

# Multiple Cell Surface Receptors for the Short Arms of Laminin: $\alpha_1\beta_1$ Integrin and RGD-dependent Proteins Mediate Cell Attachment only to Domains III in Murine Tumor Laminin

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**Abstract.** Cell surface molecules that interact with the cross formed by the three short arms of murine tumor laminin were studied using thermal perturbation, antibody and peptide blocking, and affinity chromatography. Several potential receptors for the laminin short arms were revealed that differed from those mediating cell attachment to the E8 (long arm) fragment.

Two cell lines, Rugli and L8 attached well to E1-X (short arm) fragments of laminin. This attachment was blocked by antibodies against  $\alpha_1$  integrin chains. Other cells were unable to attach strongly to E1-X, but attached to P1. This attachment was unaffected by anti- $\beta_1$  integrin antibodies, but specifically blocked by the peptide GRGDS. By contrast, binding of Rugli cells was RGD independent and blocked by anti- $\beta_1$  integrin antibodies. G7 and C2C12 myoblasts were very sensitive to GRGDS ( $ID_{50} \sim 2 \mu\text{g}\cdot\text{ml}^{-1}$ ) for attachment to P1 which implied that a non- $\beta_1$  series integrin, possibly  $\alpha_v\beta_3$ , was involved. On heat denaturation of P1<sub>3</sub> attachment remained sensitive to RGDS and  $ID_{50}$  was unchanged. On heat denaturation of E1-X, attachment

remained sensitive to RGDS but the  $ID_{50}$  increased to  $\sim 200 \mu\text{g}\cdot\text{ml}^{-1}$ .

Cellular  $\beta_1$  integrins were retained on laminin affinity columns. A  $\beta_1$  integrin with an  $\sim 190$  kD  $\alpha$ -chain could be isolated from Rugli cells whose attachment could be blocked by anti- $\alpha_1$  antibodies and not from cells blocked by RGDS peptides. Anti- $\alpha_1$  antibodies blocked Rugli attachment to native laminin, but only when the E8 cell binding sites on laminin were also blocked. Thus, a receptor related to  $\alpha_1\beta_1$  integrin can function simultaneously with a receptor for E8. Anti- $\alpha_1$  also blocked attachment to heated laminin, suggesting that the heat-stable attachment activity in laminin involved the E1-X binding site.

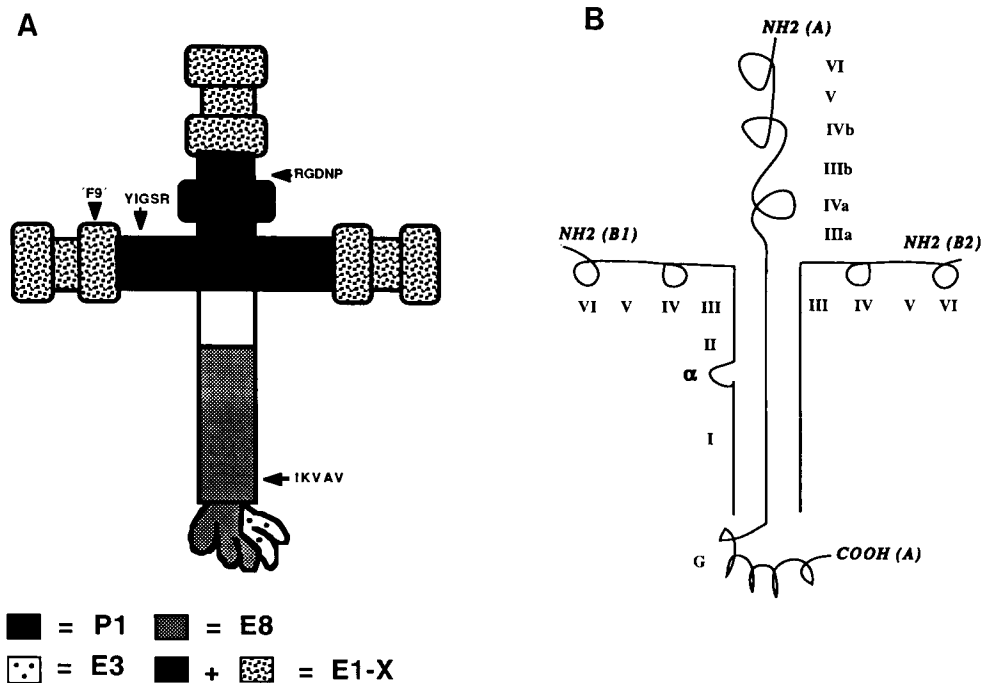
Thus, at least two putative receptors mediate attachment to the short arms of laminin. One, related to  $\alpha_1\beta_1$  integrin, recognizes RGDS-independent sites in E1-X defined by P1 (within domains III, IIIa, IIIb), and one is an RGD-dependent molecule recognizing sites in P1, and is not a  $\beta_1$  integrin.

LAMININS are major glycoproteins of the basement membrane (Chung et al., 1977; Timpl et al., 1979; Beck et al., 1990) and have many differential effects on cell behavior (Terranova et al., 1980; Baron-Van Evercooren et al., 1982; Darmon, 1982; Terranova et al., 1983; Edgar et al., 1984; Öcalan et al., 1988; Goodman et al., 1989b). To understand the molecular basis of these effects, work has focused on identifying laminin receptors and mapping their targets within laminin (Timpl et al., 1983; Edgar et al., 1984; Goodman et al., 1987; Aumailley et al., 1987; Graf et al., 1987; Dillner et al., 1988; Sonnenberg et al., 1988; Nurcombe et al., 1989).

Murine tumor laminin is a cruciform heterotrimer, possessing an A chain and two dissimilar B chains with a related

domain structure. In the current model (Sasaki et al., 1988; Beck et al., 1990) each chain forms an  $\text{NH}_2$ -terminal short arm before descending the long arm to an A-chain COOH-terminal globule.  $\text{NH}_2$ -terminal globular domains (VI) are separated by EGF-like domains (V) from a second globular region (IV on B chains IVb on A chain), followed by another EGF-repeat like domain (III on B; IIIb on A). This pattern repeats once more on the A chain (domains IVa and IIIa) before A and B helically intertwine in heptad repeats in domain II. The COOH-terminal globule has five subdomains. Major cell attachment sites in laminin are found both in the long arm elastase digestion fragment E8 and in the three short arms (E1-X, P1, and E1 fragments). Pepsin digestion fragment P1 consists of domains III from the A, B1 and B2 chain. The elastase fragment E1-X has in addition to domains III variable amounts of domains IV, IVa, IVb, V, and VI, ("E1-4" [Ott et al., 1982; Goodman et al., 1987] formally described the E1-X subpopulation bearing all these domains, i.e.,

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**Figure 1.** Location of protease fragments, domains, and biologically active peptides within murine laminin. (A) E-Fragments are derived by elastase digestion, and P-fragments by pepsin digestion of murine tumor laminin (Aumailley et al., 1990a). RGDNP begins at residue 1,122 on the A-chain (Sasaki et al., 1988); YIGSR at residue 929 on the B1 chain; the F9 peptide (Charonis et al., 1988) at residue 641 on the B1 chain; and IKVAV within peptide PA 22 at residue 2,100 on the A-chain. (B) Laminin structural domains assigned according to Sasaki et al. (1989).

>>90% of the E1-X population by rotary shadowing EM, for example E1' [Perris et al., 1989]) (see Fig. 1).

Here we focus on the nature of the E1-X and P1 binding sites. A cell binding site, cryptic on the native molecule, has been assigned to a sequence LRGDNP (cf. fibronectin attachment sequence GRGDSP [Ruoslahti and Pierschbacher, 1987]) COOH-terminal of residue 1121 in the A-chain (Sasaki et al., 1988), on the domain IIIa/IVa boundary (Aumailley et al., 1990a). Cells bind this site only after pepsin digestion of laminin (Aumailley et al., 1987; Aumailley et al., 1990a), but others can bind to a site in E1-X (Goodman et al., 1987), a region identified earlier as a cell binding domain (Liotta et al., 1981; Terranova et al., 1983). The binding activity of E1-X is stable over a wide temperature range (Goodman et al., 1987). But there is conflict as to whether the "overt" attachment sites in E1-X are artifacts of preparation (Sonnenberg et al., 1990), and in particular if they are due to contamination of E1-X by laminin, E8, or P1 fragments.

