

Integrin Recognition of Different Cell-binding Fragments of Laminin (P1, E3, E8) and Evidence that $\alpha 6\beta 1$ But Not $\alpha 6\beta 4$ Functions as a Major Receptor for Fragment E8

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Abstract. The involvement of integrins in mediating interaction of cells to well-characterized proteolytic fragments (P1, E3, and E8) of laminin was assessed by antibody blocking studies. Cell adhesion to fragment P1 was affected by mAbs against the integrin $\beta 1$ and $\beta 3$ subunits and furthermore could be prevented completely by a synthetic peptide containing the Arg-Gly-Asp sequence. Because the $\beta 3$ antibody-sensitive cell lines expressed the vitronectin receptor ($\alpha v\beta 3$) at high levels, the involvement of this receptor in cell adhesion to P1 is strongly suggested. Integrin-mediated cell adhesion to E3 is of low affinity and was inhibited by antibodies against the integrin $\beta 1$ subunit. In contrast, adhesion of some cell types to E3 was not or only partially sensitive to inhibition by anti-integrin subunit antibodies. Cell adhesion to E8 was blocked completely by integrin $\alpha 6$ or $\beta 1$ antibodies. The $\alpha 6$ -specific antibody did not inhibit cell adhesion to E3 or P1. Furthermore, the antibody only blocked adhesion to

laminin of those cells that adhered exclusively to the E8 fragment. In addition, expression of $\alpha 6\beta 1$ was closely correlated with the ability of cells to bind to the E8 fragment of laminin. These results indicate that the $\alpha 6\beta 1$ integrin is a specific receptor for the E8 fragment of laminin. Many cell types expressed, instead of or in addition to $\alpha 6\beta 1$ the recently described integrin $\alpha 6\beta 4$. Although the ligand of $\alpha 6\beta 4$ was not identified, it must be different from that of $\alpha 6\beta 1$, because cells that express $\alpha 6\beta 4$, but not $\alpha 6\beta 1$, do not adhere to E8, and cell adhesion to E8 was specifically blocked by $\beta 1$ specific antibodies. In conclusion, the data indicate that distinct integrin receptors belonging to the $\beta 1$ or $\beta 3$ subfamily are involved in adhesion of cells to the various laminin fragments. Adhesion to E3 may also be brought about by other receptor molecules, possibly proteoglycans, not belonging to the integrin family.

LAMININ is a large (850 kD) adhesive glycoprotein found exclusively in basement membranes and consists of three polypeptide chains, A (400 kD), B1 (225 kD), and B2 (205 kD) (Chung et al., 1979; Timpl et al., 1979; Sasaki et al., 1988; Timpl, 1989). The subunits are linked through disulfide bonds into a unique cross-shaped structure. A wide range of cellular and consequently tissue functions, e.g., spreading, locomotion, proliferation, and differentiation of cells (Martin and Timpl, 1987; Timpl, 1989) are thought to be dependent on interactions of these cells with laminin. Laminin also induces and promotes the outgrowth of neurites (Baron-van Evercooren et al., 1982). Studies with proteolytic fragments of laminin have identified fragment E8, the terminal half of the long arm of laminin, as the site with which neuronal cells interact (Edgar et al., 1984). Subsequent studies indicated that this fragment also serves as an attachment site for many nonneuronal cells (Aumailley et al., 1987; Goodman et al., 1987). Two other proteolytic

fragments which have cell attachment activity are fragment E1-4 (Goodman et al., 1987), corresponding to the three short arms of laminin, and fragment P1 (or E1), which is identical to E1-4 except that it lacks a substantial portion of the structures at the ends of the short arms. The P1 cell-binding site has recently been shown to be cryptic in laminin, and to require proteolytic activation for its exposure (Nurcombe et al., 1989). Still another fragment E3 has been identified as a major heparin binding domain on laminin (Ott et al., 1982) but has so far not been found to be a good cell adhesion substrate (Goodman et al., 1987).

Cell adhesion to laminin and its fragments is mediated by specific cell surface receptors. A family of adhesion receptors for extracellular matrix proteins has been identified on a variety of cells (Hynes, 1987; Ruoslahti and Pierschbacher, 1987). These receptors, referred to as integrins, are transmembrane protein complexes consisting of noncovalently associated α and β subunits. At present, 11 different α subunits

and six different β subunits have been identified. The $\beta 1$, $\beta 2$, and $\beta 3$ subunits can associate with multiple distinct α subunits and define three integrin subfamilies: the VLA protein family (Hemler et al., 1987; Hemler, 1988), the Leu-Cam family (Springer et al., 1987), and the cytoadhesins (Ginsberg et al., 1988), respectively. The VLA subfamily of integrins includes receptors for fibronectin, laminin, and collagens. Members of this family contain one of at least six distinct α subunits, noncovalently associated with a common $\beta 1$ subunit (Hemler et al., 1987; Hemler, 1988; Hemler et al., 1988). Four of these VLA proteins, VLA-1 ($\alpha 1\beta 1$), VLA-2 ($\alpha 2\beta 1$), VLA-3 ($\alpha 3\beta 1$), and VLA-6 ($\alpha 6\beta 1$), have been shown to function as receptors for laminin (Wayner and Carter, 1987; Gehlsen et al., 1988; Ignatius and Reichardt, 1988; Sonnenberg et al., 1988a; Languino et al., 1989; Turner et al., 1989; Elices and Hemler, 1989). An integrin isolated by laminin affinity chromatography from melanoma cells, which has an α subunit distinct from the six known α subunits, but an identical VLA $\beta 1$ subunit, may represent just another member of this VLA protein family involved in cell adhesion to laminin (Kramer et al., 1989). In addition to functioning as laminin receptors, VLA-1 is a receptor for collagen (Kramer and Marks, 1989; Turner et al., 1989) and VLA-3 for collagen and fibronectin (Wayner and Carter, 1987; Takada et al., 1988). The specificity of VLA-2 as a laminin receptor appeared to be determined by the cell type that expresses this molecule. Thus, on platelets VLA-2 functions as a specific collagen receptor (Kunicki et al., 1988; Staatz et al., 1989), whereas on endothelial cells and melanoma cells the same molecule also can act as a laminin receptor (Languino et al., 1989; Elices and Hemler, 1989). The only VLA protein known at present that functions as a specific laminin receptor is VLA-6.

The $\alpha 6\beta 4$ subunit of VLA-6 can also be found in a different heterodimeric complex, together with $\beta 4$ (Sonnenberg et al., 1988b; Hemler et al., 1989; Kajiji et al., 1989). The ligand of the $\alpha 6\beta 4$ complex has not been identified. However, because it shares the $\alpha 6$ subunit with VLA-6, the possibility exists that $\alpha 6\beta 4$ also functions as a laminin receptor. The $\alpha 6\beta 4$ complex can be demonstrated in epithelial cells of a variety of tissues and in Schwann cells (Sonnenberg et al., 1989). Its expression in epithelial cells is restricted to the basal site of these cells, consistent with a matrix-adhesion function for $\alpha 6\beta 4$.

A question of considerable interest is whether all of these receptors interact with the same or with distinct portions of the laminin molecule. To address this question, we have used well-characterized proteolytic fragments of laminin (Ott et al., 1982; Paulsson et al., 1985; Deutzmann et al., 1988; Mann et al., 1988), determined the ability of various cells to bind to these fragments and examined the effects of mAbs against integrin subunits on this cell adhesion. We found that, in addition to the major laminin cell-binding fragments P1 and E8, also E3 can support cell adhesion. Furthermore, we show that the VLA-6 ($\alpha 6\beta 1$) complex mediates cell adhesion to E8, that the vitronectin receptor ($\alpha v\beta 3$) and additional integrin $\beta 1$ receptor(s) are involved in cell adhesion to P1 and that at least two receptors, one of which is a $\beta 1$ integrin, may be involved in cell attachment to E3. We were unable to show that $\alpha 6\beta 4$ can bind to laminin or its fragments.

