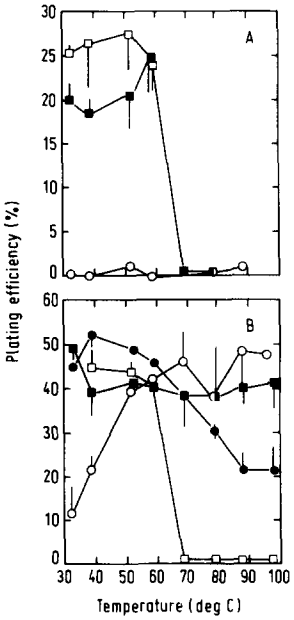


Figure 7. The effect of heat on the ability of substrate molecules to promote cell adhesion. Solutions of laminin (*solid squares*), E8 (*open squares*), E1–4 (*open circles*), and fibronectin (*solid circles*), were heated, adsorbed onto Costar wells, and used in 45-min attachment assays for myoblasts (*A*) or Rugli (*B*), as described in Materials and Methods. Error bars show standard deviations (n = 3).

attachment site lies within the E8 fragment. We describe cells that recognize only E8, both E8 and the previously described binding site in El–4 (Terranova et al., 1983; Timpl et al., 1983), or neither. E8 has previously been described as promoting outgrowth of neurites (Edgar et al., 1984) and inducing tyrosine hyroxylase activity in chromaffin cells (Acheson et al., 1986). However, as we show here, E8 is also an attachment factor for nonneuronal cells.

All the cells we examined that can adhere and spread on laminin can adhere and spread on E8. However, some cells able to adhere to whole laminin could not adhere to the El–4 fragment (myoblasts, Stromer, Hep-2); others (Rugli, Hela) could adhere to both. We studied in detail the attachment of cells possessing low affinity (fibroblasts) or strong binding affinities for one (myoblasts), or two (Rugli) laminin fragments.

When we considered the molar amounts of protein adsorbed onto the substrate, more E8 than laminin was needed to stimulate myoblast adhesion (Table II) and less E8 was needed to stimulate Rugli; the reason for this is not clear. Approximately two orders of magnitude more El–4 on the substrate was required to promote attachment similar to that of E8. This may be due to altered conformation of El–4 cell attachment sites upon adsorption, the low affinity of cellular receptors for El–4, or their lower number.

We find that myoblasts adhere more rapidly to fibronectin than to laminin. Kühl et al. (1986) have reported that adhesion of myoblasts to mixed laminin/type IV collagen substrates is faster than to fibronectin/collagen type I (Kühl et al., 1986). It is possible that the collagen orients laminin and fibronectin in a favorable cell-binding conformation. Certain cells can also express specific receptors for collagen IV and collagen I, and this may be responsible for the enhanced binding of myoblasts onto mixed substrates (Goldberg, 1979; Mollenhauer and von der Mark, 1983; Aumailley and Timpl, 1986). It will not be possible to assess these systems in more detail until the number of receptors per cell and their affinity constants are reported.

Several lines of evidence suggest that the differential activities we ascribe to E8 and El–4 are indeed due to these molecules and not to contaminants. In particular, it is probable that adhesion to El–4 is not due to contaminating E8: (*a*) pepsin treatment of E8 destroys its attachment-promoting activity and greatly degrades the A and B chains as seen in SDS-PAGE and as detected in ELISA while the same treatment of El–4 leaves the activity intact (in agreement with Terranova et al., 1983); (*b*) gel electrophoresis and rotary shadowing electron microscopy show that there is no gross contamination of E8 by El–4 or vice versa; (*c*) antibodies directed against E8 block myoblast adhesion to laminin and E8, and Rugli adhesion to E8, but not Rugli adhesion to laminin; (*d*) heating of E8 (but not of El–4 or whole laminin) destroys its cell attachment–promoting activity; and (*e*) competitive ELISA reveals that contaminating E8 in El–4 is below the 0.2% wt/wt level and in Pl below the 0.01% level.

