A Synthetic Peptide Derived from the Carboxy Terminus of the Laminin A Chain Represents a Binding Site for the $\alpha_3\beta_1$ Integrin

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Abstract. The purpose of this study was to identify the binding site(s) within laminin for the $\alpha_3\beta_1$ integrin receptor. It has been previously shown, using proteolytic fragments and anti-laminin antibodies, that the region in laminin for $\alpha_3\beta_1$ integrin binding is localized to the carboxy-terminal region at the end of the long arm (Gehlsen, K. R., E. Engvall, K. Dickerson, W. S. Argraves, and E. Ruoslahti. 1989. *J. Biol. Chem.* 264:19034–19038; Tomaselli, K. J., D. E. Hall, L. T. Reichardt, L. A. Flier, K. R. Gehlsen, D. C. Turner, and S. Carbonetto. 1990. *Neuron.* 5:651–662). Using synthetic peptides, we have identified an amino acid sequence within the carboxy-terminal region of the laminin A chain that is recognized by the $\alpha_3\beta_1$ integrin. The amino acid sequence represented by the synthetic peptide GD-6 (KQNCLSSRASFRGCVRNLRLSR residues numbered 3011 to 3032) of the globular domain of the murine A chain supports cell attachment and inhibits cell adhesion to laminin-coated surfaces. By affinity chromatography, peptide GD-6–Sepharose specifically bound solubilized $\alpha_3\beta_1$ from extracts of surface-iodinated cells in a cation-dependent manner, while it did not bind other integrins. In addition, exogenous peptide GD-6 specifically eluted bound $\alpha_3\beta_1$ from laminin–Sepharose columns but did not elute the $\alpha_5\beta_1$ integrin from a fibronectin–Sepharose column. Using integrin subunit-specific monoclonal antibodies, only those antibodies against the α_3 and β_1 subunits inhibited cell adhesion to peptide GD-6–coated surfaces. Finally, a polyclonal antibody made against peptide GD-6 reacted specifically with both murine and human laminin and significantly inhibited cell adhesion to laminin-coated surfaces but not those coated with other matrix proteins. These results identify the laminin A chain amino acid sequence of peptide GD-6 as representing a binding site in laminin for the $\alpha_3\beta_1$ integrin.

LAMININ is a multifunctional basement membrane glycoprotein that has been shown to promote the attachment and migration of cells and to participate in cellular differentiation and tumor metastasis (Chung et al., 1979; Terranova et al., 1983; Goodman et al., 1987). Interactions of cells with laminin have been shown to be mediated by both integrin-type cell surface receptors (Hemler et al., 1987; Gehlsen et al., 1989; Sonnenberg et al., 1988; Ignatius et al., 1990) and by other nonintegrin molecules including a 67-kD protein (Malinoff and Wicha, 1983; Wewer et al., 1987) and β1,4 galactosyltransferase (Begovac et al., 1991).

Integrins are a family of cell surface transmembrane receptors involved in the attachment and migration of cells on various extracellular matrix proteins and participate in cell–cell interactions (Hynes, 1987; Hemler, 1990; Ruoslahti, 1991). Several integrins are known to be receptors for laminin including $\alpha_1\beta_1$, $\alpha_2\beta_1$, $\alpha_3\beta_1$, $\alpha_6\beta_1$, $\alpha_7\beta_1$ (Tomaselli et al., 1990; Languino et al., 1989; Gehlsen et al., 1989; Sonnenberg et al., 1988, 1991; Kramer et al., 1991), and $\alpha_v\beta_3$ (Sonnenberg et al., 1990; Kramer et al., 1990). In addition, the $\alpha_1\beta_1$, $\alpha_2\beta_1$, and $\alpha_3\beta_1$ integrins also bind to other extracellular matrix proteins such as fibronectin and collagens (Hemler, 1990; Ruoslahti et al., 1991). However, well-defined binding sites within laminin for the various laminin-binding integrins have not yet been characterized.

Several regions in laminin for integrin binding have been identified using antibodies and proteolytic fragments of laminin. The region in laminin for $\alpha_1\beta_1$ binding has been localized to the murine laminin fragment P1, which is comprised of the intersection of the cross-region of laminin containing the A, B1, and B2 chains (Tomaselli et al., 1990), and $\alpha_v\beta_3$ has been shown to recognize the cryptic RGD site within the A chain of the P1 fragment (Clement et al., 1990; Kramer et al., 1990; Tashiro et al., 1991). $\alpha_6\beta_1$ has been shown to interact with the murine laminin fragment E8 which is comprised of the carboxy-terminal domain (Sonnenberg et al., 1990, 1991). Previous work on $\alpha_3\beta_1$ has indicated that the binding site within laminin is located in the carboxy-terminal region at the end of the long arm (Gehlsen et al., 1989; Tomaselli et al., 1990). This conclusion was based on results showing that purified $\alpha_3\beta_1$ bound to a chymotrypsin fragment of human laminin which is similar to the murine E8 fragment, and that antibodies whose deter-

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minants map to the carboxy-terminal domain of human laminin pepsin fragment (Engvall et al., 1986; Dillner et al., 1988) inhibit purified $\alpha_3\beta_1$ receptor binding to intact human laminin (Gehlsen et al., 1989). These results have also been confirmed using a neuronal cell line with proteolytic fragments of murine laminin and antibodies specific for integrin subunits (Tomaselli et al., 1990).

Recently, several synthetic peptides derived from the amino acid sequence of the murine laminin A, B1, and B2 chains have been described that mimic certain functional activities of intact laminin (Graf et al., 1987; Charonis et al., 1988; Tashiro et al., 1989; Skubitz et al., 1990, 1991). Interestingly, only a peptide containing an RGD sequence from the A chain near the cross region has been shown to interact with an integrin receptor, $\alpha_v\beta_3$ (Tashiro et al., 1991; Kramer et al., 1990). Skubitz et al. (1991) has described two synthetic peptides derived from the carboxy-terminal globular domain (G domain) of the murine laminin A chain that may interact with a β_1 integrin. However, the precise identification of a laminin-binding integrin involved in this interaction has not yet been determined.

The present study was undertaken in an effort to map the location of the binding site(s) within laminin for the $\alpha_3\beta_1$ integrin. These studies were done by using synthetic peptides derived from the carboxy-terminal globular domain region of the murine Engelbreth-Holm-Swarm (EHS)[1]-laminin A chain in conjunction with cell adhesion assays, receptor binding assays, peptide affinity chromatography of solubilized cell membranes, and antiintegrin antibodies. We report herein on the identification of a contiguous peptide sequence within laminin that is capable of binding the $\alpha_3\beta_1$ integrin. This amino acid sequence likely represents at least one binding site in laminin for this receptor.

Materials and Methods

Cell Lines

The human melanoma cell line C8161 was kindly provided by Dr. Mary J. C. Hendrix, University of Arizona, Tucson, AZ (Bregman and Meyskens, 1983; Welch et al., 1991). The MG-63 (human osteosarcoma) and the IMR-90 cells (human fetal fibroblasts) were purchased from the Amer. Type Culture Collection (Rockville, MD). All cell lines were cultured in DME supplemented with 10% FCS and 0.1% gentamicin (Sigma Chem. Co., St. Louis, MO). Cells were harvested using cation-free PBS containing 2 mM EDTA.

Antibodies

mAbs reactive toward specific integrin subunits were used for immunoprecipitations and for inhibition of cell attachment. Antibodies P1H5 (anti-α_2), P1B5 (anti-α_3), P4G9 (anti-α_4), P1D6 (anti-α_5), and P4C10 (anti-β_1) were either purchased from Telios Pharmaceuticals Inc. (LaJolla, CA) or generously provided by Dr. Elizabeth Wayner (Carter et al., 1990). Dr. Arnoud Sonnenberg donated rat mAb G0H3 (anti-α_6; Sonnenberg et al., 1988), Dr. David Cheresh kindly provided mAbs LM609 (anti-$\alpha_v\beta_3$) and LM142 (anti-α_v) (Cheresh et al., 1987), Dr. Erkki Ruoslahti donated mAb LM442 (anti-β_1; Cheresh, D., and E. Ruoslahti, unpublished results), mAbs 60.3 and IB4 (both anti-β_2) were from Dr. Arfors (Beatty et al., 1983; Wright et al., 1983).