Materials and Methods

Cell Adhesion Substrates and Synthetic Peptides

Laminin (Timpl et al., 1979) and laminin-nidogen complex (Paulsson et al., 1985) were purified from the mouse Englebreth-Holm-Swarm (EHS)¹ tumor as previously described. Laminin fragment P1 (Ott et al., 1982) was obtained by digestion of the laminin-nidogen complex in 0.1 M glycine-HCl buffer, pH 1.9, for 24 h at 25°C with pepsin (enzyme-substrate ratio 1:100) followed by chromatography on Sepharose CL-6B, at pH 7.2 (Mann et al., 1988). A previously used less rigorous digestion procedure at pH 3 (Timpl et al., 1979) was found to yield fragment P1 preparations that gave less consistent results in cell adhesion and RGD inhibition. Therefore only P1 prepared at pH 1.9 was used in this study. Fragment E1X-Nd, which is similar to E1-4, was obtained from an elastase digest of the same complex (Mann et al., 1988), but needed further chromatography on DEAE cellulose in 2 M urea, 0.05 M Tris-HCl, pH 8.6 (Ott et al., 1982) to reduce its contamination by fragment E8 <1%. This further purification was shown to be essential for experiments with cell lines that require only low concentrations of fragment E8 for adhesion. Preparation of laminin fragments E3, E4, E8, and E10 has been previously described (Ott et al., 1982; Paulsson et al., 1985; Mann et al., 1988). Hexosamine analysis of E10 demonstrated four glucosamines showing that its single carbohydrate receptor site (Sasaki et al., 1987) is occupied by an N-linked oligosaccharide.

All laminin fragments used were analyzed by fragment-specific radioimmunoassays (Ott et al., 1982; Paulsson et al., 1985; unpublished observations) to assess their purity. Their contamination with other laminin structures was usually <1%. Fibronectin was purchased from Gibco-BRL (Grand Island, NY). The synthetic peptide Cys-Asp-Pro-Gly-Tyr-Ile-Gly-Ser-Arg-amide (CDPGYIGSR), which corresponds to a cell attachment site in the B1 chain of laminin (Graf et al., 1987), and the synthetic peptide Gly-Arg-Gly-Asp-Ser-Pro (GRGDSP) derived from the fibronectin cell attachment site (Pierschbacher and Ruoslahti, 1984), were prepared by solid-phase synthesis. We thank Dr. S. Henke (Hoechst AG, Frankfurt, FRG) for supplying the synthetic laminin peptide.

Antibodies

Antisera were raised in rabbits against purified laminin fragments E3, E4, and E8 as previously described (Ott et al., 1982; Paulsson et al., 1985). These antisera showed comparable RIA titers for the fragment used for immunization and an at least 10–20-fold lower binding capacity for the two unrelated fragments. The mouse mAb A-1A5 against the common β subunit of the integrin VLA protein family (Hemler et al., 1983), the mouse mAb TS2/7 against $\alpha 1$ (Hemler et al., 1984) and the mouse mAb B-5G10 against $\alpha 4$ subunit (Hemler et al., 1987) were kindly provided by Dr. M. E. Hemler (Dana Farber Cancer Institute, Boston, MA). A second mAb against the $\beta 1$ subunit (A1IB2) was raised in a rat; it interferes with cell adhesion to fibronectin, laminin, and collagen (Werb et al., 1989). The rat mAb BIE5 recognizes the $\alpha 5$ subunit and inhibits adhesion of cells to fibronectin (Werb et al., 1989). Both mAb A1IB2 and BIE5 were produced at the Department of Stomatology (University of San Francisco, San Francisco, CA).

The rat mAb GoH3 was raised against the $\alpha 6$ subunit; it inhibits platelet adhesion to laminin (Sonnenberg et al., 1987; Sonnenberg et al., 1988a). Rabbit anti- $\alpha 3$ serum prepared against a synthetic peptide corresponding to the COOH-terminal 14-amino acid sequence derived from chicken cDNA clones (Hynes et al., 1989) was a gift of Dr. R. Hynes (Massachusetts Institute of Technology, Boston, MA). The mouse mAb 10G11 directed against $\alpha 2$ (Giltay et al., 1989) and the mouse mAb C17 against $\beta 3$ subunits (Tetteroo et al., 1983) were produced at the Netherlands Red Cross Blood Transfusion Service (CLB), Amsterdam, The Netherlands. The mouse mAb NK1-M9 directed against the αv subunit of the vitronectin receptor was kindly provided by Dr. C. Figdor (Netherlands Cancer Institute, Amsterdam, The Netherlands).

Cells and Cell Cultures

Cell lines used included the human ovarian carcinoma OVCAR-4, the human normal mammary HBL-100, the human mammary tumor T47D, and the human melanoma A375, and the mouse mammary tumor cell lines

1. Abbreviations used in this paper: EHS, Englebreth-Holm-Swarm tumor.

RAC-11P and RAC-34E. The cells were maintained in DME, supplemented with 10% (vol/vol) FCS.

Cell Attachment and Inhibition Assays

Microtiter plates (96-well, Greiner GmbH, Frickenhausen, FRG) were coated with laminin or laminin fragments by incubating each well of the plate for 12–16 h at 4°C with 100 μ l of protein solution diluted in PBS, pH 7.2. The wells were then washed with PBS and incubated with 150 μ l of 1% (vol/vol) BSA in PBS for 60 min to block remaining protein binding sites on the plastic. The efficiency of substrate coating was determined using 125 I-labeled laminin or fragments (Aumailley et al., 1989). In the concentration range of 0.1–10 μ g/ml, 9–10% of the added laminin and 14–16% of the P1 fragment were bound, whereas 14–17% of E3 was bound in the range of 0.1–50 μ g/ml.

Cells from subconfluent, exponentially growing cultures were detached from the flask with 0.25% (wt/vol) trypsin (Difco Laboratories, Surrey, UK), 0.02% (wt/vol) EDTA and were suspended in 10 ml of DME containing 10% (vol/vol) FCS. After centrifugation, the cells were washed twice with PBS and were finally resuspended in 150 mM NaCl at a concentration of $2\text{--}4 \times 10^7$ cells/ml. The cells were radiolabeled with 51 Cr as follows: 0.4 ml of cell suspension was mixed with 0.4 ml (400 μ Ci) of sodium 51 Cr in 150 mM NaCl and 0.4 ml of distilled water and incubated at room temperature for 30 min. The cells were then pelleted, washed twice with PBS and resuspended in DME containing 5 mg of BSA per ml to a final concentration of $1\text{--}2 \times 10^6$ cells/ml. The specific activity of the labeled cells ranged from 1–2 cpm/cell.

0.1-ml aliquots of this cell suspension were added to individual substrate-coated wells and incubated for 30–40 min at 37°C. Nonadherent cells were removed by inverting the microtiter plates onto tissue paper and adsorption of the medium. After the wells had been washed five times with DME-BSA, the bound radioactivity was determined by γ -spectrometry.

In the inhibition assays, cells were mixed with appropriate dilutions of antibodies or synthetic peptides, incubated for 60 min at room temperature and then plated onto coated wells for a 30-min attachment assay.

Labeling and Immunoprecipitations of Cell Surface Proteins

Cells were surface-labeled with 125 I using the lactoperoxidase procedure,

and then 1% NP-40 cell lysates were used for immunoprecipitation as described (Sonnenberg et al., 1987; Sonnenberg et al., 1988b). Immune complexes were collected with Protein A-Sepharose (Pharmacia, Uppsala, Sweden). Affinity-purified rabbit antiserum against mouse IgG and against rat IgG were included in immunoprecipitations, involving the mouse mAbs TS2/7, 10G11, and B-5G10 (IgG1 antibodies) and the rat mAbs GoH3 and BIE5, respectively. The immunoprecipitates were analyzed on polyacrylamide gels according to Laemmli (1970).