Here we investigate whether the E1-X cell attachment site is artifactual or present in native laminin. We illuminate the relationship between the E1-X and P1 cell attachment sites and demonstrate that cells simultaneously use both the E1-X and the E8 attachment sites.

Several members of the integrin superfamily (Tamkun et al., 1986; Hynes, 1987; Reichardt et al., 1989; Albelda and Buck, 1990) may act as receptors for laminin. Integrins are transmembrane heterodimers that interact with matrix components or with other cells, and in the cytoplasm with the cytoskeleton. They provide a transmembrane signaling system by which cells may respond to their insoluble environment. Integrins including  $\alpha_1\beta_1$  (Ignatius and Reichardt, 1988; Turner et al., 1989; Ignatius et al., 1990),  $\alpha_2\beta_1$  (Languino et al., 1989),  $\alpha_3\beta_1$  (Gehlsen et al., 1988; Gehlsen et al., 1989) and  $\alpha_6\beta_1$  (Sonnenberg et al., 1990), and a novel

$\alpha\beta_1$  complex<sup>1</sup> (Kramer and Marks, 1989; Kramer et al., 1989a,b), have been implicated as laminin receptors.

We show here that (a) there are two distinct attachment sites in E1-X one of which is present and available in intact EHS-laminin; (b) one is apparently recognized by integrins related to  $\alpha_1\beta_1$ , the other uses receptors that are RGD-dependent and non- $\beta_1$ -series integrins; and (c) cells can simultaneously use the  $\alpha_1\beta_1$ -like binding site in E1-X and the E8 site. Thus, there appear to be at least two mechanisms by which cells can attach to the three short arms of laminin, and at least two regions in the short arms used for attachment.

## Materials and Methods

Unless otherwise stated, all chemicals were obtained from Sigma Chemicals (Deisenhofen, Germany). Laminin-nidogen complex was extracted from the EHS tumour as previously described (Paulsson et al., 1987). The P1 fragment was produced by pH 1.9 pepsin digestion of laminin (Aumailley et al., 1990a). The E1-X fragment (Timpl et al., 1983; Paulsson et al., 1985; Goodman et al., 1987) was from Dr. R. Deutzmann (University of Regensburg, Germany). Peptides Gly-Arg-Gly-Asp-Ser-Pro (GRGDSP:  $\alpha_3\beta_1$  integrin fibronectin recognition site [Pytela et al., 1985a]; Boehringer-Mannheim, Mannheim, Germany), Gly-Arg-Gly-Asp-Phe-Pro (GRGFSPL: control peptide; Bachem Chemicals, Bubendorf, Switzerland) and Tyr-Ile-Gly-Ser-Arg (YIGSR; "68K specific sequence"; [Graf et al., 1987] Bachem Chemicals), were stored at  $-20^\circ\text{C}$  in sterile distilled water at  $>20 \text{ mg} \cdot \text{ml}^{-1}$ .

Murine (G7, C2C12) and rat (L8) myoblasts and human fibrosarcoma cells (HT1080) were obtained from American Tissue Culture Collection (Rockville, MD) and cultured as recommended. Rugli rat glioblastoma has been previously described (Goodman et al., 1987; Gehlsen et al., 1988, 1989).

## Anti-integrin Antibodies

Polyclonal antibodies against a 39-mer peptide from the cytoplasmic domain

1. von der Mark, H., J. Dürr, A. Sonnenberg, K. von der Mark, R. Deutzmann, and S. L. Goodman, manuscript submitted for publication.

of the  $\beta_1$  integrin subunit ("anti- $\beta_1$  antibodies") were from Dr. R. O. Hynes (Howard Hughes Cancer Institute, Massachusetts Institute of Technology, Boston, MA) or were prepared as described previously (Marcantonio and Hynes, 1988). Polyclonal antibody against rat  $\beta_1$  integrin was from Dr. D. Gullberg (University of Uppsala, Sweden [Gullberg et al., 1989]).  $\beta_1$  antibodies react in Western blots of Rugli, C2C12, G7, L8, and HT1080 cells with a single band at  $\sim 110$  kD/ $\sim 130$  kD (nonreduced/reduced [nred/red])<sup>2</sup> on SDS-PAGE, and comigrate with the  $\beta_1$  subunit of purified VLA2 (see for example, Fig. 6). Murine monoclonal antibodies against rat  $\alpha_1$  integrin (3A3; Turner et al., 1989) were provided by Dr. S. Carbenetto (Montreal General Hospital, Montreal, Canada), rat monoclonal antibodies against human and mouse  $\alpha_6$  (GOH3; Sonnenberg et al., 1988, 1990) were from Dr. A. Sonnenberg (Netherlands Red Cross Blood Transfusion Service, Amsterdam, The Netherlands).

### Attachment Assays

These were as described previously (Goodman et al., 1987; Öcalan et al., 1988). Proteins (usually at  $30 \mu\text{g} \cdot \text{ml}^{-1}$  in PBS) were coated onto 96-well tissue culture plates for 1 h at  $37^\circ\text{C}$ . Residual protein binding sites were blocked with heat-denatured BSA (2% [wt/vol] in PBS; overnight at  $4^\circ\text{C}$ ). Cells in attachment buffer (0.5% [wt/vol] BSA, DME, HEPES-NaOH (50 mM; pH 7.4)) at  $37^\circ\text{C}$  were added to the 96-well plate ( $\sim 50,000$  cells in  $50 \mu\text{l}$  attachment buffer). After 1 h at  $37^\circ\text{C}$ , the plate was washed on an ELISA-plate washer (Titertek plate washer S8; Flow Laboratories; Mecklenheim, Germany) with PBS, absolute numbers of cells attached were quantitated using a lysosomal hexosaminidase assay (Landegren, 1984) with reference to serially diluted external standards.

The binding of cells to BSA-blocked wells (taken as nonspecific background and subtracted from the data presented here) was consistently under 0.2% of the maximum specific binding (excepting B16F10 where the backgrounds were variable and up to 20% of the experimental maximum. This does not affect the interpretation of the results described here). Experiments were repeated at least three times, and the results shown are from typical representative experiments. Error bars show twice the standard deviation of (usually) four replicate measurements. Rare exceptions are noted in the figure legends.

For P1, the highest activity was obtained when ELISA plates were used ('Maxisorp'; cat. 442404; Nunc; Wiesbaden-Bierbrich, Germany), other tissue culture plastics from Nunc and Falcon Labware gave variable results. For laminin, fibronectin, and other laminin fragments all plates gave similar activities.

For E1-X, the number of cells attaching at a given coating concentration under standard conditions varied between E1-X preparations (see for example, Figs. 3 and 4). This may be due to heterogeneity in E1-X (see Introduction). Thus, the absolute numbers of cells attaching in different experiments need not be directly comparable. However the relative effects (induced by heat or by blocking reagents) are completely consistent.

### Thermal Inactivation Experiments

These were as described previously (Goodman et al., 1987). Protein solutions ( $500 \mu\text{l}$ :  $10 \mu\text{g} \cdot \text{ml}^{-1}$  in PBS) stored on ice in Eppendorf microcentrifuge tubes (Brinkman Instruments, Inc., Palo Alto, CA) were transferred to a heating block. After 10 min at the desired temperature, the tubes were transferred to ice until used for coating and cell attachment assays.