Polyclonal antibodies were generated against the synthetic peptides coupled to the carrier protein, keyhole limpet hemocyanin, as previously described (Skubitz et al., 1990). Briefly, rabbits were immunized with the peptides–keyhole limpet hemocyanin, and sera was tested for specificity by ELISA on immobilized peptide or on murine EHS-laminin. IgG was purified by ammonium sulfate precipitation and DEAE chromatography. Purity of IgG was determined by SDS-PAGE and subsequent ELISA. In addition, peptide GD-6-specific polyclonal antibodies were affinity purified by chromatography on EHS-laminin-Sepharose as described previously (Skubitz et al., 1990).

Peptide Synthesis

A series of peptides were synthesized from both the A and B1 chains of laminin in the region of the carboxy-terminal portion of the long arm (Fig. 1), since the $\alpha_3\beta_1$ integrin has been shown to bind to this domain (Gehlsen et al., 1989; Tomaselli et al., 1990). The criteria for peptide selection was based on the net positive charge and hydrophilicity of specific regions of murine EHS-laminin such that the laminin-derived peptides would have an increased potential for cell surface interactions. Peptide sequences were derived from the published sequences of the EHS-laminin A and B1 chains (Sasaki et al., 1988) and synthesized by the Merrifield solid-phase method as previously described (Charonis et al., 1988). Peptides were purified by HPLC and verified by amino acid analysis using an amino acid analyzer (Beckman Instrs., Inc., Fullerton, CA) and sequence determination done by sequential Edman degradation on a gas phase sequenator. Peptides containing free sulfhydryl groups were deblocked during the process of the removal of the peptides from the resin using hydrofluoric acid and acetic acid. In addition, sulfhydryl groups in several of the peptides were treated by alkylation (Kouzi-Koliakos et al., 1989).

Radiolabeling of Cell Surface Proteins and Immunoprecipitations

Cells were surface labeled with ^{125}I as previously described (Gehlsen et al., 1988, 1989). Briefly, 100 U lactoperoxidase/ml (Sigma Chem. Co.), 1 mCi/ml ^{125}I (New England Nuclear, Burbank, CA), and 4 μl of a 30% solution of hydrogen peroxide were added to 10^7 cells for 10 min on ice. Unbound ^{125}I was removed by sequential washings with PBS and cells were extracted as described below. Lysates or eluates to be immunoprecipitated were first preabsorbed with 1 ml of goat anti-mouse IgG-Agarose (Sigma Chem. Co.). Radioactive counts were determined and equivalent counts incubated with 1 μg each of the various antiintegrin antibodies described above. Goat anti-mouse IgG-Agarose was then added to the mixture and incubated overnight at 4°C with shaking. For the rat mAb against α_6, goat anti-rat IgG-Agarose was used. The immunocomplexes were centrifuged and the beads washed five times with extraction buffer (see below) containing 500 mM NaCl and 1% Tween-20. The immunocomplexes were removed from the Agarose with 4× sample buffer (0.625 M Tris-HCl, pH 6.8, 1% glycerol, 0.25% SDS, an 0.06% bromophenol blue), boiled for 5 min, and analyzed by 7.5% SDS-PAGE as previously described (Gehlsen et al., 1989). Gels were dried and autoradiographed using X-OMAT film (Eastman Kodak Co., Rochester, NY).

Cell Adhesion Assays

Cell adhesion to both murine and human laminin, proteolytic fragments, or chemically synthesized peptides was done as previously described (Wilke and Skubitz, 1991). Briefly, various concentrations of the proteins or peptides to be immobilized were dissolved in either PBS or a 0.05-M sodium bicarbonate buffer, pH 8.6. Protein solutions were then added to Immulon 1 plates (Dynatech Labs., Inc., Chantilly, VA) and adsorbed overnight at room temperature. Plates were then extensively washed with PBS and unbound sites blocked with 5 mg/ml BSA or ovalbumin. Cells were harvested and the concentration adjusted to 50,000 cells/150 μl. 150-μl aliquots were added to each well and incubated at 37°C for 1 h. In the peptide or antiintegrin mAb inhibition studies, the cells were preincubated for 30 min at 37°C with the "inhibitors", and then both cells and inhibitors were added to the wells and incubated for an additional hour at 37°C. Plates were washed three times with PBS and adherent cells were fixed and stained with 3.75% paraformaldehyde and 0.1% toludine blue. Wells were again washed five times with PBS and adherent cells were quantitated using a microtiter plate reader (TiterTec MultiScan Plus; Flow Labs., Inc., McLean, VA) at an absorbance of 600 nm.

Inhibition of C8161 cell adhesion to various surfaces with purified IgG against peptide GD-6 was performed as previously described (Skubitz et al., 1990). Briefly, 100 μl of DME/Hepes containing 2 mg/ml BSA and various concentrations of the purified IgGs were added to microtiter wells that had been preadsorbed with 0.35 μg of laminin, 0.04 μg of peptide GD-6, or 0.25 μg of fibronectin. The IgG was incubated in the wells for 1 h at 37°C.

1. *Abbreviation used in this paper*: EHS, Engelbreth-Holm-Swarm.

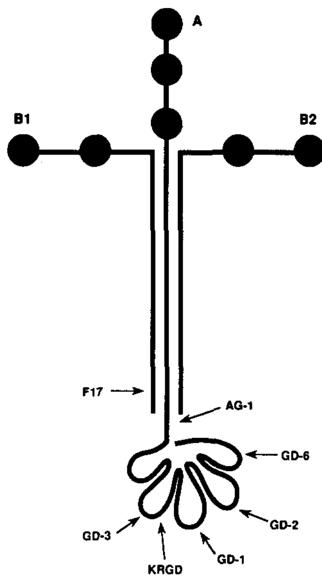

Figure 1. Diagrammatic model of laminin indicating the location of the peptides. The A, B1, and B2 chains of laminin are shown with the globular domains indicated (●). Other structural features are not shown for simplification. Peptides GD-1, GD-2, GD-3, GD-6, AG-1, and KRGD are derived from the A chain of laminin and peptide F17 is derived from the B1 chain of laminin.

100 μl of radiolabeled cells at 10^5/ml were added to each well and incubated for 30 min at 37°C, at which time the wells were washed and adherent cells were solubilized and quantitated in a scintillation counter as described previously (Charonis et al., 1988).

Affinity Chromatography

Peptides were coupled to activated CH-Sepharose according to the manufacturer's instructions (Pharmacia LKB Biotechnology Inc., Piscataway, NJ). In addition, a mock-coupled column was made without peptides for use as a control column. Briefly, 10 mg of HPLC-purified peptides were dissolved in coupling buffer (15 mM sodium carbonate and 35 mM sodium bicarbonate, pH 8.6) and then the peptide solution was added to 3 ml of preswollen beads and mixed overnight at 4°C. Unbound peptide was removed by washing the beads with coupling buffer and the remaining reactive groups were hydrolyzed at pH 8.0 with 0.1 M Tris-HCl for 2 h. Surface-iodinated cells were extracted as described previously (Gehlsen et al., 1988, 1989), using extraction buffer (50 mM Tris-HCl, pH 7.4, 50 mM octyl-β-D-glucopyranoside, 15 mM NaCl, 1 mM $MgCl_2$, 1 mM $MnCl_2$, and 1 mM PMSF). Cleared lysates were then incubated with the various peptide–Sepharose beads as a slurry with constant mixing overnight at 4°C. The peptide-Sepharose beads were then packed into a column and the columns were washed with extraction buffer, and eluted with 20 mM EDTA in extraction buffer lacking the cations. Columns were subsequently eluted with 1 M NaCl to isolate any remaining bound proteins. Peak elution fractions were then electrophoresed by SDS-PAGE.