Results

Cell Adhesion to Laminin Fragments

The human cell lines that were used in this study were selected because of their unique binding properties to different proteolytic fragments of laminin (for nomenclature and localization of the fragments, see Fig. 1). The proteolytic fragments were generated by digestion of mouse laminin of the EHS tumor with elastase or with pepsin. Because the conditions of proteolysis may slightly vary in treating different samples of laminin, we have tested various batches of these fragments. The cell adhesion activities of the E3 and E8 fragments were essentially the same when different batches of these fragments were used. For fragment P1, consistent results were obtained with material obtained by digestion at pH 1.9.

OVCAR-4 cells showed good binding to the E8 fragment of laminin but adhesion to P1 has also been demonstrated in some experiments. T47D cells had a clear preference for binding to E3 and apparently did not or only weakly bind to other laminin fragments (Fig. 2). The adhesion of OVCAR-4 cells to E8 and the adhesion of T47D cells to E3 showed similar concentration dependence as the binding of these cells to intact laminin. This suggests that the cell-

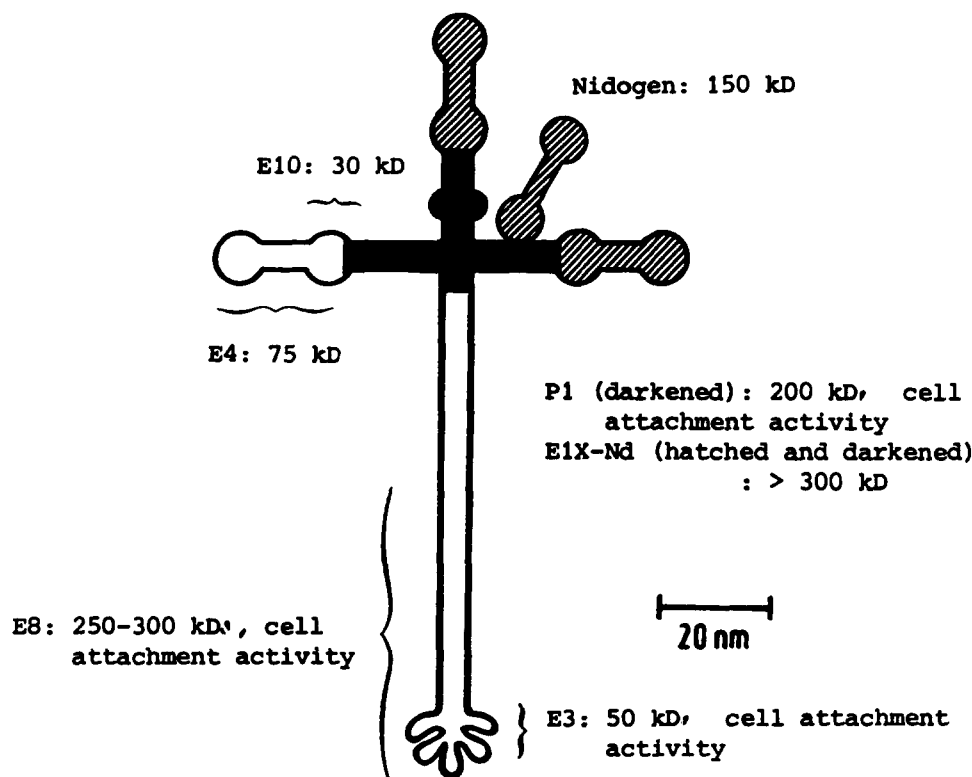


Figure 1. Schematic representation of the localization of laminin fragments in the cross-shaped molecule based on previous studies (Ott et al., 1982; Paulsson et al., 1985; Mann et al., 1988; Deutzmann et al., 1988; Sasaki et al., 1988).

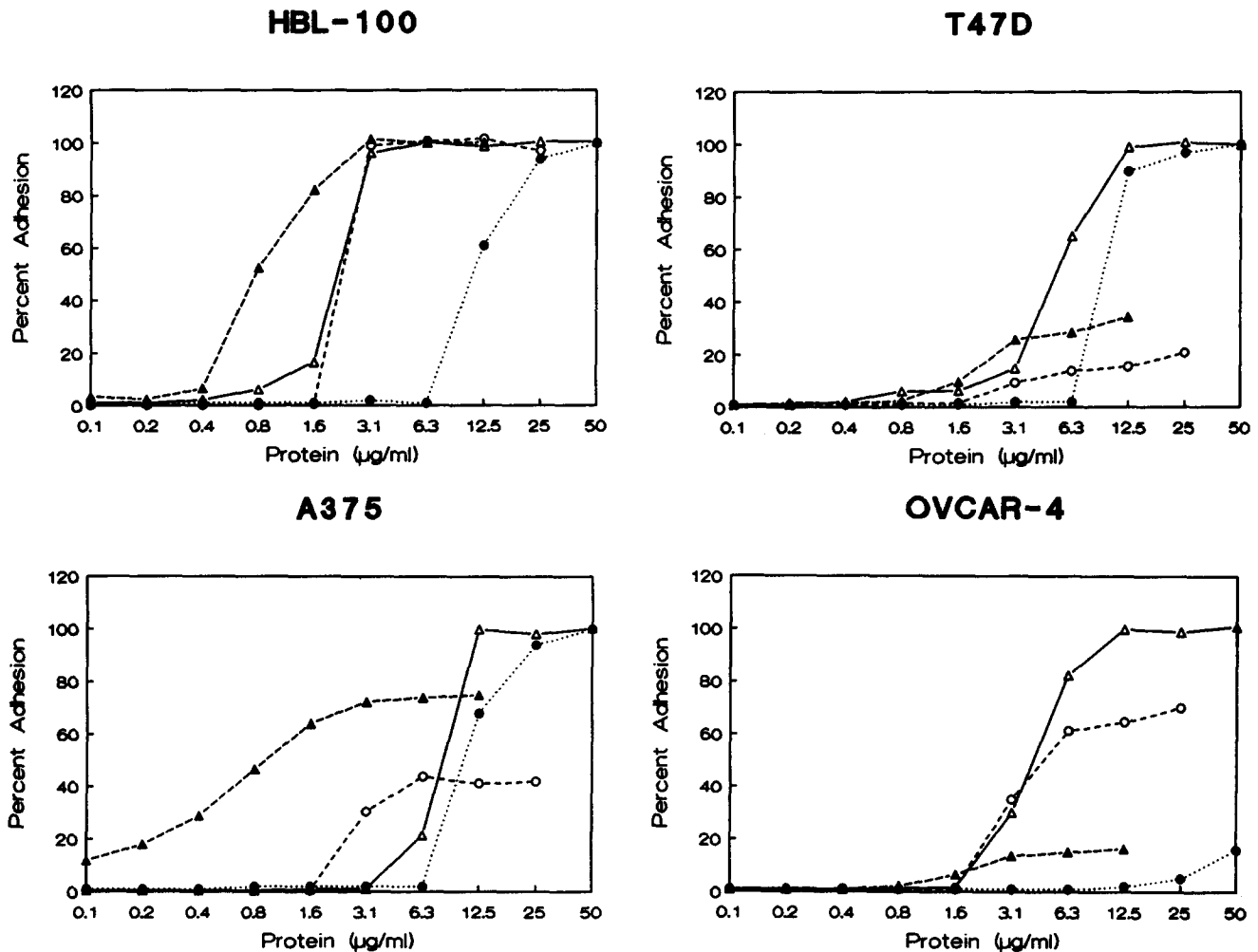


Figure 2. Adhesion of cells to substrates coated with different concentrations of laminin and laminin fragments. A 96-well microtiter plate was coated with laminin (Δ), E8 (\circ), P1 (\blacktriangle), and E3 (\bullet) at the concentrations indicated. Normal human mammary HBL-100, human mammary carcinoma T47D, human melanoma A375, and human ovarian carcinoma OVCAR-4 cells were labeled with ^{51}Cr and allowed to attach at the coated wells as described in Materials and Methods. Total cells bound to wells coated with 50 μg of laminin per ml are indicated as 100%. The data are presented as the mean of triplicate determinations.