### Antibody and Peptide Blocking Experiments

In these experiments P1 and E1-X were used heated or native at a coating concentration of  $30 \mu\text{g} \cdot \text{ml}^{-1}$ . (a) Antibody blocking experiments were performed by preincubating cells with diluted purified antibody or with diluted hybridoma supernatant, or with diluted serum ascites ( $1 \text{ h} \times 37^\circ\text{C}$ ), before transfer to protein coated plates and attachment assays. The final concentrations of the antibodies used was  $100 \mu\text{g} \cdot \text{ml}^{-1}$  (anti- $\beta_1$  external and internal domains [Gullberg et al., 1989]),  $10 \mu\text{g} \cdot \text{ml}^{-1}$  (3A3; anti- $\alpha_1$  [Turner et al., 1989]), 1:100 diluted ascites (GOH3; anti- $\alpha_6$  [Sonnenberg et al., 1990]). (b) For substrate blocking assays, protein coated plates were preincubated ( $1 \text{ h} \times 37^\circ\text{C}$ ) with serially diluted anti-E8 rabbit polyclonal antisera (Goodman et al., 1987), before washing with PBS and use in attachment assays. An antibody concentration giving  $>95\%$  inhibition of attachment to E8 was selected (1:75 dilution of serum), protein coated plates were preincubated ( $1 \text{ h} \times 37^\circ\text{C}$ ) with this concentration of anti-E8 antise-

rum or of control anti- $\beta_1$  integrin (cytoplasmic domain) antiserum, before antibody blocking experiments as described in (a). (c) For peptide blocking assays, cells were transferred for attachment assay onto plates containing peptides diluted (at least 1:50) with attachment buffer. Rare variations in these dilutions are given in the figure legends.

### Affinity Chromatography

Divalent cation-dependent laminin and collagen binding proteins were isolated essentially according to Gehlsen et al. (1988). Cells in roller bottles were harvested by scraping with a rubber policeman, and washed with serum-free medium. The cell pellet (5–10 g wet weight) was extracted twice ( $1 \text{ h} \times 4^\circ\text{C}$ ) with 2 vol of detergent buffer (buffer A: 50 mM octylglucoside, 1 mM  $\text{MnCl}_2$ , 150 mM NaCl, 1 mM NEM, 1 mM PMSF, 0.1% (wt/vol) sodium azide, 50 mM Tris, pH 7.4). After clearing by high speed centrifugation the supernatant was passed via a guard column of Sepharose CL-4B (Pharmacia, Uppsala) over murine tumor laminin-coupled Sepharose or over human collagen I-coupled Sepharose (both columns CNBr-Sepharose;  $\sim 1\text{--}2 \text{ mg protein ml}^{-1}$  packed bed;  $1 \times 6 \text{ cm}$ ) preequilibrated in buffer B (buffer A but containing 25 mM octylglucoside and 0.1 mM NEM and PMSF). After washing with buffer B, the columns were eluted with buffer B containing 10 mM EDTA and lacking  $\text{MnCl}_2$ , and the EDTA eluant immediately "neutralized" with excess  $\text{MnCl}_2$ . All columns were run at  $20^\circ\text{C}$ . Eluants were concentrated (Centricon; Amicon Corp., Danvers, MA) and stored at  $-20^\circ\text{C}$ , before analysis by SDS-PAGE (Laemmli, 1970) on 7% gels, and Western blotting (Towbin et al., 1979).

### Iodination

Cells were iodinated in situ or in suspension after EDTA treatment (5 mM in PBS, 10 min at  $37^\circ\text{C}$ ) by the coupled lactoperoxidase/glucose oxidase method (Hubbard and Cohn, 1972) to  $\sim 6 \text{ dpm/cell}$ , before detergent extraction as described above for affinity chromatography. Affinity-purified

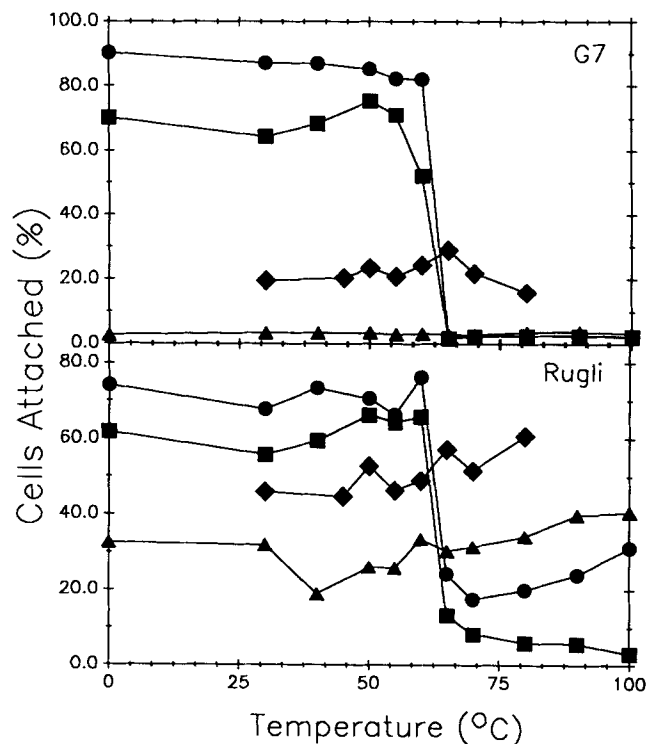
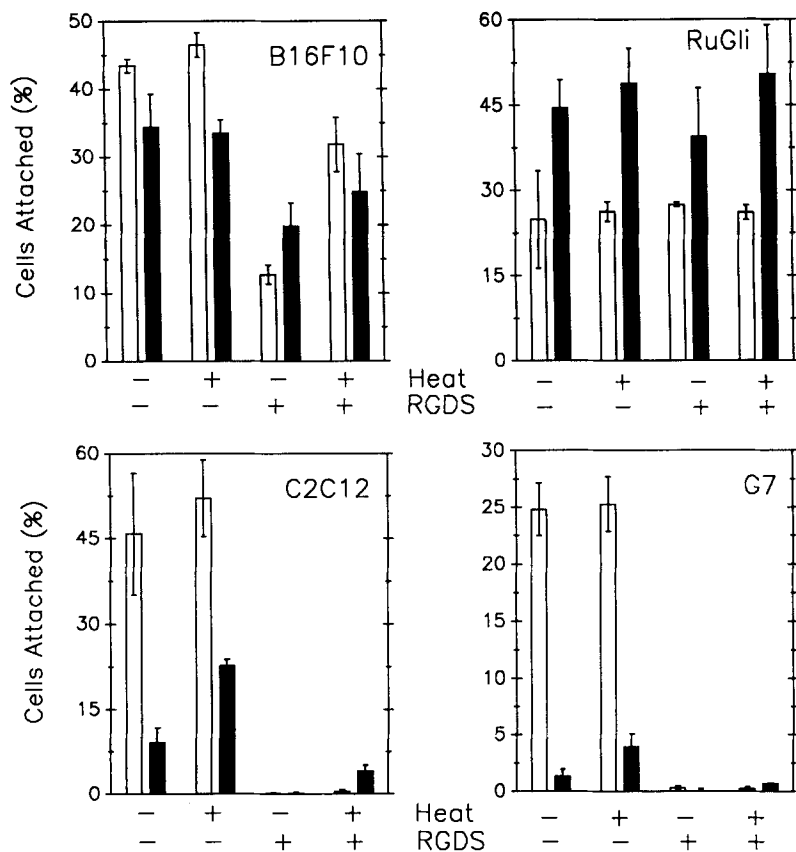


Figure 2. The E8 binding sites of laminin are thermally sensitive. The P1 and E1-X sites are stable. Laminin (circles), E8 (squares), P1 (diamonds), and E1-X (triangles) were diluted to  $10 \mu\text{g} \cdot \text{ml}^{-1}$  before heating to the temperature shown for 10 min. Coating and attachment assays with G7 myoblasts (top) or Rugli cells (bottom) were in duplicate for 1 h at  $37^\circ\text{C}$ . The mean of the duplicate is shown. The activity of E8 drops precipitously between 60 and  $65^\circ\text{C}$ , while P1 and E1-X activity remains stable.