Affinity chromatography on Sepharose columns coupled with the pepsin fragment of human laminin or the cell attachment fragment of fibronectin was done as previously described elsewhere (Gehlsen et al., 1988, 1989).

Radio-receptor Binding Assay

The ability of the various laminin-derived synthetic peptides to support purified $\alpha_3\beta_1$ integrin binding was tested in a solid-phase radio-receptor binding assay as previously described (Gehlsen et al., 1989). Briefly, surface-iodinated C8161 cells were extracted as described above and the cleared extracts passed over a human laminin–Sepharose column. Purified $\alpha_3\beta_1$ was eluted with 20 mM EDTA and the purified receptor verified by immunoprecipitation with integrin subunit-specific mAbs followed by SDS-PAGE. The EDTA in the receptor-containing eluate was then neutralized with 15 mM $MgCl_2$ and 15 mM $MnCl_2$. The receptor eluate was adjusted to 200,000 cpm/150 μl and then added to microtiter wells that had been previously coated with either 2 μg/ml of human laminin or 0.5 μg/ml of the various peptides. The plates were incubated for 2 h at room temperature, the wells were washed three times with extraction buffer, and the bound receptor was solubilized in TBS containing 1% SDS. Solubilized receptor was quantitated by counting receptor-associated radioactivity in a gamma counter (AutoGamma 5650; Packard Instr. Co., Inc., Meriden, CT).

Results

Identification of a Synthetic Peptide from the Laminin A Chain That Supports $\alpha_3\beta_1$ Integrin Binding

We and others have previously determined that $\alpha_3\beta_1$ integrin binds on or near the carboxy-terminal region at the end of the long arm of both murine (EHS) or human laminin (Gehlsen et al., 1989; Tomaselli et al., 1990; Goodman et al., 1991; Sonnenberg et al., 1991). To delineate the binding site(s) in laminin for $\alpha_3\beta_1$, we synthesized a series of peptides from this region of laminin. Peptide sequences were selected for synthesis based on hydrophilicity and net charge criteria (Table I), such that these peptides would be predicted

Table I. Laminin-derived Peptides, Their Sequences, Location, and Ability to Support $\alpha_3\beta_1$ Integrin Binding

Peptide	Amino acid sequence*	Location*	Net charge‡	Hydrophilicity§	$\alpha_3\beta_1$ bound‖	References
					%	
GD-1	KATPMLKMRTSFHGCIK	2,615–2,631 (A)	+5	−5.1	21	Skubitz et al., 1991
GD-2	KEGYKVRLDLNITLEFRTTSK	2,890–2,910 (A)	+2	−10.1	10	Skubitz et al., 1991
GD-3	KNLEISRSTFDLLRNSYGVRK	2,443–2,463 (A)	+3	−8.6	12	Skubitz et al., 1991
GD-6	KQNCLSSRASFRGCVRNLRLSR	3,011–3,032 (A)	+6	−8.9	79	Wilke et al., 1991
HGD-6	KQKCLRSQTSFRGCLRKLALIK	3,009–3,030 (A)	+7	−9.25	87	N/A
SGD-6	CRNRGRCNSSLFQVRSRKLLSA	N/A	+6	−8.9	N/D	N/A
HSGD-6	KQCLKSQRSFTRGLCRLKAKIL	N/A	+7	−9.25	N/D	N/A
AG-1	KLLISRARKQAASIK	2,087–2,101 (A)	+5	−0.5	14	Harvath et al.¶
F17	LERKYENDQKYLEDKA	1,722–1,737 (B1)	−1	−33.9	9	N/A
KRGD	VEKRGDREEA	2,515–2,523 (A)	−1	N/A	11	N/A

* Amino acid sequence designation is based on single letter codes. G, glycine; A, alanine; V, valine; L, leucine; I, isoleucine; F, phenylalanine; Y, tyrosine; W, tryptophan; M, methionine; C, cysteine; S, serine; T, threonine; H, histidine; K, lysine; R, arginine; D, aspartate; E, glutamate; N, asparagine; Q, glutamine; P, proline. Sequences were derived from murine EHS-laminin (Sasaki et al., 1988), except for KRGD, which was derived from the carboxy-terminal human laminin A chain (Olsen et al., 1989). N/A, not applicable.

‡ Net charge is calculated by assuming a +1 net charge for lysine (K) and arginine (R) residues, and a net −1 charge for glutamic acid (E) and aspartic acid (D) at neutral pH. Histidine is assumed to be uncharged at this pH.

§ Calculated by the method of Kyte and Doolittle (1982). According to this method, more hydrophilic peptides correspond to the more negative numerical values.

‖ $\alpha_3\beta_1$ bound represents the percentage of purified $\alpha_3\beta_1$ bound to peptide-coated surfaces compared with that bound to human laminin-coated wells (100%), as described in Materials and Methods. N/D, not done.

¶ Harvath, L., N. E. Brownson, and A. P. N. Skubitz. 1991. Laminin peptides stimulate neutrophil chemokinesis. *J. Cell Biol.* 115(3, Pt. 2):113*a*. (Abstr.)

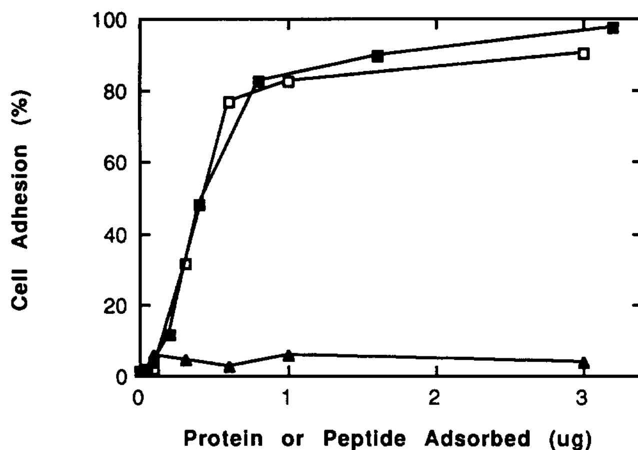

Figure 2. C8161 melanoma cell adhesion to peptide GD-6. Radiolabeled C8161 cells were incubated for 2 h in microtiter wells adsorbed with various concentrations of peptide GD-6 (■), laminin (□), or ovalbumin (▲). Cell adhesion is expressed as a percentage of the total number of cells added. Each value represents the mean of four separate determinations. Three separate experiments gave similar results.

