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Novel Peptide Ligands for Integrin $\alpha 6\beta 1$ Selected from a Phage Display Library[1]

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Integrin $\alpha 6\beta 1$ is a major adhesion receptor for the basement membrane, specifically binding to laminin-1. To identify the peptide sequences recognized by $\alpha 6\beta 1$, we screened a 15-mer phage display library by panning with $\alpha 6\beta 1$ purified from human placenta. DNA sequencing of 73 randomly picked phage revealed that three clones were dominantly enriched after repeated panning with $\alpha 6\beta 1$. None of the peptide sequences displayed on these phage showed significant homology to laminin-1. A synthetic peptide modeled after the sequence displayed by one of these phage, designated P3, was found to strongly inhibit the binding of laminin-1 to $\alpha 6\beta 1$. This inhibitory effect of the P3 peptide seems to be specific for $\alpha 6\beta 1$, since it did not affect the binding of fibronectin to integrin $\alpha 5\beta 1$. A synthetic peptide with a scrambled P3 amino acid sequence barely inhibited the binding of laminin-1 to $\alpha 6\beta 1$. When coated on a substratum after conjugation with bovine serum albumin, the P3 peptide was capable of promoting cell spreading in an $\alpha 6\beta 1$-dependent manner, although the peptide with the scrambled sequence showed activity similar to that of a control peptide. These results taken together indicate that the P3 peptide is a novel ligand for integrin $\alpha 6\beta 1$ with potent cell spreading activity.

Key words: cell adhesion, integrin $\alpha 6\beta 1$, laminin-1, phage display library, synthetic peptides.

Adhesion to the basement membrane is required for the induction and maintenance of epithelial differentiation (*1*). The basement membrane consists of type IV collagen, laminins, heparan sulfate proteoglycan, and other noncollagenous glycoproteins, of which laminins play a predominant role in cell adhesion to the basement membrane. Laminins are a group of large heterotrimeric glycoproteins consisting of three subunit polypeptides termed α, β, and γ. Laminin-1, also referred to as EHS-laminin, is a major laminin present in the epithelial basement membrane, and consists of $\alpha 1$ (400 kDa), $\beta 1$ (200 kDa), and $\gamma 1$ (200 kDa) subunits, which are assembled into a characteristic cruciform structure (*2, 3*). Cell adhesion to laminins is mediated by a variety of cell-surface receptors including a range of integrins, 67 kDa laminin/elastin-binding protein, 35 kDa carbohydrate-binding protein, cell-surface galactosyltransferase, and 110 kDa amyloid precursor protein (*4-6*), among which integrins play central roles in laminin-mediated cell adhesion and subsequent signal transduction across the plasma membrane.

Integrins comprise a large family of heterodimeric transmembrane molecules consisting of an α subunit noncovalently associated with a β subunit. Eight distinct forms of integrins, *i.e.* $\alpha 1\beta 1$, $\alpha 2\beta 1$, $\alpha 3\beta 1$, $\alpha 6\beta 1$, $\alpha 7\beta 1$, $\alpha 6\beta 4$, and $\alpha v\beta 3$, have been shown to mediate cell adhesion to laminins, and each of them has a distinct ligand-binding specificity. Among them, $\alpha 6\beta 1$ appears to serve as a major receptor for laminin-1 in many cell types, since the function-blocking anti-$\alpha 6$ monoclonal antibody, GoH3, strongly inhibited the laminin-1-mediated adhesion of various cell types (*7-9*). The binding site for integrin $\alpha 6\beta 1$ has been mapped to the distal half of the long arm of laminin-1, typically obtained as a 240 kDa fragment upon elastase digestion (*10, 11*). This fragment, designated E8, has potent ability to promote cell attachment and spreading, and to induce neurite outgrowth (*12-14*). Further dissection of the binding site for integrin $\alpha 6\beta 1$ has been hampered, however, by the loss of the adhesive activity upon denaturation and/or separation of the $\alpha 1$-derived 140 kDa fragment from the $\beta 1$-$\gamma 1$ dimer-derived 80 kDa fragment (*15*). Many synthetic peptides modeled after the E8 fragment have been examined as to their cell-adhesive activity, but none of them possessed activity comparable to that of the E8 fragment or intact laminin-1 (*16-20*).