2. Abbreviations used in this paper: nred, nonreduced; red, reduced.



**Figure 3.** Cell attachment to P1 is blocked by RGDS; on E1-X it may or may not be depending on cell type. B16F10, Rugli, C2C12 and G7 cell attachment to substrates coated with  $30 \mu\text{g}\cdot\text{ml}^{-1}$  P1 (open bars) or E1-X (solid bars) was measured in the presence (RGDS+) or absence (RGDS-) of GRGDS ( $500 \mu\text{g}\cdot\text{ml}^{-1}$ ) before (HEAT-) and after (HEAT+) heating to  $80^\circ\text{C}$  (cf. Fig. 2). Error bars =  $2 \times \text{SD}$  of four replicates.

proteins in buffer A were iodinated for 2 min at  $0^\circ\text{C}$  to  $\sim 2 \times 10^6 \text{ dpm}/\mu\text{g}$  by the iodogen method (Fraker and Speck, 1978), and free iodine was removed by passage over a Sepharose-G-25 column ( $1 \times 5 \text{ cm}$ ) pre-equilibrated in buffer A.

## Results

### Cell Attachment Site in Laminin-E8 is Heat Labile in P1 and E1-X Heat Stable

Rugli attached and spread on laminin, E8, P1, and on E1-X. G7 attached and spread on laminin, E8 and P1 but not on E1-X. The attachment activity of P1 was heat stable as was that of E1-X (Fig. 2). By contrast, the activity of E8 was abolished by heating to between  $60$  and  $65^\circ\text{C}$ . The attachment activity of laminin for G7 myoblasts also disappeared between  $60$  and  $65^\circ\text{C}$ . The activity of laminin for Rugli dropped (at between  $60$  and  $65^\circ\text{C}$ ) to a constant value,  $20$ – $40\%$  of the activity of intact laminin.

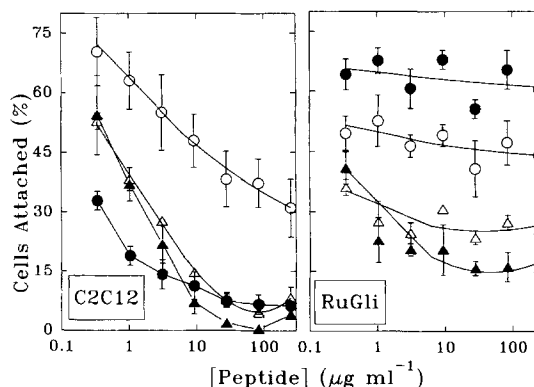
### Two Patterns of Cell Attachment to P1 and E1-X

Cell response to P1 and E1-X fell into two classes (Fig. 3). In one, cells recognized P1 well and E1-X poorly: for C2C12 and G7 mouse myoblasts  $25$ – $50\%$  added cells attached to P1 and  $<10\%$  attached to E1-X. The other class, B16F10, Rugli, and L8 (Fig. 3; Fig. 7), bound both fragments strongly (i.e., consistently  $>20\%$  added cells attached). On E1-X, Rugli flattened and spread well within  $45 \text{ min}$  (Fig. 9). By contrast, on P1, the cells attached but were more rounded. As some cell lines that attached to P1 could not bind to E1-X while others could bind both, we speculated that cells could attach using at least two regions in E1-X.

Multiple cell receptors for E1-X were revealed by observing attachment after heat treatment. There were two classes of cellular response. Heat treatment of E1-X either increased its cell attachment-promoting activity (i.e., for myoblasts; see also Fig. 5), or left it unchanged (i.e., for Rugli, B16F10); it did not increase or decrease P1-dependent attachment activities (Fig. 3). We next investigated the relationship between the sites in E1-X and P1, and the receptors that bound them.

### The Recognition of P1 and E1-X Can Be Mediated by RGD-dependent Receptor(s)

Recognition of RGD sequences characterizes cell attach-



**Figure 4.** Cell adhesion to P1 and E1-X uses receptors with different RGDS sensitivities. Plates were coated with  $30 \mu\text{g}\cdot\text{ml}^{-1}$  of native (closed symbols) or heated (open symbols) E1-X (circles) or P1 (triangles). C2C12 myoblasts and Rugli cells were allowed to attach in the presence of increasing amounts of GRGDS.

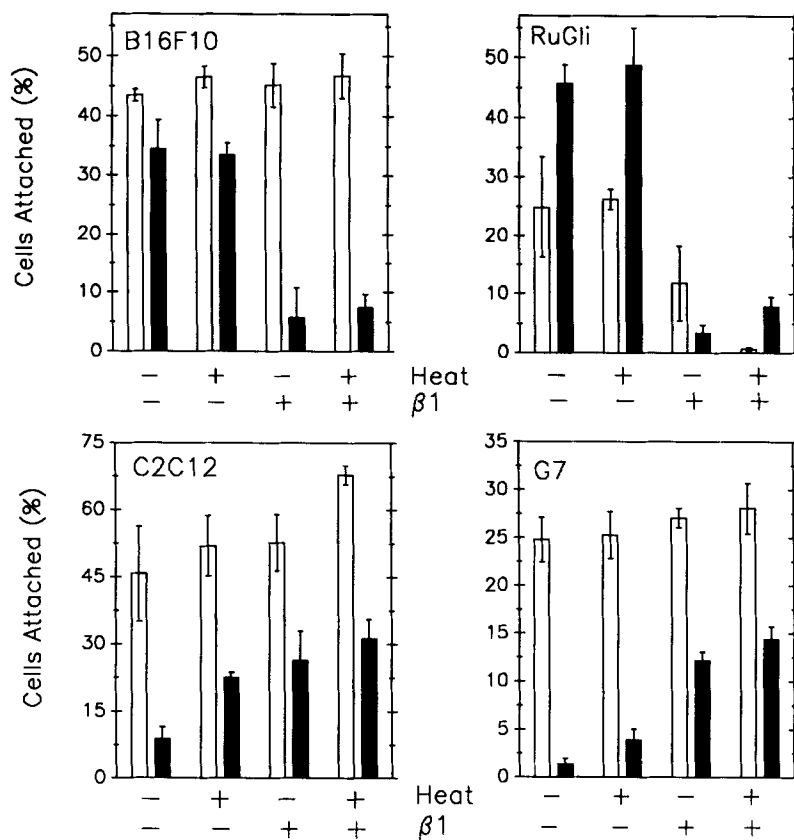


Figure 5.  $\beta_1$  integrins mediated RGD-independent cell attachment to E1-X but not to P1. Cells were allowed to attach to fragments P1 (open bars) or E1-X (solid bars) unheated (HEAT-) or heated to 80°C for 10 min (HEAT+; see legend to Fig. 2) in 100  $\mu\text{g}\cdot\text{ml}^{-1}$  polyclonal antibodies against  $\beta_1$ -integrin extracellular domains ( $\beta_1+$ ), or  $\beta_1$  cytoplasmic domain ( $\beta_1-$ ).

ment to several integrins and RGD peptides can affect attachment to P1 (Aumailley et al., 1990). The A-chain of murine laminin bears an RGD sequence (from cDNA: residues 1120-1125; LRGDNP (Sasaki et al., 1988)). GRGDS abolished cell attachment to P1 and E1-X of C2C12, G7, and of HT1080 (not shown), and inhibited B16F10 (Fig. 3). By marked contrast, attachment of Rugli to E1-X and P1 was not significantly affected by GRGDS (Fig. 3). We studied these responses in detail.

#### Unchanged Sensitivity of Myoblast RGDS-dependent Receptors for Native and Heated P1: E1-X Receptors Become Less Sensitive to RGDS

To identify if the same RGDS-dependent receptors were being used by cells attaching to heated and unheated P1 and E1-X we examined the concentration dependence of the inhibition of attachment on these substrates (Fig. 4).

For C2C12 myoblasts attachment to E1-X, P1, and heated P1 was blocked by GRGDS (concentration for 50% maximum inhibition ( $\text{ID}_{50}$ )  $\sim 2 \mu\text{g}\cdot\text{ml}^{-1}$ ), suggesting that similar receptors were being used. On heating E1-X, the sensitivity to GRGDS dropped by 2 orders of magnitude ( $\text{ID}_{50} \sim 200 \mu\text{g}\cdot\text{ml}^{-1}$ ), suggesting that a different RGDS-dependent receptor was being used, or that the RGD sequence conformation was changed on heating and thus altered its affinity for the "native P1" receptor. Rugli attachment to E1-X was essentially insensitive to GRGDS ( $\text{ID}_{50} \gg 10 \text{ mg}\cdot\text{ml}^{-1}$ ), while attachment to P1 was only partially inhibited ( $\sim 50\%$ :  $\text{ID}_{50} \sim 2 \mu\text{g}\cdot\text{ml}^{-1}$ ). There was minimal inhibition ( $\text{ID}_{50} \gg 10 \text{ mg}\cdot\text{ml}^{-1}$ ) with the control peptides GRGFSPL and YIGSR (not shown) (see Basson et al., 1990). Thus, at least two different attach-

ment specificities were expressed on the cells we studied, one RGD dependent, and one RGD independent.