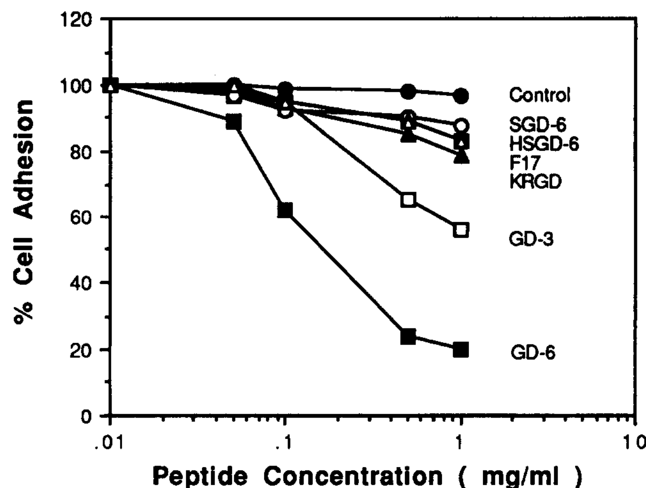

Figure 3. Inhibition of C8161 cell adhesion to human laminin by peptide GD-6. The synthetic peptides were each incubated with C8161 human melanoma cells at various concentrations for 30 min before adding the cells in the continued presence of the peptides into microtiter wells as described in Materials and Methods. Data are expressed as a percentage of the number of cells that adhere to human laminin in the absence of peptides: GD-3 (□), GD-6 (■), SGD-6 (○), HSGD-6 (■, upper), F17 (△), and KRGD (▲). Data represent duplicate experiments done in triplicate. Control experiments were done in the absence of peptide (●). SD was <6% for all groups.

to be from sites on laminin that would be accessible for the interaction with cell surface receptors. These peptides were tested for their ability to support receptor attachment using a radio-receptor binding assay. Table I lists the results of these experiments for 7 of the 24 laminin-derived peptides tested. One peptide, designated GD-6 from murine laminin, supported the binding of high levels of purified $\alpha_3\beta_1$. This peptide is from the carboxy terminus of the EHS-laminin A chain and has the amino acid sequence KQNCLSSRASFR-GCVRNLRLSR (Wilke and Skubitz, 1991). Peptide GD-6 and peptide HGD-6 from the homologous region of human laminin bound purified $\alpha_3\beta_1$ at a level comparable to 80% of that which bound to human laminin, suggesting that the peptide GD-6 sequence represents a major ligand binding site for the $\alpha_3\beta_1$ integrin. The other laminin-derived and control peptides did not significantly support $\alpha_3\beta_1$ integrin binding, although, many of the peptides had similar net cationic charges (Table I).

Peptide GD-6 Supports Cell Attachment and Inhibits Laminin-mediated Cell Adhesion

Peptide GD-6 has previously been shown to directly promote the attachment of human keratinocytes in a concentration-dependent manner when used as an adhesion substrate (Wilke and Skubitz, 1991). In addition, peptide GD-6 inhibits keratinocyte cell attachment to EHS-laminin–coated substrates (Wilke and Skubitz, 1991). We have extended this work to human melanoma cells (C8161), human osteosarcoma cells (MG-63), and human fetal fibroblasts (IMR-90), and have found that peptide GD-6 also supports the adhesion of these cells. For example, C8161 human melanoma cells adhered to peptide GD-6 and human laminin in a concentration-dependent manner (Fig. 2). The cells adhered to surfaces adsorbed with peptide GD-6 at levels comparable to intact laminin, with maximal attachment occurring at a coating concentration of 1 μg per well. Cells did not adhere to ovalbumin (Fig. 2). The cells adhered to several other laminin-derived peptides including GD-1 and GD-3 (data not shown) that have previously been described (Skubitz et al., 1991). These results suggest that other melanoma cell binding sites exist in laminin and that other cell surface receptor mechanisms for laminin-mediated adhesion are probably involved.

Peptide GD-6 and several other peptides were then tested for their ability to inhibit C8161 melanoma cell adhesion to human laminin-coated surfaces. Peptide GD-6 inhibited cell attachment to human laminin in a concentration-dependent manner with maximal inhibition of ~80% observed at a concentration of 500 μg/ml (Fig. 3). Peptide GD-3 had a modest inhibitory effect on cell adhesion (~30% at 500 μg/ml), while the laminin-derived RGD-containing peptide (KRGD) and peptide F17 from the B1 chain had only a minimal inhibitory effect at similar or higher concentrations. Control peptides (SGD-6 and HSGD-6) and several other laminin-derived peptides containing similar net charge and distribution of charged residues (Table I) did not significantly inhibit cell adhesion to human laminin-coated substrates (data not shown). The inability of peptide GD-6 to completely inhibit cell adhesion is most likely due to the presence of other domains on laminin that are able to promote the adhesion of these cells.

Purification of $\alpha_3\beta_1$ Integrin by Affinity Chromatography on Peptide GD-6–Sepharose

As another means of showing that $\alpha_3\beta_1$ integrin binds to laminin through the sequence of peptide GD-6, detergent extracts of surface-labeled cells were analyzed by affinity chromatography on peptide GD-6 coupled to Sepharose. C8161 human melanoma cells were selected since we have determined that they express relatively similar levels of many of the known integrins (Seftor et al., 1992). These cells were

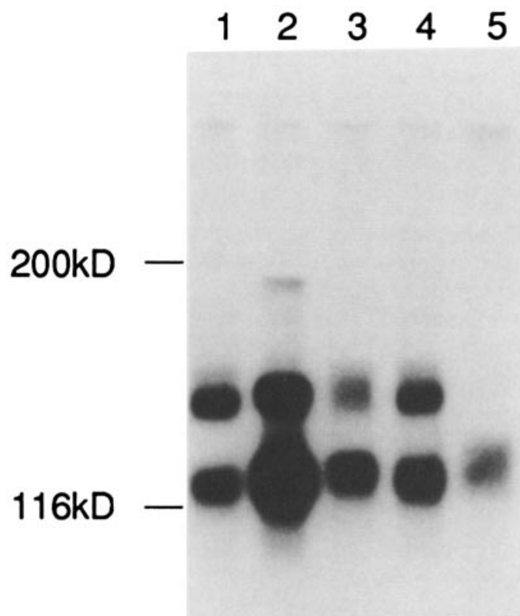

Figure 4. Integrin expression on C8161 cells. Detergent extracts from surface labeled C8161 cells were immunoprecipitated with anti-integrin mAbs to α_2 (lane *1*), β_1 (lane *2*), α_3 (lane *3*), α_5 (lane *4*), and α_v (lane *5*). Samples were analyzed by 7.5% SDS-PAGE under nonreducing conditions, and autoradiographed as described in Materials and Methods.

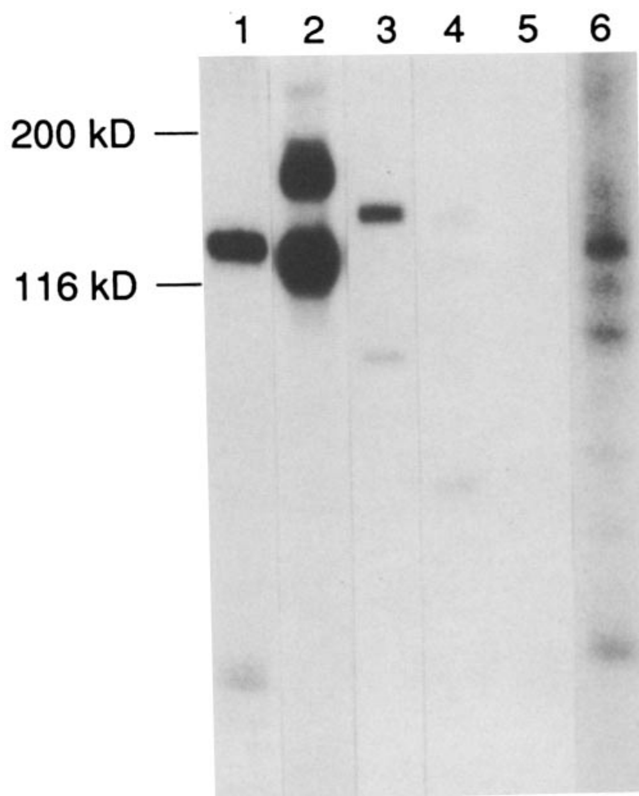

Figure 5. Affinity chromatography on peptide affinity columns. Extracts of radiolabeled C8161 human melanoma cells were passed over various peptide–Sepharose columns. The EDTA eluted peak fractions from the peptide columns are shown for peptide GD-3 (lane *1*); peptide GD-6 (lane *2*); KRGD (lane *3*); peptide F17 (lane *4*); a mock-coupled column (lane *5*); and a control peptide, scrambled human GD-6 (SHGD-6, lane *6*). Samples were analyzed by 7.5% SDS-PAGE under nonreducing conditions and autoradiographed as described in Materials and Methods. Molecular sizes are indicated.

surface iodinated and proteins extracted with octylglucoside as described in Materials and Methods. The extracts were then immunoprecipitated with the antiintegrin subunit-specific mAbs and these human melanoma cells were found to express $\alpha_1\beta_1$, $\alpha_2\beta_1$, $\alpha_3\beta_1$, $\alpha_5\beta_1$, and $\alpha_v\beta_3$ (Fig. 4). Notably, the $\alpha_1\beta_1$, $\alpha_2\beta_1$, $\alpha_3\beta_1$, and $\alpha_6\beta_1$ integrins have been shown to be laminin-binding integrins (Tomaselli et al., 1990; Languino et al., 1989; Gehlsen et al., 1989; Sonnenberg et al., 1988).