binding sites on these two fragments and on intact laminin are very similar or even identical. Important for the detection of E3-mediated cell adhesion was the use of polystyrene microtiter plates coated with E3. Tissue culture plates that have been used in previous studies gave variable results and were found to poorly bind cells. Cells of two other cell lines, HBL-100 and A375, showed binding to P1. The adhesion of these cells to P1 proved to be better than to intact laminin at lower concentrations of protein (Fig. 2). First, when we consider the molar amounts, two to fourfold fewer P1 than laminin molecules were required to support adhesion of A375-cells. This remains true after correction for the difference in coating efficiency for these two molecules. Secondly, particularly with A375 cells but also to a lesser extent with HBL-100 cells, the decline of adhesion at diminishing concentrations was much less abrupt than in the case of laminin.

The greater adhesion-promoting activity of the P1 fragment may indicate the presence of a binding site on P1 that is cryptic in the intact laminin molecule and requires proteolytic activation for its exposure. The existence of such a cryptic binding site was already suggested by Nurcombe et al.

(1989). In addition, HBL-100, and to a lesser extent A375 cells, bound to fragment E8 and showed variable adhesion between different experiments to fragment E3. This variability may be explained by a low affinity of adhesion, because relatively large amounts of E3 were needed to obtain binding. In fact, for HBL-100 cells ~ 10 times as much adsorbed E3 protein was required as substrate to produce similar attachment as to laminin, which is 170-fold more on a molar basis. Laminin fragments E4, E10, and E1X-Nd were found to be inactive substrates for all four cell lines (not shown).

Specificity of Cell Adhesion to Laminin and Laminin Fragments Examined by Antibody Inhibition

The specificity of cell adhesion to the various laminin fragments was examined by antibody blocking experiments using polyclonal antisera against fragments E3, E4, or E8. Strong inhibition of cell adhesion was observed only by antibodies against the corresponding purified fragments (Table I). For example, binding of T47D cells to E3 could be blocked by antibodies against E3, but not by antibodies against E4 or E8.

Table 1. Inhibition of Cell Attachment of Various Cell Lines to Laminin and to Laminin Fragments P1, E3, and E8 by Antilaminin Fragment Antisera

Cell lines	Substrate*	Inhibition of cell adhesion by†		
		Anti-E3	Anti-E4	Anti-E8
%				
HBL-100	Laminin	2	1	0
	P1	2	2	1
	E3	99	9	8
	E8	14	16	98
T47D	Laminin	22	1	19
	E3	98	1	0
OVCAR-4	Laminin	99	13	90
	E8	6	4	87
A375	Laminin	68	9	69
	P1	7	17	19
	E3	99	10	11
	E8	8	7	99

* Laminin and laminin fragments were prepared at concentrations that were shown in Fig. 1 to give plateau binding of cells.

† Cells were labeled with ^{51}Cr and incubated on the coated surfaces in the presence or absence of antiserum against laminin fragments. The inhibitory effect of the antisera is presented as a mean of triplicate determinations and as the percentage of decrease in cell adhesion of that observed in the absence of antibodies. The antisera were used at dilutions of 1:400 (HBL-100 and A375) and 1:200 (T47D and OVCAR-4).

Similarly, binding of OVCAR-4 cells to E8 could be blocked by antibodies against E8, but not by antibodies to E3 or E4. It is also noteworthy that although adhesion of HBL-100 to E3 was variable, this adhesion appeared to be specific as judged from the results of the blocking experiments. None of the polyclonal antisera had a strong effect on cell adhesion to P1.

When the same inhibition experiments were performed with intact laminin or laminin-nidogen as substrates instead of its fragments, inhibition was less specific in that adhesion of OVCAR-4 cells to laminin, which occurs via E8, was also blocked by anti-E3 antibodies. We assume that this is due to steric hindrance of the binding site on E8 by antibodies fixed to E3, since these structures are close to each other in laminin (Deutzmann et al., 1988). Similar inhibition data have been reported for neurite stimulation by laminin which is mainly due to a fragment E8 structure (Edgar et al., 1984).

In contrast, some of the inhibition patterns observed with individual laminin fragments were not observed with intact laminin substrates. For example, the effect of the E3 antibodies on the adhesion of T47D cells to intact laminin was relatively weak when compared with the profound effect of the E3 antibodies on cell adhesion to the isolated E3 fragment. This may suggest that T47D cells also recognize another cell-binding site on laminin, distinct from the one on the E3 fragment. Alternatively, the epitopes on E3 that are involved in binding of the inhibitory antibodies may not be properly exposed on the intact laminin molecule. The same may also be true for HBL-100 cells, because adhesion to laminin cannot be blocked by either one or by the combination of E3- and E8-specific antibodies.

Inhibition of Cell Adhesion by Synthetic Peptides

Two synthetic peptides, GRGDSP (Pierschbacher and Ruos-

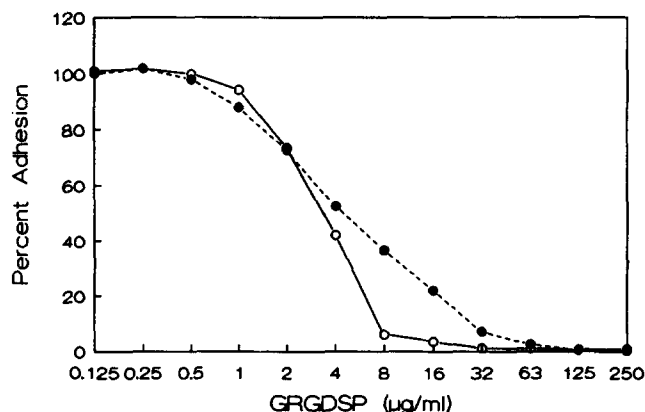


Figure 3. Effect of synthetic peptide GRGDSP on the attachment of A375 and HBL-100 cells to P1. ^{51}Cr -labeled HBL-100 (○) and A375 (●) cells were preincubated with or without GRGDSP peptide and added to the P1 substrates. Total cells bound to P1 in the absence of peptide are indicated as 100%. Data are presented as the mean of triplicate determinations.

lahti, 1984) and CDPGYIGSR-amide (Graf et al., 1987), which were reported to interfere with cell attachment on various extracellular substrates, were studied for their capacity to inhibit adhesion of cells to substrates made from the laminin-nidogen complex or the laminin fragments P1, E8, and E3. The only clear effect was observed with GRGDSP competing against a P1 substrate (Fig. 3). This was observed for both HBL-100 and A375 cells with 50% inhibition requiring only low peptide concentrations ($\sim 5 \mu\text{g/ml}$). GRGDSP did not block cell attachment on laminin-nidogen, E3, or E8, even when used at 30–40-fold higher concentrations (not shown). Peptide CDPGYIGSR-amide, which corresponds to a laminin B1 chain sequence present in fragment P1, showed no or only marginal inhibition on all three substrates when examined in the concentration range 10–1,000 $\mu\text{g/ml}$.

Inhibition of Cell Adhesion to Fragment P1 by Integrin $\beta 1$ and $\beta 3$ Antibodies

The inhibition of cell adhesion to P1 by GRGDSP suggested that integrins were involved in this process. At least two integrins, the vitronectin and the fibronectin (VLA-5) receptor, are known to have ligand specificity for the RGD sequence in matrix proteins. Therefore, we examined whether antibodies against these two receptors interfered with cell adhesion to P1.