#### Recognition of P1 and E1-X by $\beta_1$ Series Integrins: Dependence on Cell Type

We next examined whether cell attachment to P1 and E1-X was mediated by  $\beta_1$  series integrins (Fig. 5). On the E1-X fragment, anti- $\beta_1$ -extracellular antibodies strongly blocked ( $>80\%$ ) the attachment of Rugli and B16 cells; for Rugli, blocking was weaker on heated E1-X. By contrast, on P1 fragment (Fig. 5), of the five cells tested, only attachment of Rugli and HT1080 (not shown) was reduced by the  $\beta_1$  extracellular-specific antibody. With control antibody directed against the cytoplasmic domain of the  $\beta_1$ -subunit there was no blocking.

In spite of their specificity by immunoblotting and immunoprecipitation (see Fig. 6), for G7 and C2C12, experimental protein A-purified antibodies stimulated attachment to E1-X, but not to P1, over control. We presume the effect is nonspecific but its basis is not clear. Others have reported such stimulation by anti- $\alpha_2$  and  $\alpha_5$  antibodies (Languino et al., 1989; Sonnenberg et al., 1990).

In summary, anti- $\beta_1$  antibodies had no effect on attachment of mouse muscle cells to P1, but strongly blocked attachment of Rugli and B16 to E1-X and P1.

#### Affinity-isolated Laminin Binding Proteins are $\beta_1$ Integrins

We isolated proteins whose binding to immobilized laminin depended on divalent cations (Fig. 6) (Turner et al., 1987; Gehlsen et al., 1989). Major protein complexes eluted with

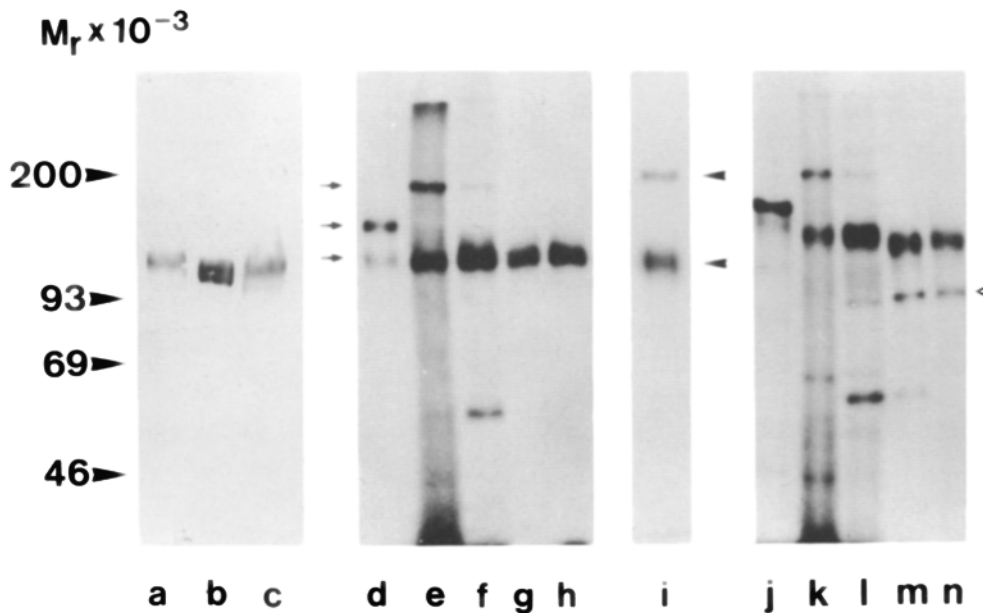


Figure 6.  $\beta_1$  integrins are specifically retained by laminin columns in the presence of divalent cations. Detergent lysates of Rugli, G7, and C2C12, were subjected to affinity chromatography on laminin-Sepharose, eluted with EDTA, and resolved on 7% SDS-PAGE. (a-c) Nonreducing gels were Western blotted and probed with antibodies against  $\beta_1$  integrins (lane a, Rugli; lane b, G7; lane c, C2C12). Eluants were iodinated and nonreducing (f-h) or reducing (l-n) gels were autoradiographed (lanes f and l, Rugli; lanes g and m, G7; lanes h and n, C2C12). The prominent band  $\sim 55/\sim 60$  kD (nred/red) was not surface iodinated and its nature is unknown. Rugli cells were surface iodinated before laminin

affinity chromatography (Gehlsen et al., 1988) and SDS-PAGE under nonreducing conditions (lane i). Only two bands are visible (arrowheads), migrating at the same positions as the  $\beta_1$  integrin (lower) and as the upper band of the rat collagen binding integrin (upper). An alpha-chain from myoblasts runs as a closely spaced doublet over  $\beta_1$  (nonreduced) and  $\sim 95$  kD (reduced: open arrowhead). VLA-2 from human platelets (lane d), and collagen I-binding integrin from Rugli (lane e; probably rat VLA-1 homologue [Ignatius et al., 1990; Tawil et al., 1990]) were markers for rat  $\alpha_1$  (upper arrow),  $\alpha_2$  (middle arrow), and  $\beta_1$  (bottom arrow) integrin. All eluants are rich in  $\beta_1$  integrins. However, only in Rugli eluants are bands characteristic of  $\alpha_1$  visible. The position of  $C^{14}$  molecular weight standards (Amersham, Büchler, Germany) are shown (left).

EDTA from laminin columns, that were identifiable as  $\beta_1$  series integrins. Western blots with  $\beta_1$  integrin-specific antibodies of the C2C12, G7, and Rugli EDTA eluants detected one band (Fig. 6, lanes a-c). GRGDSP ( $100 \mu\text{g}\cdot\text{ml}^{-1}$ ) failed to elute material from these columns (not shown).

After iodination of the affinity purified receptors, SDS-PAGE, and prolonged autoradiography of the gels to amplify the detection of eluted proteins, no bands migrating over the  $\beta_1$  chain were visible under nonreducing conditions (Fig. 6, lanes f-h). For Rugli an additional band of varying intensity at  $\sim 190$  kD (Fig. 6, lane f) was seen. This band comigrated with the upper band of a  $\beta_1$  integrin affinity-purified from Rugli over collagen I (Fig. 6, lane e) i.e., VLA-1- $\alpha$ , and distinct from the  $\alpha$ -chain of platelet  $\alpha_2\beta_1$  integrin  $\sim 165$  kD/ $\sim 150$  kD (nred/red). The band at  $\sim 190$  kD comigrated with the Rugli collagen I-binding integrin upper band under both reducing and nonreducing conditions (Fig. 6). Each EDTA-eluant in silver stained gels gave a band migrating around 110 kD/130 kD (nred/red). This band comigrated with the species blotted by  $\beta_1$ -specific antibodies. This was the major band visible for myoblasts C2C12 and G7 (attaching weakly to E1-X). The  $\beta_1$  band resolved as a clear doublet in silver gels.<sup>1</sup> C2C12, G7 and Rugli eluants under reducing conditions revealed in addition to a 130 kD  $\beta_1$  integrin band, a band migrating  $\sim 95$  kD (Fig. 6, lanes l-n), believed to be a novel  $\alpha$ -chain.<sup>1</sup>

Surface-iodinated Rugli cells passed over the laminin column yielded the  $\beta_1$ -chain, and the upper  $\sim 190$ -kD band (Fig. 6, lane i). The affinity-purified preparations were an estimated  $\sim 70\%$  pure. Although Rugli, G7, and C2C12 variously express  $\alpha_2\beta_1$ ,  $\alpha_3\beta_1$ ,  $\alpha_5\beta_1$ , and a little  $\alpha_6\beta_1$ , these alpha chains are not in affinity-purified laminin receptor prepara-

tions from these cells. Only the  $\sim 130$ - $95$ -kD (red)  $\beta_1$  integrin, but not the  $\sim 130$ - $190$  kD (red)  $\beta_1$  integrin are retained on E8 fragment affinity columns.<sup>1</sup>