In parallel experiments, the radiolabeled cell extract was passed over peptide affinity columns and bound proteins were eluted with 20 mM EDTA and analyzed by SDS-PAGE under nonreducing conditions as described in Materials and Methods (Fig. 5). Two major proteins having apparent molecular sizes from 120 to 150 kD bound to the peptide GD-6 affinity column (Fig. 5, lane *2*). In affinity chromatography experiments using the RGD-containing peptide, KRGD, only the $\alpha_v\beta_3$ integrin bound (Fig. 5, lane *3*) as determined by immunoprecipitation (data not shown). Peptide F17 weakly bound proteins different from those seen with the other peptide columns (Fig. 5, lane *4*), whereas a mock-coupled column and a control peptide column (SHGD-6) bound no integrin-like cell surface proteins (Fig. 5, lanes *5* and *6*, respectively). Peptide GD-3 bound a 130-kD protein that appears to be different than those proteins that bound to the other peptide columns (Fig. 5, lane *1*). This 130-kD protein has been determined in preliminary immunoprecipitation studies not to be a known integrin (data not shown), and we are presently characterizing this protein by amino-terminal amino acid sequencing. Several other control peptides (SGD-6) and other laminin-derived peptides (GD-1, GD-2, and AG-1) did not bind proteins related to integrins using the above methods (data not shown). These chromatography experiments have been repeated numerous times using different batches of peptides, including the human GD-6 homologue (HGD-6), and different extracts of C8161 cells and other cell types, all with similar results.

The proteins that eluted from the GD-6 column with EDTA were analyzed by immunoprecipitation using integrin α- and β-chain-specific mAbs. Only α_3 and β_1 integrin subunits were immunoprecipitated from the peptide GD-6 column eluate (Fig. 6, lanes *3*, *5*, and *7*). The α-subunit labeled less well than the β-subunit and are thus seen as faint bands at the appropriate mobility. This trend is common for iodination of integrin subunits. Neither protein was precipitated with mAbs specific for α_1, α_2, α_4, α_5, α_6, α_v, β_2, or β_3 integrin subunits.

Elution of $\alpha_3\beta_1$ from a Laminin–Sepharose Column by Exogenous Peptide GD-6

The specificity of peptide GD-6 for $\alpha_3\beta_1$ was also shown in another experiment whereby a radiolabeled C8161 cell extract was passed over a human laminin–Sepharose column. The column was washed with extraction buffer; then 500 μg/ml of peptide GD-6 in extraction buffer was used to elute bound material. Exogenous peptide GD-6 released virtually

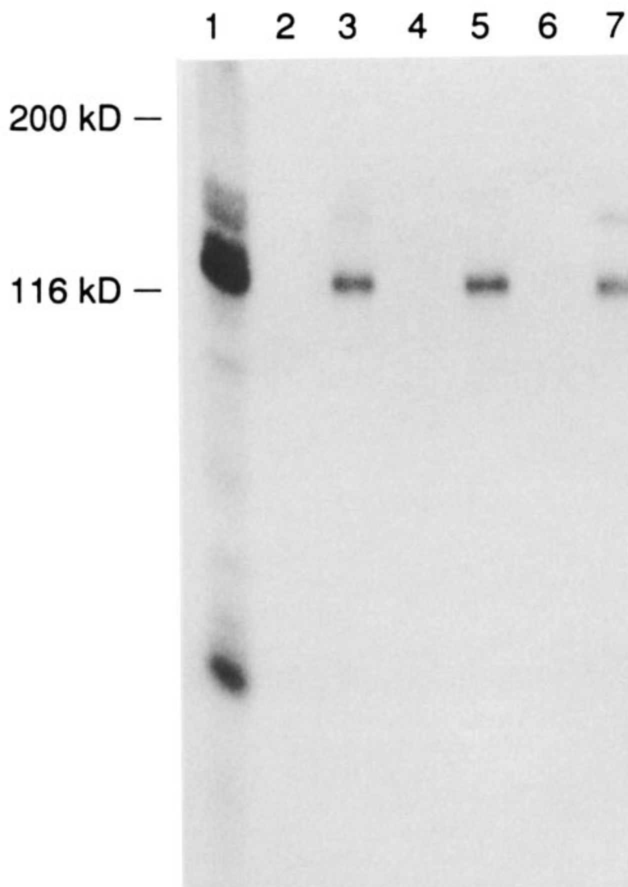

Figure 6. Immunoprecipitation analysis of proteins eluted from the peptide GD-6 affinity column. The proteins eluted by EDTA from the peptide GD-6 column were immunoprecipitated with antiintegrin mAbs. Peak fraction starting material is shown in lane *1*. Immunoprecipitation with anti-β_2 (lane *2*); anti-β_1 (P4C10, lane *3*); anti-α_2 (lane *4*); anti-α_3 (lane *5*); anti-α_4 (lane *6*); and another anti-β_1 (mAb LM442, lane *7*). Immunocomplexes were separated in a 7.5% SDS-PAGE and autoradiographed as described in Materials and Methods.

all bound $\alpha_3\beta_1$ from the human laminin–Sepharose affinity column (Fig. 7, lanes *8–13*). Subsequent elution of the laminin column with 1 M NaCl released negligible amounts of material (data not shown). In a parallel experiment, exogenous peptide GD-6 did not elute bound proteins from a fibronectin fragment column (Fig. 7, lanes *1–6*), although subsequent elution of this column with 20 mM EDTA released the expected $\alpha_5\beta_1$ integrin (Fig. 7, lane *7*) as determined by immunoprecipitation analysis (data not shown). In a separate experiment, $\alpha_3\beta_1$ that was bound to laminin-Sepharose was eluted with 20 mM EDTA and the pooled fractions were electrophoresed (Fig. 7, lane *14*) to demonstrate the specificity of the human laminin column. Determination that only $\alpha_3\beta_1$ bound to human laminin-Sepharose was done by immunoprecipitation of the material shown in lane 14 with various integrin-specific mAbs; these results have been previously reported elsewhere (Gehlsen et al., 1989). As a control, peptide KRGD did not elute $\alpha_3\beta_1$ from the laminin–Sepharose column, but did elute $\alpha_5\beta_1$ from the fibronectin–Sepharose column. Also, peptide F17 did not elute $\alpha_3\beta_1$ from the laminin–Sepharose column, even at a peptide concentration of 1 mg/ml. Furthermore, peptide GD-3 and the scrambled version of peptide GD-6 (HSGD-6 and SGD-6) did not elute $\alpha_3\beta_1$ from the human laminin–Sepharose column (data not shown).

Inhibition of Cell Adhesion to Peptide GD-6 by Antiintegrin Antibodies

As shown above, peptide GD-6 promoted the adhesion of C8161 cells when adsorbed to microtiter wells (Fig. 2). The adhesion of C8161 cells to surfaces adsorbed with peptide GD-6 was examined in the presence of various antiintegrin subunit-specific antibodies. Anti-α_3 (PIB5) and anti-β_1 (P4C10) mAbs significantly inhibited cell adhesion to peptide GD-6, whereas anti-α_2, -α_4, -α_5, -α_6, -α_v, and -β_2 mAbs had no inhibitory effect (Fig. 8). As an additional control, the anti-α_3 mAb did not block C8161 cell adhesion to peptide KRGD-coated surfaces (data not shown).