As shown in Fig. 4, complete inhibition of cell adhesion to P1 was observed for HBL-100 cells with mAb AIIB2, directed against the common $\beta 1$ subunit of the VLA protein family and for A375 cells with a combination of mAb AIIB2 (anti- $\beta 1$) and mAb C17 which recognizes the $\beta 3$ subunit of the vitronectin receptor. The individual antibodies did not (AIIB2) or only partially (C17) inhibit adhesion of A375 cells to P1. OVCAR-4 cells, which bind only weakly to P1, were sensitive to inhibition by mAb C17, but not by mAb AIIB2 (not shown).

The mAb BIE5, against the $\alpha 5$ subunit of the fibronectin receptor, did not inhibit on cell adhesion to P1. In contrast, BIE5 appeared to increase adhesion of A375 cells to P1. The reason for this is unknown. When BIE5 was combined with either C17 or AIIB2, also no inhibitory activity of

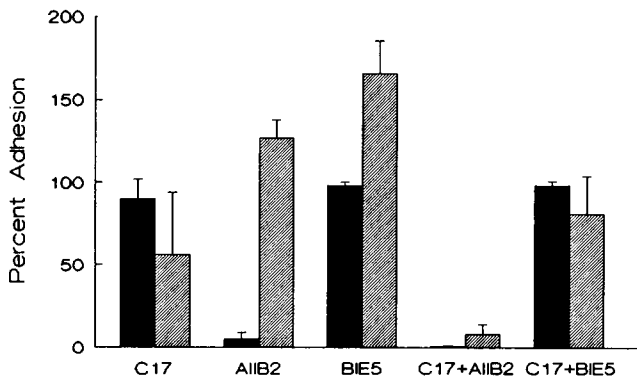


Figure 4. Inhibition of cell attachment to P1 by antibodies against integrin subunits. A 96-well microtiter plate was coated with 10 $\mu\text{g/ml}$ P1. ^{51}Cr -labeled HBL-100 (■) and A375 (▨) cells were preincubated with or without antibody and adhesion assays were performed as described in Materials and Methods. The antibodies were mAb C17, directed against $\beta 3$, mAb AIIB, directed against $\beta 1$, and mAb BIE5, directed against $\alpha 5$. Total cells bound to P1 in the absence of antibody are indicated as 100%. Adhesion was measured in triplicate, and the results from four separate sets of experiments were averaged. Error bars represent SEM of the averaged results from the four experiments.

this antibody could be demonstrated. The BIE5 antibody, however, efficiently blocked adhesion of T47D and OVCAR-4 cells to fibronectin (not shown). Adhesion of HBL-100 and A375 cells to fibronectin could not be prevented by BIE5 or by a combination of BIE5 and C17.

From these results, we conclude that both $\beta 1$ and $\beta 3$ integrin receptors can mediate cell adhesion to P1 and that the $\beta 1$ receptor is not identical to the fibronectin receptor.

Inhibition of Cell Adhesion to Fragment E8 by Integrin $\alpha 6$ and $\beta 1$ Antibodies and Variable Effect of Integrin $\beta 1$ Antibodies on Cell Adhesion to Fragment E3

We have recently shown that a rat mAb (GoH3) against the $\alpha 6$ subunit of human VLA-6 blocked the interaction of platelets with laminin (Sonnenberg et al., 1988a). To determine whether this antibody also interfered with adhesion of cells from the HBL-100, OVCAR-4, T47D, and A375 cell lines to laminin and its cell-binding fragments, inhibition experiments were carried out. As shown in Fig. 5, the antibody specifically blocked cell adhesion to E8. Adhesion to E3 or P1 (latter not shown) was not prevented by the mAb GoH3. Notably, the inhibitory activity of the $\alpha 6$ -specific antibody for E8-mediated cell adhesion was observed for all cell lines that showed binding to this fragment. When studied with laminin or laminin-nidogen substrates, the antibody only blocked the adhesion of those cells which exclusively adhered to the E8 fragment of laminin, e.g., OVCAR-4 cells. The adhesion of HBL-100 and A375 cells, which recognize several cell-binding sites on laminin, was either not or only partially affected by the GoH3 antibody. Also, adhesion of T47D cells, which probably bind exclusively via the E3 cell-binding site to laminin was not affected by the mAb GoH3. Thus, it seems that GoH3 blocks cell adhesion to laminin only when the interaction of cells is mainly due to the E8-cell-binding site.

The $\beta 1$ -specific antibody, mAb AIIB2, gives exactly the

same results as GoH3 for the E8 cell-binding site. As GoH3 and AIIB2 are directed against different parts of the $\alpha 6\beta 1$ complex, i.e., to the $\alpha 6$ -chain and to the $\beta 1$ -chain, respectively, binding of cells to E8 is probably mediated by $\alpha 6\beta 1$ and not by $\alpha 6\beta 4$, which also occurs on these cells (see below). In addition, the $\beta 1$ -specific antibody blocked adhesion of A375 and HBL-100 cells to E3 for 56 and 72%, respectively, and completely prevented the adhesion of these cell lines as well as that of the OVCAR-4 cell line to intact laminin. Adhesion of T47D cells to either E3 or intact laminin was not affected by the AIIB2 antibody. These results suggest that there are two cell-binding sites present on the E3 fragment, one that is sensitive to inhibition by the $\beta 1$ -specific antibody, AIIB2, and another one that is not.

Analysis of Integrin Receptors

The antibody inhibition experiments clearly indicated that VLA-6 is an important mediator of cell binding to laminin. Other VLA proteins that have been shown to be capable of acting as laminin receptors are VLA-1 (Ignatius and Reichardt, 1988), VLA-2 (Languino et al., 1989; Elices and Hemler, 1989), and VLA-3 (Wayner and Carter, 1987; Gehlsen et al., 1988). To investigate the expression of these molecules and other integrin receptors and to relate this expression to the ability of cells to bind to the different laminin fragments, immunoprecipitation experiments of surface-labeled components were carried out with subunit-specific antibodies. As shown in Fig. 6, both HBL-100 and OVCAR-4 cells, which bind well to the E8 fragment, clearly express VLA-6. Less VLA-6 was detected on A375 cells, which adhered more weakly to fragment E8, and almost no VLA-6 was found on T47D cells, which adhered poorly to this fragment. Thus, the level of VLA-6 expression is closely correlated with the ability of cells to adhere to fragment E8.

No such correlation was found between $\alpha 6\beta 4$ expression and adhesion of cells to E8. For example, T47D cells expressed high levels of $\alpha 6\beta 4$, but poorly adhered to E8. This finding further supports the notion that $\alpha 6\beta 4$ does not function as a receptor for the E8 fragment.

There are also marked differences in the expression of VLA-1 to VLA-5 proteins on the cells of the four cell lines. The HBL-100 and A375 cells express all five VLA proteins, whereas T47D and OVCAR-4 cells only express some of these molecules. In addition, the level of each of the VLA proteins varied on the cells of the various cell lines. We found no obvious correlation between the expression of VLA subunits 1-5 and the ability of cells to bind to fragments E3, E8 or P1.

Expression of the vitronectin receptor was examined by immunoprecipitation with antibodies against both αv and $\beta 3$ subunits. Two cell lines, OVCAR-4 and A375, expressed the vitronectin receptor at high levels, whereas two other cell lines, T47D and HBL-100, showed significantly less expression of this receptor (Fig. 7). The expression of the vitronectin receptor on A375 and OVCAR-4 cells is in good agreement with the effects of the $\beta 3$ -specific antibody C17 on the adhesion of these cells to P1. We assume, however, that A375 cells, in addition to the vitronectin receptor, express a second P1 receptor that belongs to the $\beta 1$ subfamily of integrins, since complete inhibition of A375 cell adhesion to P1 requires combination of C17 and AIIB2.