#### $\alpha_1\beta_1$ -like Integrin Mediates Rat Cell Attachment to E1-X and to P1

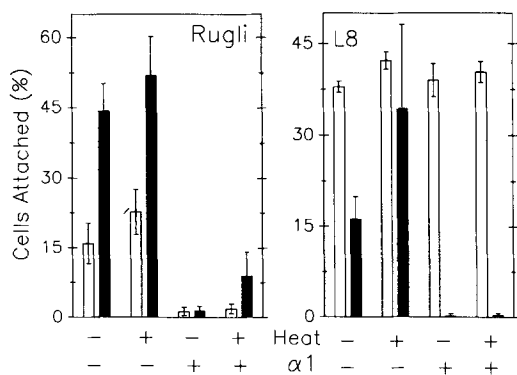
The 3A3 antibody, specific for rat  $\alpha_1$  integrin, blocked ( $>90\%$ ) Rugli and L8 (rat) cell attachment to E1-X. It also blocked Rugli, but not L8 attachment to P1 (Fig. 7). By contrast, control antibodies against  $\alpha_6$  integrin subunits (GOH3; [Sonnenberg et al., 1988]) had no effect on attachment of Rugli, L8, C2C12, G7, HT1080, or B16, either to P1, E1-X or to their heated analogues (not shown) (note that GOH3 does not cross-react with rat).

As  $\beta_1$ -specific antibodies also blocked Rugli attachment to E1-X and P1 (Fig. 5), it seemed plausible that integrin chains related to  $\alpha_1\beta_1$  were involved in Rugli attachment to P1 and E1-X.

#### The Cell Attachment Sites Remaining in Laminin after Heating Are Recognized by $\alpha_1$ Integrins

We assumed that residual cell attachment activity in heated laminin (Fig. 2) corresponded to the thermally stable sites in E1-X. We were able to demonstrate this by challenging Rugli attachment to laminin and to heated laminin with the 3A3 anti- $\alpha_1$  antibody.

3A3 inhibited cell attachment to heated laminin ( $>85\%$ ; Figs. 8 and 9), but on native laminin (and on E8) there was little inhibition, and the cells attached and spread well (Fig. 9). This supports the view that on destruction of the terminally labile E8 attachment sites, the residual attachment ac-



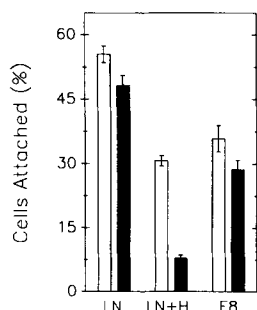
**Figure 7.** Attachment to E1-X and to P1 is blocked by anti- $\alpha_1$  antibody. Cells attached to P1 (open bars) or E1-X (solid bars) unheated (HEAT-) or heated to 80°C for 10 min (HEAT+) in the presence (+) or absence (-) of 10  $\mu\text{g}\cdot\text{ml}^{-1}$  antibody 3A3 against  $\alpha_1$  integrin ( $\alpha_1$ ).

tivity for Rugli in laminin is mediated by the same attachment site found in E1-X, an  $\alpha_1\beta_1$  integrin target.

The morphology of spread cells on laminin in the presence of 3A3 indicated that an  $\alpha_1\beta_1$ -like integrin was involved in recognition of intact laminin. Rugli spread with well-defined lamellae and close cell-cell contacts on laminin, with few cells showing branching "neurite-like" processes (Fig. 9 A, ~9%). On heated laminin, although cells were less well spread, a similar fraction had this morphology (Fig. 9 C, ~11%). By contrast, on laminin in the presence of 3A3 many attached cells bore branching, neurite-like processes (Fig. 9 B, ~40%). Thus, 3A3 evidently manipulated a cell-substrate interaction, presumably over  $\alpha_1\beta_1$  integrin, as cells were attaching to laminin.

#### Rugli Cells Use an $\alpha_1\beta_1$ -like Integrin While Attaching to Native Laminin

It was possible that binding sites for an  $\alpha_1\beta_1$ -like integrin had been generated artifactually in laminin by heating or by the protease treatment necessary to produce E1-X and P1. We tested this by preblocking the laminin-E8 region before Rugli attachment to laminin (Fig. 10). Preincubation of E8 substrates with an anti-E8 antiserum blocked attachment of Rugli ( $\text{ID}_{50}$  ~300-fold dilution of serum). By contrast, laminin substrates could not be blocked >25% by the antiserum (similar  $\text{ID}_{50}$ : Fig. 10, A and B). As previously discussed (Goodman et al., 1987), this indicates the presence of Rugli cell attachment sites distant from E8 within laminin. Anti-E8 antiserum had no effect on Rugli attachment on E1-X, nor did control antisera affect attachment to laminin, E8 or to E1-X (Fig. 10 B). 3A3, directed against the  $\alpha_1$  integrin chain, completely abolished cell attachment onto E1-X, but had little effect on attachment on control antisera-treated E8 and laminin. By marked contrast, Rugli attachment onto laminin was abolished by 3A3 when the substrates were pretreated with the anti-E8 antisera. These results indicate that Rugli used two sites for attachment to laminin, one in E1-X region of the molecule recognized by the  $\alpha_1$  integrin chain, and one in E8 which will be described in detail elsewhere.<sup>1</sup>



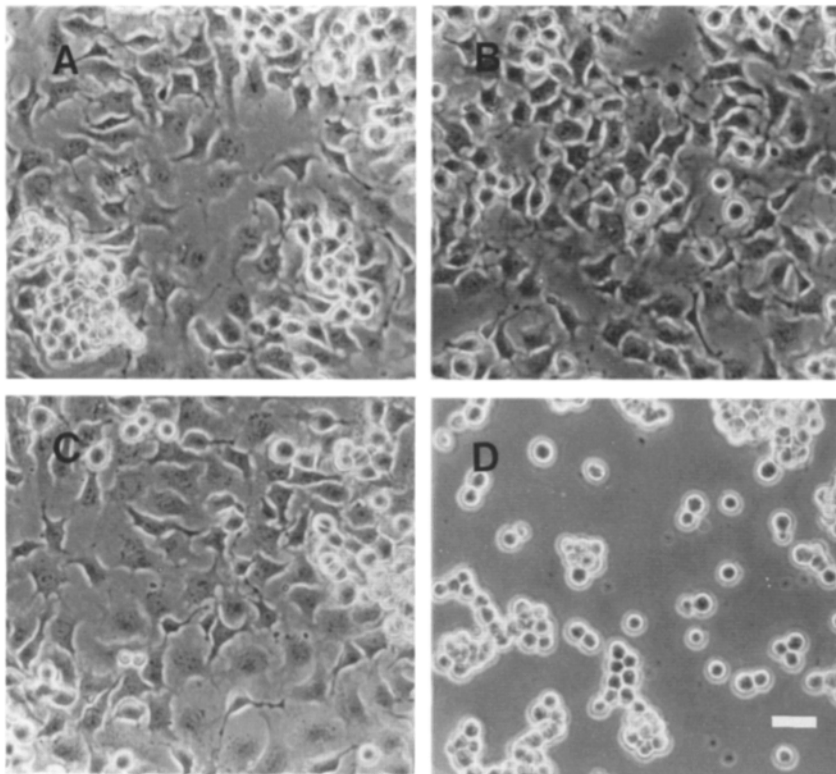
**Figure 8.** Attachment to heated laminin but not to unheated laminin is mediated by  $\alpha_1$  integrins. Rugli cells were allowed to attach to laminin (LN), laminin heated to 80°C for 10 min (LN+H), or to the E8 subfragment (E8) for 1 h in the presence of control antibody (open bars, control: GOH3 ascites diluted 1:250) or of monoclonal antibody against  $\alpha_1$  integrin (solid bars). Rugli are unaffected by 3A3 in attachment to laminin, strongly blocked in attachment to heated laminin (see Fig. 2), and weakly blocked in attachment to E8 (all coatings at 10  $\mu\text{g}\cdot\text{ml}^{-1}$ ).