Inhibition of Laminin-mediated Cell Adhesion by anti–GD-6 Antibodies

Additional evidence that the sequence of peptide GD-6 is an important cell adhesion site in laminin was obtained by generating polyclonal antisera against peptide GD-6. The antisera was purified by ammonium sulfate precipitation and DEAE-chromatography; then the IgG was affinity purified on an EHS-laminin–Sepharose column. This purified IgG reacted specifically with peptide GD-6, murine laminin, and the human laminin, but did not react with other peptides (AS3) and proteins such as fibronectin or ovalbumin (Fig. 9). Furthermore, the anti–GD-6 IgG almost completely (88%) inhibited C8161 cell adhesion to surfaces absorbed with peptide GD-6 and significantly inhibited cell adhesion (60%) to intact EHS-laminin or human laminin (Fig. 10), but not to fibronectin. These results indicate that the site in laminin represented by peptide GD-6 is accessible to IgG and cell surface integrin receptors. These data support previous reports which suggest that $\alpha_3\beta_1$ binds to the carboxy-terminal globular domain of laminin (Gehlsen et al., 1989; Tomaselli et al., 1990) and may also recognize additional sites within this region. As a control, normal rabbit IgG did not inhibit laminin- or peptide GD-6–mediated cell adhesion.

Discussion

We report herein on an amino acid sequence, KQNCLSSRA-SFRGCVRNLRLSR, named GD-6, derived from the laminin A chain carboxy terminus that represents a major site for interaction with the $\alpha_3\beta_1$ integrin. The following data suggest that this amino acid sequence is a major site for $\alpha_3\beta_1$ integrin binding to laminin. Peptide GD-6 can support the adhesion of many cell types when immobilized to microtiter wells (Wilke and Skubitz, 1991; and our present work), and can inhibit cell attachment in a concentration-dependent manner to surfaces absorbed with either human or murine laminin. When various laminin-derived peptides were screened by an in vitro $\alpha_3\beta_1$ receptor binding assay, peptide GD-6 supported the binding of purified $\alpha_3\beta_1$. Furthermore, peptide GD-6–Sepharose specifically bound $\alpha_3\beta_1$ from C8161 cell extracts in a cation-dependent fashion. In addition, peptide GD-6 completely eluted $\alpha_3\beta_1$ from human laminin-Sepharose, but did not cause the release of the $\alpha_5\beta_1$ integrin

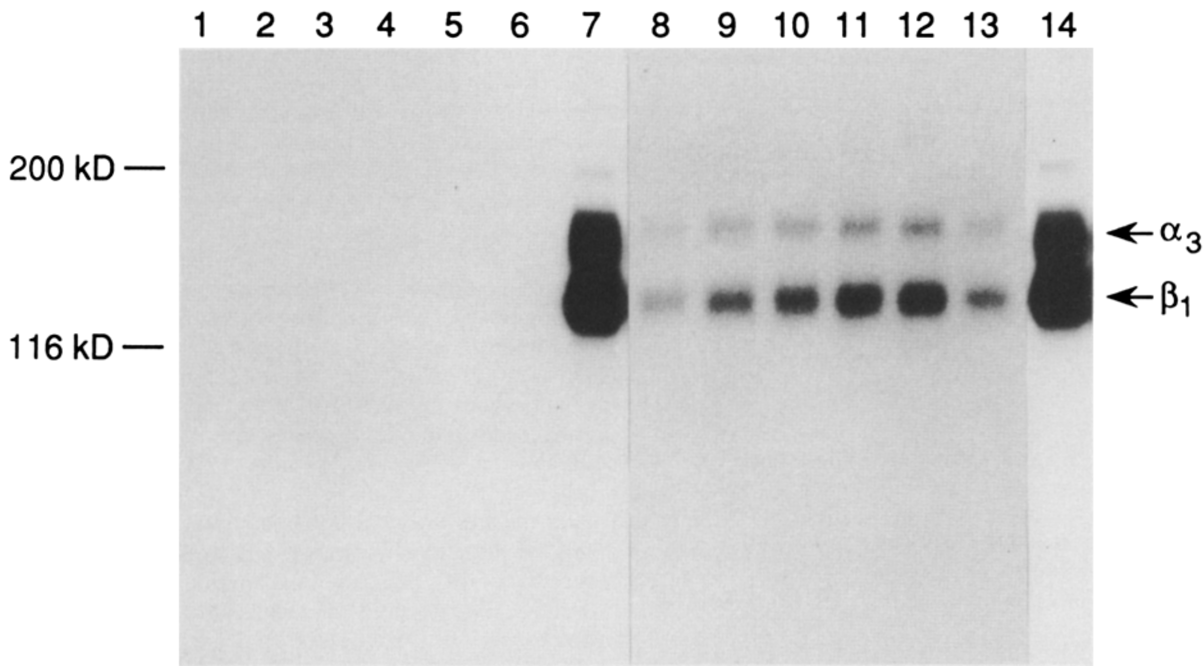

Figure 7. Elution of $\alpha_3\beta_1$ from a laminin–Sepharose affinity column by peptide GD-6. Both fibronectin– and laminin–Sepharose columns were eluted with 500 μg/ml of peptide GD-6. Lanes *1–6* show the elution profile from the fibronectin column using peptide GD-6, followed by an elution with EDTA (lane *7*). Lanes *8–13* show the proteins eluted by peptide GD-6 from the laminin–Sepharose column. In a parallel experiment, material eluted with EDTA from a separate laminin–Sepharose column is shown in lane *14*. Fractions were electrophoresed and autoradiographed as described above.

from fibronectin-Sepharose. Several control peptides, two consisting of peptide GD-6 in a scrambled sequence (SGD-6 and HSGD-6), and several other laminin-derived peptides having a similar basic charge, had no activity in any of the assays described. Moreover, mAbs specific for α_3 or β_1 inhibited cell adhesion to surfaces adsorbed with peptide GD-6. Finally, antisera raised against peptide GD-6 bound to murine and human laminin and significantly blocked cell attachment to both murine and human laminin. Taken together, these results strongly suggest that the amino acid sequence of peptide GD-6 represents at least one binding site in laminin for $\alpha_3\beta_1$.

Peptide GD-6 has previously been shown to promote the attachment of human keratinocytes (Wilke and Skubitz,

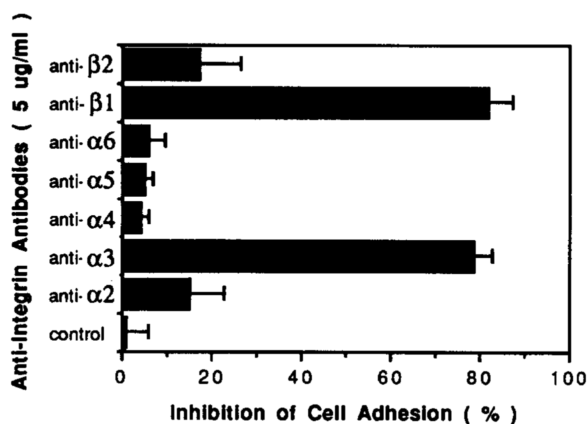

Figure 8. Inhibition of cell adhesion to peptide GD-6 using antiintegrin antibodies. Cells were incubated in peptide GD-6–coated wells (1 μg/well) in the presence of various antiintegrin mAbs—anti-β_2, anti-β_1, anti-α_2, anti-α_3, anti-α_4, anti-α_5, and anti-α_6—at a concentration of 5 μg/ml for 1 h at 37°C. Control represents cell adhesion in the absence of mAbs. Data represent duplicate experiments done in triplicate and the SD.