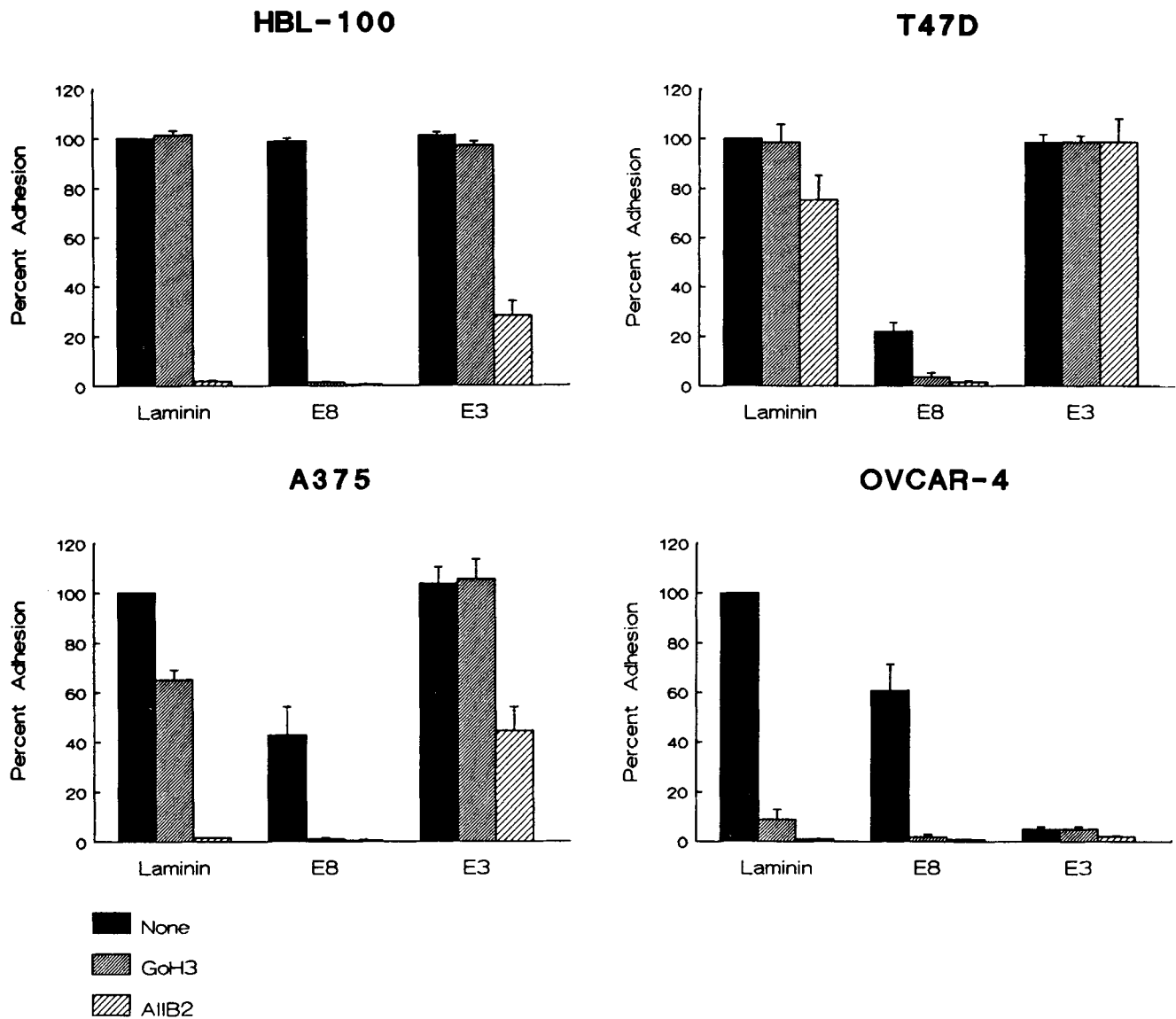


Figure 5. Inhibition of cell attachment to laminin, E3 and E8, by antibodies against integrin subunits. ^{51}Cr -labeled cells were preincubated with or without antibody and added to laminin, E8, and E3 substrates. The antibodies were mAb GoH3, directed against $\alpha 6$ (■) and mAb AIIB2, directed against $\beta 1$ (▨). Total cells bound to laminin in the absence of antibody (■) are indicated as 100%. Adhesion was measured in triplicate, and the results from three separate sets of experiments were averaged. Error bars represent SEMs of the averaged results from the three experiments.

Further Distinction between $\alpha 6\beta 1$ and $\alpha 6\beta 4$ Integrin Involvement in Fragment E8 Adhesion

In previous studies, we have shown that the mouse mammary tumor cell line RAC-11P expresses $\alpha 6\beta 4$, but not $\alpha 6\beta 1$ integrins (Sonnenberg et al., 1988b; Sonnenberg et al., 1989). A spontaneous transformant of this cell line, RAC-34E, however, was shown to express the $\alpha 6\beta 1$ complex in addition to $\alpha 6\beta 4$. Consistent with our above observations that only cells expressing $\alpha 6\beta 1$ adhere to the E8 fragment of laminin, we found that RAC-34E but not RAC-11P cells bind to E8. RAC-11P cells adhered well to laminin and its E3 fragment. In contrast, no binding to E3 was found of RAC-34E cells (Fig. 8). For reasons unknown, adhesion of RAC-34E cells to both laminin and E8 could only be partially inhibited by mAb GoH3.

Discussion

Adhesion of cells to laminin is a complex process involving many different attachment sites. The results reported in this manuscript clearly establish the presence of at least three distinct cell-binding sites and correlate them, with some exceptions, to integrin receptor recognition.

The E8 Cell-binding Site

The E8 cell-binding site was recognized previously as an important site for neurite outgrowth (Edgar et al., 1984), myoblast locomotion (Goodman et al., 1989), cell adhesion (Aumailley et al., 1987; Goodman et al., 1987) and is apparently involved in maintaining cell polarity (Klein et al., 1988). Also, in our study several cell lines were found to adhere to