#### Discussion

In this work we consider cell attachment to the three short arms of laminin. We and others have suggested cryptic (Aumailley et al., 1987, 1990) and noncryptic (Terranova et al., 1983; Goodman et al., 1987, 1989) binding sites for cells exist in the three short arms, but their interrelationship has not been clear. By comparing the blocking activities of anti-integrin antibodies and RGD peptides with laminin affinity chromatograms of cell surface receptors, we have now been able to unequivocally distinguish two classes of receptors recognizing two regions of the short arms. We show (a) that there is a nonartificial cell binding site in the E1-X region of native EHS laminin, (b) that this site and a site in E8 can be simultaneously used by a cell attaching to intact laminin, and (c) that the cryptic P1 site and the E1-X cell attachment site are indeed distinct.

One receptor is related to the  $\alpha_1\beta_1$  integrin because: (a) antibody that recognizes rat  $\alpha_1$  (Turner et al., 1989) blocks rat cell attachment to E1-X, to P1, to heated laminin, but to whole laminin only when the E8 site has been blocked; (b) antibodies directed against  $\beta_1$  integrin block attachment in  $\alpha_1$ -sensitive cells on E1-X and P1; (c) in laminin affinity isolates from Rugli, but not from G7 or C2C12 (i.e., only from cells attaching in an  $\alpha_1$ -dependent manner to E1-X) is a  $\beta_1$  integrin found with a noncovalently associated band at ~190kD/190kD (nred/red), a characteristic of the  $\alpha_1$  chain of VLA-1 (Ignatius and Reichardt, 1988; Turner et al., 1989; Ignatius et al., 1990); (d) immunoprecipitation of cell lysates with  $\beta_1$ -specific antibodies also coprecipitates this band.<sup>1</sup> The anti- $\alpha_1$  antibody used, 3A3, has been used to clone the rat VLA-1- $\alpha_1$  homologue (Ignatius et al., 1990). It specifies rat  $\alpha_1\beta_1$  by antibody affinity chromatography, immunoprecipitation, and cell attachment studies (Turner et al., 1989; this study).

The  $\alpha_1\beta_1$ -dependent site in E1-X is distinct from the cryptic P1 site (Nurcombe et al., 1989) because only the cryptic P1 site is sensitive to RGD-containing peptides. The cryptic site is not created by heating E1-X or laminin; attachment to heated E1-X and P1 remains either anti- $\alpha_1\beta_1$  sensitive, or RGD sensitive, but does not change sensitivity. RGD-sensitivity characterizes several integrins notably  $\alpha_5\beta_1$  and  $\alpha_v\beta_3$  (Pierschbacher and Ruoslahti, 1984; Ruoslahti and Pierschbacher, 1987). The molecular context of RGD affects its recognition (Akiyama et al., 1985; Akiyama and Yamada, 1985; Yamada and Kennedy, 1987). Thus the RGD target receptors we report here are probably integrins.  $\beta_3$  rather



**Figure 9.** Rugli cell attachment to heated and unheated laminin in the presence and absence of anti- $\alpha_1$  integrins. The experiment is described in Fig. 8. Rugli cells after attachment for 60 min to (A and B) laminin or (C and D) heated laminin, in the presence of (A and C) GOH3 antibody (ascites 1:100) or (B and D) 3A3 antibody ( $10 \mu\text{g}\cdot\text{ml}^{-1}$ ). The plates are shown before washing. The rounded and clumped cells are not attached. Note the many branching neurite-like processes in B. Bar,  $10 \mu\text{M}$ .

than  $\beta_1$  series integrins may be involved because of the following. (a) The high sensitivity to RGD ( $\text{ID}_{50} \sim 2 \mu\text{g}\cdot\text{ml}^{-1}$  GRGDS) are characteristic of  $\beta_3$  integrins (Pierschbacher and Ruoslahti, 1987; Cheresh et al., 1989) (sensitivity of  $\alpha_5\beta_1$ :  $\text{ID}_{50} \sim 100\text{--}500 \mu\text{g}\cdot\text{ml}^{-1}$  [Yamada and Kennedy, 1987; Aumailley et al., 1990a]). (b) Anti- $\beta_1$ -antibodies have no effect on RGD-dependent cell attachment to P1. Cells attaching RGD dependently on P1 express  $\beta_3$  series integrins (von der Mark, H., unpublished observations), yet only  $\beta_1$  integrins can be isolated on laminin affinity columns. This correlates with the cryptic nature of the RGD-dependent P1 attachment site of laminin: it is only revealed by proteolysis. (c) The most likely receptor is  $\alpha_v\beta_3$ , the vitronectin receptor, because we find that heating P1 (that may change P1 conformation and so the molecular context of the RGDN sequence) reduces the sensitivity to RGD of cell attachment (Pierschbacher and Ruoslahti, 1987; Perris et al., 1989). Some cell attachment to P1 can only be blocked by combining antibodies to  $\beta_1$ - and  $\beta_3$ -chains (Sonnenberg et al., 1990; Aumailley, M., manuscript in preparation). Attachment characteristics suggest that B16 and L8 cells may be in this class. Weak attachment of C2C12 and G7 on E1-X that is blocked by RGDS may be due to E1-X forms lacking all globular domains (and resembling P1: see Introduction for a description of E1-X).

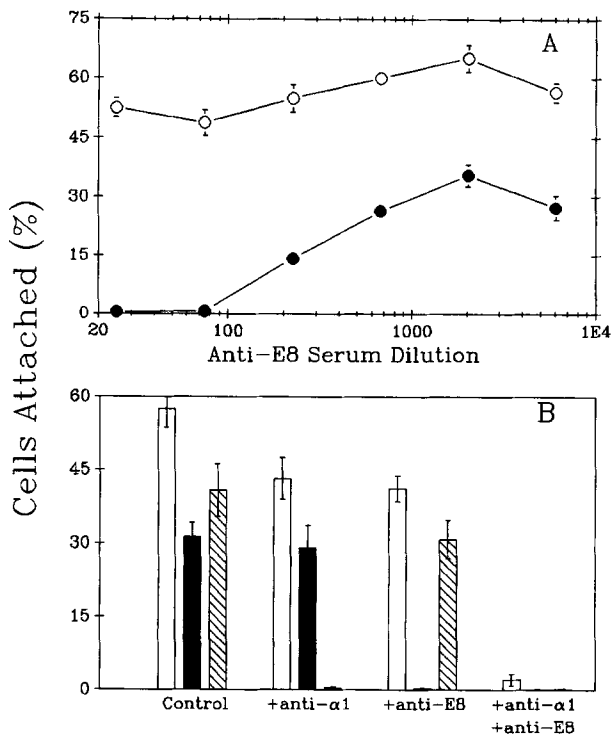
E1-X attachment sites are not artifacts of proteolysis or purification. Antibodies that block the E8 sites do not affect attachment on E1-X or on laminin when the cell bears E1-X receptors. Conversely, antibodies that block the E1-X site do not affect attachment on E8 or on laminin when the cell bears E8 receptors. Heating, which destroys the E8 sites, leaves E1-X and P1 still active. Reports that there is no cell attach-

ment activity in intact E1-X (Sonnenberg et al., 1990) may thus have been greatly exaggerated due to variations in the coating or proteolysis protocols. Other workers confirm that E1-X can support cell attachment (Perris et al., 1989; Hall et al., 1990). Different plastic surfaces can evoke different cell attachment activities from the same fragments. This is not due to different amounts of protein adsorption (Goodman, S. L., unpublished observations). We speculate that different plastics may selectively alter the conformation of the adsorbed protein (Eirich, 1977).