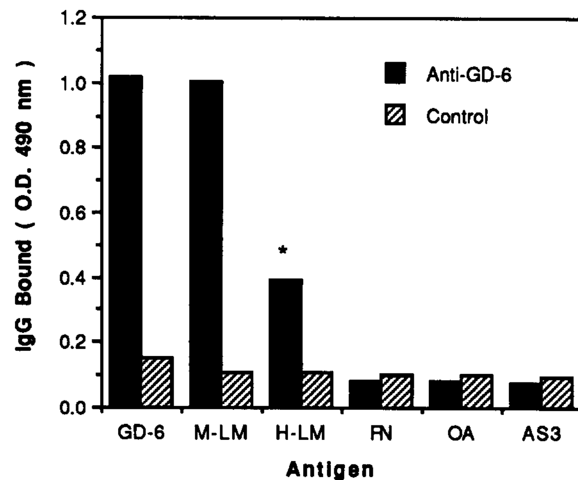

Figure 9. Specificity of rabbit polyclonal IgG against peptide GD-6. Microtiter wells were coated with peptides GD-6 and AS3 at 1 μg/well, and with the proteins murine laminin (*M-LM*), human laminin (*H-LM*), fibronectin (*FN*), and ovalbumin (*OA*) at 3 μg/ml. Peptide AS3 is a complementary peptide to F9 (Skubitz et al., 1990) and is derived from laminin. ELISAs were performed using 100 μg/ml of affinity-purified IgG and the mean OD 490 nm is shown for both the anti–GD-6 and normal rabbit sera. * represents statistically significant binding compared to controls and other proteins.

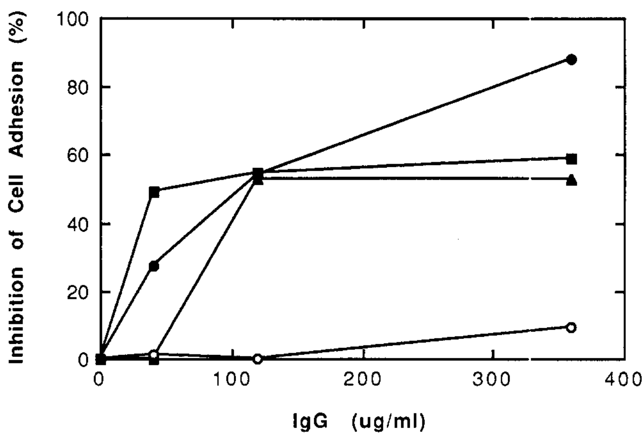

Figure 10. Inhibition of cell attachment by anti-GD-6 antibodies. Microtiter wells were coated with either EHS-laminin (0.7 μg/ml, ■), human laminin (2 μg/ml, ▲), fibronectin (5 μg/ml, ○), or peptide GD-6 (0.8 μg/ml, ●), and were incubated for 30 min with various concentrations of affinity-purified antipeptide GD-6 IgG. C8161 cells were then added to the wells and incubated in the presence of the IgG for 30 min at 37°C. Wells were washed and adherent cells quantitated. The percent inhibition of cell adhesion was determined as described in Materials and Methods. Background inhibition in the presence of normal rabbit IgG has been subtracted from each value. Data represent three experiments done in triplicate.

1991). In this study, we report that other cell types including MG-63, C8161, and IMR-90 cells also adhere to peptide GD-6. These cells have been well characterized with respect to their integrin repertoire, and they all have been shown to express the $\alpha_3\beta_1$ integrin, and other laminin-binding integrins on their surfaces (Gehlsen et al., 1989; Languino et al., 1989). Human keratinocytes have also been shown to express $\alpha_3\beta_1$, and recently, epiligrin, a new potential ligand for $\alpha_3\beta_1$ has been isolated from keratinocyte matrices (Carter et al., 1991). However, further characterization of epiligrin is required to determine if this molecule is related to other matrix proteins that bind $\alpha_3\beta_1$.

Recently, there has been a discrepancy in the literature as to whether or not $\alpha_3\beta_1$ is a true laminin receptor. Carter et al. (1991) has suggested that $\alpha_3\beta_1$ is not a receptor for laminin based on their inability to inhibit HT1080 cell adhesion to EHS-laminin using the anti-α_3 mAb P1B5. However, earlier reports by several groups have clearly shown that $\alpha_3\beta_1$ is indeed a laminin-binding integrin and that mAb P1B5 could, in fact, partially inhibit laminin-mediated cell adhesion (Tomaselli et al., 1990; Gehlsen et al., 1988, 1989; Carter et al., 1990). mAb P1B5 did not completely inhibit laminin-mediated cell adhesion in our previous and present studies, and the inability of this mAb to completely inhibit cell adhesion to either peptide GD-6 or to laminin suggests that the C8161 cells used in our studies may contain additional laminin receptor binding mechanisms. Alternatively, it is possible that the epitope recognized by this antibody may not interfere with receptor–ligand interactions as well as other known antiintegrin antibodies. Furthermore, it is conceivable that this integrin may contain several interactive regions for the different ligands or binding sites that it recognizes and, therefore, this mAb may only partially block one such interaction.

Evidence that $\alpha_3\beta_1$ can recognize several distinct sites within the ligands to which it binds was presented by Elices et al. (1991), who showed that $\alpha_3\beta_1$ could bind to fibronectin-Sepharose in an RGD-dependent manner only after $\alpha_5\beta_1$ was removed from the cell extracts. However, binding of $\alpha_3\beta_1$ to human laminin has been shown to be RGD-independent (Gehlsen et al., 1988; Elices et al., 1991; Sonnenberg et al., 1991). $\alpha_3\beta_1$ was not elutable from human laminin-Sepharose by RGD-containing peptides, nor did purified $\alpha_3\beta_1$ bind to RGD-coated surfaces. Therefore, it is not yet clear which mechanism this receptor uses to bind to the RGD site in fibronectin and yet apparently not bind to the RGD site in laminin. In addition, Elices et al. (1991) showed that $\alpha_3\beta_1$ could be eluted from laminin and fibronectin-Sepharose using high concentrations of NaCl, thus suggesting that both cation-dependent and ionic interactions may function in $\alpha_3\beta_1$ binding to its ligands. We have preliminary evidence that suggests that $\alpha_3\beta_1$ may acquire a different binding state following the receptor's binding to laminin, which might allow this receptor to remain bound to laminin even in the presence of EDTA; but then can be eluted by 1 M NaCl (Gehlsen, K. R., unpublished results). Several other integrins including $\alpha_2\beta_1$, $\alpha_4\beta_1$, and $\alpha_{IIb}\beta_3$ also appear to recognize distinct sites within the same or different ligands and also may be able to alter their binding affinities after ligand or antibody interactions (Elices and Hemler, 1989; Mould et al., 1991; Phillips et al., 1991).

Peptide GD-6 contains two cysteine residues and thus it was possible that the interaction of GD-6 with $\alpha_3\beta_1$ could have been through free sulfhydryl groups. To exclude this type of interaction we synthesized peptide GD-6 that had either unmodified or blocked cysteines. The results for all forms of peptide GD-6 were identical in the experiments described herein. In addition, it is unlikely that EDTA would have eluted this $\alpha_3\beta_1$ receptor from a peptide GD-6–Sepharose column, if the interactions were due to disulfide bridging. Therefore, future studies will focus on determining the mechanism by which certain integrins recognize multiple ligands through apparently distinct sequences.