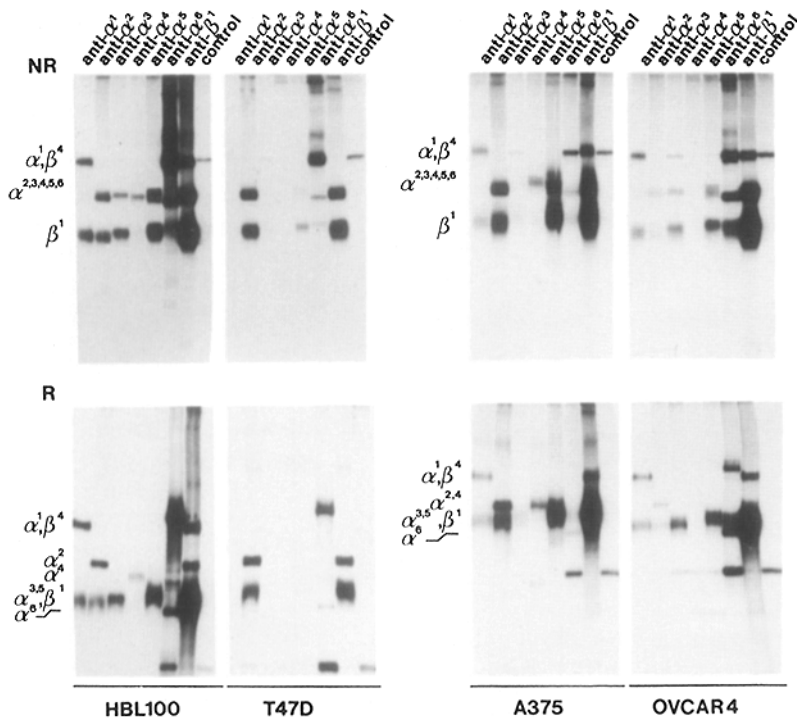


Figure 6. Analysis of VLA/integrin heterodimers on HBL-100, T47D, A375 and OVCAR-4 cells. Lysates of ^{125}I -labeled HBL-100, T47D, A375, and OVCAR-4 cells were immunoprecipitated with anti- $\alpha 1$ (TS2/7), anti- $\alpha 2$ (10G11), anti- $\alpha 3$ ($\alpha 3$ peptide, rabbit anti-serum), anti- $\alpha 4$ (B-5G10), anti- $\alpha 5$ (BIE5), anti- $\alpha 6$ (GoH3), anti- $\beta 1$ (A-1A5), and control (normal rabbit serum). Samples were analyzed on SDS-5% polyacrylamide gels under reducing and nonreducing conditions. Notably, immunoprecipitation of VLA-3, using the polyclonal anti- $\alpha 3$ peptide serum was very inefficient. Higher yields of VLA-3 were obtained when immunoprecipitation was carried out with an mAb against $\alpha 3$ (not shown). Analysis of VLA expression by flow-cytometry confirmed the immunoprecipitation results and showed that expression of VLA-3 on HBL-100 and OVCAR-4 cells is five to six times higher than that of VLA-6 on these same cells.

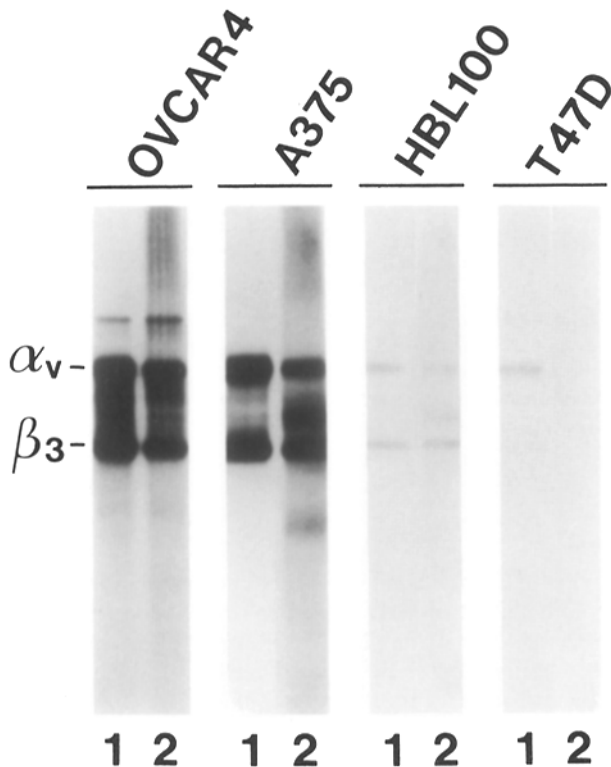


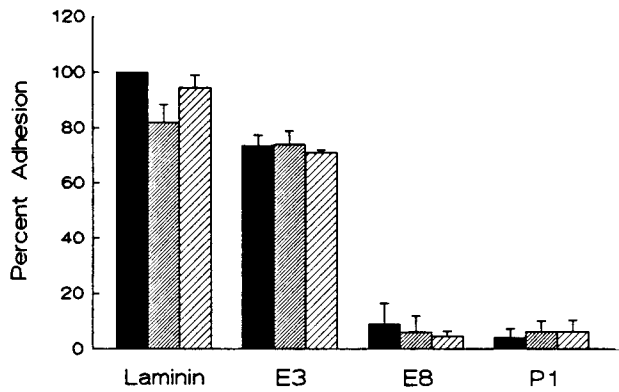
Figure 7. Analysis of the vitronectin receptor from HBL-100, T47D, A375 and OVCAR-4 cells. Lysates of ^{125}I -labeled HBL-100, T47D, A375, and OVCAR-4 cells were immunoprecipitated with mAb NKI-M9 (lane 1) and mAb C17 (lane 2), directed against the αv and $\beta 3$ subunits of the vitronectin receptor, respectively. Samples were analyzed on a SDS-5% polyacrylamide gel under nonreducing conditions.

this site. The interaction of these cells with E8 could be inhibited by mAb GoH3, directed against the $\alpha 6$ subunit of VLA-6, and by mAb AIIB2, directed against the common $\beta 1$ subunit of the VLA protein family. Moreover, we found that the expression of VLA-6 is closely correlated with the ability of cells to adhere to the E8 fragment. These results strongly suggest that VLA-6 is an important mediator of cell binding to E8. Three other VLA proteins, VLA-1, VLA-2, and VLA-3, have been implicated in cell adhesion to laminin. VLA-1 has been suggested to interact with the E1-4 fragment of laminin (Hall, D., L. Reichardt, and C. H. Damsky, unpublished results). Three of the cell lines used in this study express the VLA-1 protein, but these cell lines did not bind to E1X-Nd; a fragment similar to E1-4. Therefore, we cannot confirm that VLA-1 acts as a receptor for the E1-4 fragment of laminin. The binding site for VLA-2 on the laminin molecule has so far not been mapped, but that for VLA-3 was shown to be located at or close to the terminal globule of the laminin long arm (Gehlsen et al., 1989). VLA-3, therefore, in principle, can mediate binding of cells to the same fragment as VLA-6, i.e., E8. We have not examined the binding properties of cell lines that express the VLA-3 molecule as the only receptor for E8. Only cell lines that express both VLA-3 and VLA-6 were investigated. Because adhesion of these latter cells could be completely blocked by the $\alpha 6$ -specific mAb, GoH3, it is assumed that VLA-3 cannot play a major role in cell adhesion to E8, when VLA-6 is also present.

The P1 Cell-binding Site

The P1 cell-binding site is a cryptic cell-binding site, not recognized by cells on the intact laminin molecule; that is

RAC-11P



RAC-34E

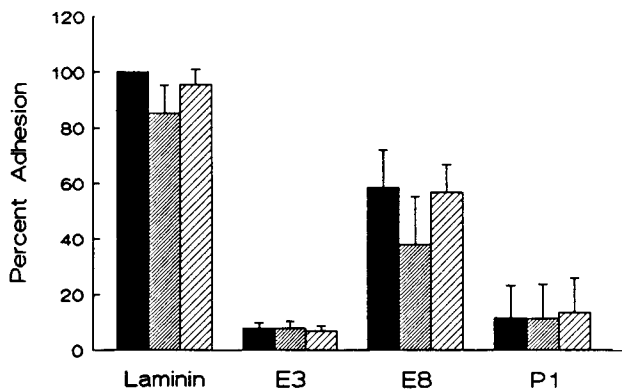


Figure 8. Effect of integrin $\alpha 6$ specific antibodies on the attachment of RAC-11P and RAC-34E cells to laminin and to fragments of laminin. ^{51}Cr -labeled cells were preincubated with or without antibody and added to laminin, E8, E3, and P1 substrates. Antibodies were mAb GoH3, directed against $\alpha 6$ (▨), and mAb JsE3, a control rat antibody of the same isotype as GoH3 (▩). Total cells bound to laminin in the absence of antibody (■) are indicated as 100%. Adhesion was measured in triplicate, and the results from four separate sets of experiments were averaged. Error bars represent SEMs of the averaged results from the four experiments.