A working hypothesis developed to explain our attachment data is that two separate cell attachment sites are present in laminin and that two complementary cell surface receptors interact with them or that there is one receptor whose specificity is modulated for E8 and El–4. This hypothesis is supported by antibody blocking and thermal inactivation experiments. Specific antiserum against E8 (Paulsson et al., 1985) abolished the adhesion of myoblasts both to laminin and to E8, and the adhesion of Rugli to E8, but only slightly blocked Rugli adhesion to whole laminin. This confirmed the attachment data; myoblasts can only use E8 for attachment, Rugli recognize both E8 and El–4. The high plating efficiencies of Rugli on E8 and El–4 suggest that at least a majority of the cells are expressing receptors for both domains.

What structure in the long arm bears the cell-binding domain? When laminin or E8 were heated and used as substrates for myoblast adhesion, a sharp cutoff in activity when the temperature rose from 57 to 68°C was observed. Ott et al. (1982) and Paulsson et al. (1985) found that denaturation of the α-helices in the long arm occurs in the same temperature range. However, both Edgar et al. (1984) and Acheson et al. (1986) report that antibodies directed against an E8 terminal globular fragment, E3, perturb cell laminin interactions. Using rotary-shadowing electron microscopy, we have observed aggregation of E8 about the globular domains between 60 and 70°C. Thus, whether the cell-binding site lies in the arm or the globular domain remains undefined.

The ability of laminin to support Rugli cell adhesion after heating decreased only slightly. This appears to be due to an increase in the binding activity of El–4 when heated from 39 to 57°C. These data support the observation of Johansson et al. (1981) that binding of hepatocytes to substrates of boiled laminin remains unchanged. Does the jump in El–4 activity on heating reflect its changing from a ligand for laminin receptors to one for fibronectin receptors (cf. the fibronectin and vitronectin receptors; see Ruoslahti et al., 1985 for discussion)? This seems unlikely as neither murine fibroblasts nor myoblasts could bind even to heat-treated El–4.

It has been suggested that certain avian cells bind both fibronectin and laminin over a similar system, the CSAT/integrin receptor (Horwitz et al., 1985). All the cells we examined bound to fibronectin regardless of their specificity for laminin and its fragments. Thus, either a CSAT/integrin-like molecule (Pytela et al., 1985; Horwitz et al., 1985; Horwitz et al., 1986) mediates cell attachment to fibronectin and is modified to expand its specificity, or at least one other discrete and independent receptor for laminin exists. Given the data presented here the latter possibility must be favored, the E8 receptor being a candidate. Our unpublished preliminary studies suggest that both whole laminin and E8 bind the 68-kD muscle receptor (von der Mark and Kühl, 1985); the situation with El–4 is not yet resolved. Antibodies that block cell binding to E8 have little effect on cell binding to fibronectin or to El–4. As yet there has been no report of an RGDS-like sequence in laminin. Concerning Rugli binding, the thermal inactivation profile of fibronectin (acting through an RGDS sequence) is very different both from that of E8 and of El–4, perhaps arguing against the involvement of RGDS-like sequences (Pierschbacher and Ruoslahti, 1984) in E8–cell interactions.

What might the biological function of a second class of receptors for laminin be? One possibility is that it reflects the process of malignant transformation. The transformed cell line Rugli possesses two laminin receptors, whereas secondary myoblasts possess only one. Greater expression of one 68-kD laminin-binding molecule may allow metastasizing

cells to better adhere to and migrate through exposed basement membrane (Terranova et al., 1983; Barsky et al., 1984). The presence of other laminin receptors may alter the affinity of cells for laminin or modulate either the locomotory systems of the cells or their interaction with other extracellular matrix components. Whether multiple receptor classes for laminin are unique to tumor cells and lead to aberrant behavior is an interesting but still open question. It will be crucial to identify the changes in cell biochemistry and behavior that follow the binding of these fragments to their receptors.

In summary, we have presented evidence for a binding site on the E8 region of laminin that can support the adhesion and spreading of several nonneuronal cells. Our data suggest that cells can differentially recognize, and thus express, receptors for this site and for another on E1-4. If cell binding to these different receptors is accompanied by different biological responses, a molecule such as laminin may be able to exert complex regulatory effects from the solid phase. Such spatially restricted regulation would be of obvious value during developmental processes and its failure could influence tumor development.

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Note Added in Proof: We have observed that RGDS-containing peptides (gift of E. Ruoslahti, Scripps Clinic, La Jolla, CA) specifically block Rugli and myoblast adhesion to fibronectin but do not affect their adhesion to laminin, E8, or E1-4.

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