Exogenous peptide GD-6 was able to inhibit C8161 cell adhesion to human laminin in a concentration-dependent manner. This inability of peptide GD-6 to completely inhibit cell attachment to human laminin is most likely due to the presence of multiple cell binding sites on laminin and other laminin binding receptors on the cells. It has previously been shown that not only do integrins, such as $\alpha_1\beta_1$, $\alpha_2\beta_1$, $\alpha_3\beta_1$, $\alpha_6\beta_1$, $\alpha_7\beta_1$, and $\alpha_v\beta_3$ bind laminin, but also that other non-integrin molecules bind laminin as well. There are reports suggesting that cell surface heparin-like molecules or heparan sulfate proteoglycans can interact with specific regions on laminin (Charonis et al., 1988; Skubitz et al., 1991). Other cell surface molecules also have been reported to bind laminin including a 67-kD protein (Terranova et al., 1983) and β1,4 galactosyltransferase (Begovac et al., 1991). Recently, Skubitz et al. (1991) has described several peptide sequences from the G domain of laminin that support cell adhesion and inhibit laminin-mediated cell attachment. Interestingly, two of the reported peptides, GD-3 and GD-4, were shown to possibly interact with the β_1 integrin subunit while peptides GD-1 and GD-2 bound heparin and may adhere to cells through proteoglycans. We have tested these peptides in our assays and, as presented, when peptide GD-3 was coupled to Sepharose, the column bound a 130-kD pro-

Murine: K Q N C L S S R A S F R G C V R N L R L S R

Human: K Q K C L R S Q T S F R G C L R K L A L I K

Merosin: K Q F G L T T S I P F R G C I R S L K L T K

Figure 11. Comparison of the murine, human, and merosin GD-6 sequences. The amino acid sequence for the murine-derived GD-6 peptide was compared to these sequences found in the same regions of the human laminin A chain and in the human merosin A chain. Identical amino acid residues are boxed.

tein which did not immunoprecipitate with our anti-β_1 antibody. However, it is possible that this protein may represent a variant of the known β_1 subunit, or it may be an as yet uncharacterized integrin subunit. Further studies into the specificities of the described globular domain peptides are presently being done to resolve these issues. These data also provide evidence suggesting that cells contain several different mechanisms for adhesion to laminin and to other basement membrane proteins. Since multiple peptide sequences derived from laminin have been reported (Graf et al., 1987; Charonis et al., 1988; Tashiro et al., 1989; Skubitz et al., 1991), the understanding that multiple mechanisms for laminin interactions with cells becomes more likely.

It is now apparent that there are several distinct isoforms of laminin (Sasaki et al., 1988; Olsen et al., 1989; Ehrig et al., 1990; Sanes et al., 1990). Both murine EHS-laminin and human forms of laminin support C8161 cell adhesion. The GD-6 peptide sequence used in this study was derived from the murine EHS-laminin sequence. To determine if the human counterpart of peptide GD-6 would also bind $\alpha_3\beta_1$, we synthesized the human peptide (HGD-6; Table I, Fig. 11). When peptide HGD-6 was used in parallel experiments with peptide GD-6, the results of the direct cell adhesion, inhibition assays, and affinity chromatography assays, were identical to those presented herein for peptide GD-6. The homologous merosin sequence is also shown for comparison (Fig. 11). There is 64% identity between the murine laminin and human laminin sequences, 45% identity between merosin and the murine laminin sequence, and 50% identity between the human laminin and merosin sequences. Since $\alpha_3\beta_1$ was described as a receptor that binds to human placental-derived forms of laminin (Gehlsen et al., 1988, 1989), it will be important to test the various corresponding globular domain "GD" peptides derived from both merosin and human laminin in order to determine if they also interact with integrins.

The carboxy-terminal, or G domain, of the laminin A chain has 5 looplike structures of $\sim$30% identity (Deutzmann et al., 1988). The peptide GD-6 sequence of laminin is located in the last looplike structure. Previous studies have shown that $\alpha_3\beta_1$ binds to the pepsin fragment of human laminin and also to the E8 fragment of murine laminin (Gehlsen et al., 1989; Tomaselli et al., 1990). It has been shown that the E8 fragment is comprised of the first three looplike structures of the G domain and is lacking the E3 fragment (the last two looplike structures), which contains the sequence of peptide GD-6 (Deutzmann et al., 1988). It is possible that the murine laminin E8 preparations contain additional sites that could support $\alpha_3\beta_1$ binding, or that the preparations are not completely pure since elastase digestion may be incomplete, resulting in parts of the last two loop-like structures being present in the E8 preparations. Alternatively, sites similar to the peptide GD-6 sequence may exist within the first three looplike structures of murine laminin and evidence for such sites is seen when comparing the GD-6 sequence with the GD-1 sequence. There is a stretch of five amino acids that are nearly identical (SFRGC) and these sequences also contain similar regions of charge distribution. Peptide GD-1 has also been shown to support cell adhesion (Skubitz et al., 1991). Future experiments will determine if such similar sequences are important for integrin binding. Thus, the similarities among these looplike structures may provide for additional receptor binding sites and that in the intact forms of laminin, the GD-6 site is possibly a major site for the binding of the $\alpha_3\beta_1$ integrin.

The G domain not only binds $\alpha_3\beta_1$, as previously shown (Gehlsen et al., 1989; Tomaselli et al., 1990), but also supports the binding of $\alpha_6\beta_1$ (Sonnenberg et al., 1990, 1991). Recently, it has been reported that $\alpha_6\beta_1$ binds to both the murine laminin E8 fragment and the human laminin pepsin fragment (Sonnenberg et al., 1991). However, when cells were used that contain both $\alpha_3\beta_1$ and $\alpha_6\beta_1$, it was necessary to first remove the $\alpha_3\beta_1$ from the detergent cell extracts before $\alpha_6\beta_1$ binding to human laminin could be detected. These results support the present study, such that the murine E8 fragment may not contain the GD-6 sequence and this might explain why $\alpha_3\beta_1$ binds less well to this fragment than does $\alpha_6\beta_1$. This study also presents data showing that $\alpha_3\beta_1$ binds to human laminin with a much higher affinity than does $\alpha_6\beta_1$. This result might suggest that the human laminin used in our studies may contain the GD-6 site, whereas, the murine E8 fragment does not contain this site. Support for this conclusion is derived from the fact that the anti-GD-6 antisera reacts with our human laminin preparations. These results, taken together, suggest that the GD-6 sequence may be the primary laminin binding site for $\alpha_3\beta_1$, and that there may also exist yet other interactive sites for this receptor. The consequences of having multiple sites in laminin for receptor interaction may allow for an increased cell binding affinity. In addition, having multiple receptor binding sites and receptors creates the possibility for a diversity of laminin-mediated effects on cellular behavior. Such regulation of cellular responses may relate to the type and number of specific laminin-binding receptors expressed on a cell surface.

It now appears that not only can integrins distinguish between specific regions of laminin, but there is evidence that certain integrins can recognize different species homologues of laminin as well (Brown and Goodman, 1991; Goodman et al., 1991). For example, there is data to suggest that the $\alpha_3\beta_1$ integrin on human cells has a higher affinity for human forms of laminin compared with the binding of $\alpha_6\beta_1$, whereas $\alpha_6\beta_1$ from human cells and platelets binds preferentially to EHS-laminin (Sonnenberg et al., 1991). Furthermore, $\alpha_3\beta_1$ has not been observed in cell-substratum adhesion plaques when human cells are plated on murine laminin (Carter et al., 1990; Sonnenberg et al., 1991), but can clearly be seen when human cells are seeded on human laminin (Gehlsen, K. R., unpublished results). These data suggest that $\alpha_3\beta_1$ and $\alpha_6\beta_1$ may have different affinities for different laminins due to specific species differences found

within the binding sequences. Therefore, future experiments to determine if $\alpha_6\beta_1$ and $\alpha_3\beta_1$ share common binding sites within laminin need to be done.

In conclusion, the data presented strongly suggest that the amino acid sequence of peptide GD-6 represents at least one site in laminin for binding of the $\alpha_3\beta_1$ integrin. Identification of this and other integrin-binding sites in laminin will help in our understanding of laminin-mediated regulation of cellular function.

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