The 3A3 antibody blocks Rugli and L8 rat cell attachment to E1-X and binds a rat VLA-1  $\alpha$ -chain homologue in a  $\beta_1$  integrin (Turner et al., 1989). We isolated a  $\beta_1$  integrin with an  $\sim 190\text{-kD}$  (nred/red)  $\alpha$ -chain from Rugli cell extracts on both laminin and collagen affinity columns. VLA-1 ( $\alpha$ -chain  $\sim 190 \text{ kD}$  (nred/red)) is described as a laminin and collagen receptor (Turner et al., 1989; Hall et al., 1990), however, we have as yet been unable to isolate sufficient  $\sim 190 \text{ kD}$  for microsequencing to confirm its identity as the rat  $\alpha_1$ -homologue (Tawil et al., 1990). It will be interesting to discover whether there is heterogeneity in the "VLA-1-like" collagen and laminin binding populations. A  $\beta_1$ -associated band at  $95 \text{ kD}$  (red) in laminin affinity eluants may be the  $\alpha$ -chain mediating Rugli and muscle cell attachment to E8. This band but not the  $\sim 190\text{-kD}$  (nred/red)  $\alpha$ -chain are retained on E8 affinity columns.<sup>1</sup> It has been reported that murine B16 cells express a VLA-1 that is weakly retained on laminin affinity columns, but apparently it is not used in attachment on laminin (Ramos et al., 1990), which our results tend to support.

Recent reports suggest that regulation of attachment to laminin may be yet more complex. Rat hepatocytes use  $\alpha_1\beta_1$  to attach both to P1 and E8 (Forsberg et al., 1990); we





**Figure 10.** Rugli cells use an  $\alpha_1\beta_1$ -like integrin in attachment to intact laminin. (A) Laminin (open circles) or E8 (solid circles) substrates were incubated with the indicated dilutions of anti-E8 antisera before washing and cell attachment assay with Rugli. Binding to E8 is abolished by the antisera, binding to laminin is only little affected. (B) Laminin (open bars), E8 (solid bars) or E1-X (hatched bars) coated substrates were incubated with 75-fold dilution of antisera against the cytoplasmic domain of  $\beta_1$  integrin (Control; +anti- $\alpha_1$ ) or against the E8 fragment (+anti-E8; +anti- $\alpha_1$  +anti-E8) (as used in A). Cells were preincubated with GOH3 ascites (Control; +anti-E8) or with 3A3 antibody (+anti- $\alpha_1$ ; +anti- $\alpha_1$  +anti-E8). Attachment assays were performed using these cells and the washed substrates. Error bars show 95% confidence limits ( $n = 7$ ).

agree it can recognize P1, but find little indication that  $\alpha_1\beta_1$  is involved in attachment to E8. The basis for these variations is not yet clear.

Both RGD-dependent and  $\alpha_1\beta_1$ -dependent binding sites are in P1, domains III, IIIa, and IIIb (see Fig. 1). Thus, four regions of E1-X may influence cell attachment, an  $\alpha_1\beta_1$  binding site independent of the RGDN site of the A-chain (Aumailley et al., 1990; this study), YIGSR (Graf et al., 1987; Basson et al., 1990), and the F9 peptide (Charonis et al., 1988).

Our data suggest that both  $\alpha_1\beta_1$  integrin and the E8 receptors can function simultaneously during attachment to laminin. We can eliminate the possibility that two populations of cells, one bearing only E8 and one only E1-X-specific receptors exist. If this were the case the effects of combined blocking antibodies should be additive, but in fact they are synergistic (Fig. 10). This seems to imply for attachment that the  $\alpha_1\beta_1$  and the E8 receptors are functionally equivalent and thus redundant, and deviates from the prevailing view that the E8 domain is necessarily the primary cell attachment target (Aumailley et al., 1987, 1990; Goodman et al., 1987; Sonnenberg et al., 1990). The activities we describe may be important in recognition of laminin degrada-

**Table I. Partial Binding Specificities of  $\beta_1$  and  $\beta_3$  Series Integrins**

	LN			FN				
	E8	E1-X	P1	V	IIIc	ColI	ColIV	VN
$\alpha_1\beta_1$		■**	■**					
$\alpha_2\beta_1$	■§					■§		
$\alpha_3\beta_1$	■			■		■		
$\alpha_4\beta_1$				■†				
$\alpha_5\beta_1$				■**				
$\alpha_6\beta_1$	■††, †††	■†††						
$\alpha_7\beta_1$ §§§	■§§	■§§						
$\alpha_v\beta_3$				■††				■****

\* This study.

† Turner et al., 1989.

§ Languino et al., 1989; Elices and Hemler, 1989.

|| Wayner and Carter, 1987.

† Guan and Hynes, 1990.

\*\* Pytela et al., 1985a; Tamkun et al., 1986.

†† Aumailley et al., 1990b; Sonnenberg et al., 1990.

§§ See footnote 1.

||| Kramer et al., 1989b.

††† Sonnenberg et al., 1990.

\*\*\*\* Pytela et al., 1985b.

††† Hall et al., 1990.

§§§ Tentative assignment of the  $\alpha_7$  chain.

tion products arising during development or basement membrane remodeling (Liotta et al., 1984), or in recognition of laminin isoforms (Hunter et al., 1989; Leivo et al., 1989; Paulsson and Saladin, 1989).

At least four sites in laminin can be used for cell attachment, one in the P1 region of E1-X, one only revealed in P1 itself, one in E8 and one in E3 (Sonnenberg et al., 1990). At least six members of the integrin family,  $\alpha_1\beta_1$  (this study; Ignatius and Reichardt, 1988; Turner et al., 1989),  $\alpha_2\beta_1$  (Languino et al., 1989; Elices and Hemler, 1989),  $\alpha_3\beta_1$  (Gehlsen et al., 1988; Wayner and Carter, 1987),  $\alpha_6\beta_1$  (Sonnenberg et al., 1990; Aumailley et al., 1990),  $\alpha_v\beta_3$  (Sonnenberg et al., 1990), and another  $\beta_1$ -series integrin (Kramer et al., 1989a,b)<sup>1</sup> mediate attachment (summarized in Table I). Six laminin receptors (so far) is a surprise. Presumably, cell response to laminin depends on the receptor(s) used. For example, E8 but not other fragments promote locomotion (Goodman et al., 1989; Perris et al., 1989), while P1 promotes growth (Panayotou et al., 1989; Engel, 1989); these fragments are recognized by different receptors. Thus, multiple receptors may allow cells to respond uniquely to a homogeneous matrix environment (e.g., to locomote or stop, to differentiate or grow). Another possibility is that spatially restricted isoforms of laminin may only express a subgroup of integrin binding sites. They could thus mark matrix domains for cells bearing a subset of laminin receptors (Languino et al., 1989; Korhonen et al., 1990; Sanes et al., 1990). In other words, laminin isoforms and laminin receptor expression may play a role in pattern formation. More precise definition of both matrix and receptors in situ may answer these questions. While this work was in a late stage of preparation, we learned of another cell type, the JAR choriocarcinoma line, that also attaches in an  $\alpha_1\beta_1$ -dependent manner to E1 (the elastase-derived P1 analogue [Hall et al., 1990]), and to E1-X, but their attachment requires high coating concentrations of E1-X ( $\sim 120 \mu\text{g}\cdot\text{ml}^{-1}$  vs.  $\sim 30 \mu\text{g}\cdot\text{ml}^{-1}$ ). This may reflect differences in the E1-X

preparation (which can be heterogeneous), or quantitative and qualitative variations in the surface expression of VLA-1 (i.e., species-dependent differences in its structure; JAR cells are human, Rugli and L8 are from rat).

In conclusion,  $\alpha_1\beta_1$  integrin and at least one other RGD-dependent receptor mediate attachments to domains III of laminin. These integrins do not regulate attachment to the E8 fragment. The subsequent functions of these receptors and the reasons for their differential expression must await further experiments.<sup>3</sup>

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3. After submission of this manuscript, Rossino et al. (Rossino, P., I. Gavazzi, R. Timpl, M. Aumailley, M. Abbadine, F. Giancotti, L. Silengo, P. C. Marchisio, and G. Tarone. 1990. *Ext. Cell Res.* 189:100-108) reported that P1 and laminin affinity columns can adsorb  $\alpha_1\beta_1$ -like molecules from rat PC12 cells, strongly supporting the data presented here.

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