suggested by (a) the observed better adhesion of A375 and HBL-100 cells to P1 than to laminin, (b) the absence of adhesion-promoting activity of the larger E1X-Nd fragment, which comprises the P1 structure and (c) the fact that adhesion to P1, but not to laminin could be blocked by the synthetic Arg-Gly-Asp- (RGD) containing peptide. The proposed existence of a cryptic P1 cell binding site is in agreement with observations made by Nurcombe et al. (1989). They showed that although the P1 fragment inhibited specific binding of ^{125}I -labeled laminin to HT1080 cells, this inhibition was noncompetitive. In contrast, inhibition of binding between fragment E8 and laminin was competitive. The P1 cell-binding site can be exposed by proteolytic degradation either directly of the laminin-nidogen complex or via sequential proteolysis of the E1X-Nd fragment. Because a synthetic Arg-Gly-Asp- (RGD) containing peptide efficiently inhibited

the attachment of cells to P1 and because an RGD sequence has been identified in one of the cysteine-rich domains of the A chain (Sasaki et al., 1988), this sequence probably represents the cell-binding site of P1. This has recently been confirmed in biochemical studies (Aumailley et al., 1990). Whether the P1 cell binding site is exposed and used *in vivo* is not known yet. The results of the antibody blocking experiments indicated that at least two cell surface receptors can mediate adhesion to P1. One of these is the vitronectin receptor, a promiscuous receptor that recognizes RGD sequences in a variety of adhesive glycoproteins (Cheresh, 1987; Cheresh and Spiro, 1987; Charo et al., 1987). The second receptor that mediates cell adhesion to P1 belongs to the $\beta 1$ subfamily of integrins. The possibility has been considered that the $\beta 1$ receptor is identical to VLA-5; however, antibodies against $\alpha 5$, which could block interactions of cells to fibronectin, failed to inhibit cell binding to P1. Also, the fact that two cell lines, OVCAR-4 and T47D, showed good adhesion to fibronectin, but do not or only poorly adhere to P1, argues against a possible role of the fibronectin receptor in cell binding to P1.

In a recent study, the tenascin receptor has been shown to mediate RGD-dependent cell adhesion (Bourdon and Ruoslahti, 1989). This receptor is a typical $\alpha \beta 1$ integrin that is not related to any of the six known VLA proteins. It will be interesting to determine whether this receptor facilitates binding of cells to P1.

The E3 Cell-binding Site

The recognition by cells of the E3 fragment of laminin was unexpected because in past studies this fragment was found not to promote cell adhesion or neurite stimulation (Edgar et al., 1984; Goodman et al., 1987). In our experience, cells adhere poorly to E3 coated on tissue culture plates, which were used in earlier studies. This is most probably due to a poorer coating of E3 to these plates at higher concentrations than to polystyrene plates (10 instead of 14% of added E3 is bound). The choice of microtiter plate may be particularly important for detecting E3-mediated cell adhesion, because even if adequate polystyrene plates are used, relatively large amounts of this fragment are needed. At present, it is not clear which receptor(s) are involved in cell adhesion to E3. The finding that adhesion of HBL-100 and A375 cells to E3 could be blocked by the anti- $\beta 1$ antibody, AIIB2, suggests that one of these receptors is a $\beta 1$ integrin. The T47D cells also adhere to E3, but the adhesion of these cells cannot be blocked by AIIB2. Therefore, T47D cells must utilize a different receptor for binding to E3. The possibility that $\alpha 6 \beta 4$ functions as a receptor for E3 seems less likely because HBL-100 cells express high levels of $\alpha 6 \beta 4$, but adhesion of these cells to E3 can be completely blocked by a $\beta 1$ -specific mAb, and RAC-34E cells also expressing high levels of $\alpha 6 \beta 4$, do not adhere to E3 at all. Because E3 contains a heparin-binding site (Ott et al., 1982), it appears more likely that proteoglycans can function as receptor(s) for the E3 binding site. In support of this, we have recently found that interaction of T47D cells with E3 could be prevented by low concentrations of heparin or dextran sulphate (Sonnenberg, A., and R. Timpl, unpublished observations). The best studied proteoglycan is syndecan, an integral membrane proteoglycan on epithelial cells which contains both heparan

sulfate and chondroitin sulfate (Rapraeger and Bernfield, 1985). Although this molecule mediates cell adhesion to a variety of extracellular matrix components, no binding was so far observed to laminin (Saunders and Bernfield, 1988).

Inactive Cell-binding Fragments of Laminin

Fragment E1X-Nd corresponds to the previously described fragment E1-4/E1 (Paulsson et al., 1985; Goodman et al., 1987). When properly purified, E1X-Nd was an inactive adhesion substrate, even for rat Rugli cells, which have been found to adhere to E1-4/E1 (Goodman et al., 1987). We could show that a contamination by fragment E8 is responsible for cell adhesion activity of less pure E1X-Nd preparations, particularly with those cells that bind to low concentrations of E8 by the VLA-6 receptor. Another inactive fragment, E10, corresponds to a globular domain in the short arm B1-chain segment of laminin (Sasaki et al., 1987; Mann et al., 1988). A synthetic 14-mer peptide corresponding to an internal E10 sequence and possessing a potential N-glycosylation site (Sasaki et al., 1987) was shown to block heparin and cell binding (Charonis et al., 1988).

The full occupation of the carbohydrate receptor site as shown here for fragment E10 could be responsible for its inability to bind to cells and to heparin (Mann et al., 1988) and thus indicates a possible way to regulate the biological activity of laminin by N-glycosylation. Fragment E4 was also inactive in promoting cell adhesion confirming previous observations (Goodman et al., 1987). It was recently shown to possess a major binding site for laminin self-assembly (Bruch et al., 1989), which may be the principal function of the fragment E4 structure.

The $\alpha 6 \beta 4$ Complex Does Not Serve as a Receptor for E8

Although the ligand of $\alpha 6 \beta 4$ has not been identified, we conclude that it must be different from that of $\alpha 6 \beta 1$, the laminin E8 receptor. This conclusion is based on the following two findings: (a) human T47D and mouse RAC-11P cells express $\alpha 6 \beta 4$ at high levels but do not adhere to E8, and (b) mAb A1B2, directed against the common $\beta 1$ subunit, was capable of blocking adhesion of all human cells to E8, including those that express both $\alpha 6 \beta 4$ and $\alpha 6 \beta 1$. Differences in specificity of ligand binding due to the presence of a different β subunit in the heterodimeric integrin has recently also been demonstrated for the vitronectin receptor (Cheresh et al., 1989); the same receptor as the one identified in this report to bind to P1. The vitronectin receptor ($\alpha v \beta 3$) functions as a receptor for vitronectin, fibrinogen, and von Willebrand factor, whereas the novel vitronectin receptor ($\alpha v \beta x$) binds vitronectin and fibronectin, but not fibrinogen or von Willebrand factor. Another integrin α subunit, which has been reported to form complexes with either of two β subunits, is the mouse homologue of the $\alpha 4$ subunit of VLA-4 (Holzmann and Weissman, 1989). No difference in receptor specificity was found for these two integrin forms of VLA-4.

It is perhaps important to mention that the conclusion that $\alpha 6 \beta 4$ has a different ligand-binding specificity than $\alpha 6 \beta 1$ does not imply that $\alpha 6 \beta 4$ cannot interact with another domain on laminin. Furthermore, there is the possibility that $\alpha 6 \beta 4$ interacts with variant forms of laminin. Recently, three variant forms, s-laminin (Hunter et al., 1989), heart laminin

(Paulsson and Saladin, 1989), and merosin (Ehrig et al., 1990) have been described and it will be interesting to determine whether either of these can bind to $\alpha 6 \beta 4$.

In conclusion, the approach used in this study has allowed the clear definition of three distinct cell-binding sites (E3, E8, and P1) on laminin and has given insight into the role of integrins in the recognition of these sites. For the P1 fragment, RGD sequences were shown to be involved in receptor recognition of this structure; it now will be important to identify the amino acid sequences within the E3 and E8 fragments that are recognized by the different adhesion receptors.

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