Recently, random phage display libraries have been successfully introduced to identify the peptide sequences involved in various protein-protein interactions including those between integrins and their ligands (*21*). Screening of random phage display libraries with integrins $\alpha 5\beta 1$ (*22, 23*), $\alpha IIb\beta 3$ (*24*), and $\alpha v\beta 3$ (*25*) resulted in the predominant selection of peptide sequences containing the Arg-Gly-Asp (RGD) tripeptide. In the present investigation, we

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Abbreviations: BSA, bovine serum albumin; PMSF, phenylmethylsulfonyl fluoride; WGA, wheat germ agglutinin; TBS, 25 mM Tris-HCl, pH 7.5, 130 mM NaCl; TBS(+), TBS containing 1 mM $CaCl_2$ and 1 mM $MgCl_2$; TBS(Mn), TBS containing 1 mM $MnCl_2$; PBS, phosphate-buffered saline (8.1 mM Na_2HPO_4, 1.5 mM KH_2PO_4, 137 mM NaCl, 2.7 mM KCl, pH 7.4); DMEM, Dulbecco's modified Eagle's medium.

screened a phage library displaying degenerate pentadecapeptides for those specifically recognized by integrin $\alpha6\beta1$. One of the peptides thus selected was found to specifically inhibit laminin-1 binding to integrin $\alpha6\beta1$, and to possess potent cell-adhesive activity.

EXPERIMENTAL PROCEDURES

Materials—The 15-mer phage display library constructed in the fUSE5 phage vector was a gift from Dr. Hideyuki Saya (Kumamoto University Medical School, Kumamoto). *Escherichia coli* strain K91kan was provided by Dr. George P. Smith (University of Missouri, Columbia, MO). Peptides were synthesized by Sawadei Technologies (Tokyo) or in our laboratory with an Applied Biosystems peptide synthesizer, Model 431A, followed by purification by reverse-phase high-performance liquid chromatography. Synthetic hexapeptides, GRGDSP and GRGESP, were purchased from Iwaki Glass (Tokyo). CNBr-activated Sepharose 4B, WGA-Sepharose, and heparin-Sepharose CL-6B were from Pharmacia LKB Biotechnology (Uppsala, Sweden). Protein A was from Sigma (St. Louis, MO). Mouse Engelbreth-Holm-Swarm (EHS) tumor was kindly provided by Dr. George R. Martin (National Institute of Aging, Baltimore, MD), and propagated in C57/BL mice. Laminin-1 was isolated from the EHS tumor tissue based on the method of Paulsson *et al.* (*26*), and further purified by affinity chromatography on heparin-Sepharose (*27*). Laminin-1 was radioiodinated with $Na^{125}I$ using IODOBEADS (Pierce, Rockford, IL). Plasma fibronectin was prepared from outdated human plasma by gelatin-affinity chromatography (*28*) and then radioiodinated as described above.

Antibodies—Antibodies against integrins were obtained from the following sources: Rat monoclonal anti-$\alpha6$ (GoH3) was from Cosmo-Bio (Tokyo), mouse monoclonal anti-$\alpha3$ (P1B5) from Gibco-BRL (St. Gaithersburg, MD), mouse monoclonal anti-$\beta1$ (4B4) from Coulter (Hialeah, FL), and rabbit polyclonal antibodies against the cytoplasmic domains of $\alpha5$ and $\beta1$ subunits, RM66 and RM22, respectively, from Bioline Diagnostici (Torino, Italy). Control rabbit IgG was from Jackson Immunoresearch Laboratories (St. West Grove, PA). A polyclonal antibody against the cytoplasmic domain of the $\alpha6$ subunit was prepared by immunizing rabbits with the synthetic peptide, CIHAQPSDKERLTSDA (designated "6C"). The anti-peptide 6C antibody was purified by affinity chromatography on a column of the antigenic peptide immobilized on CNBr-activated Sepharose 4B.

Purification of Integrin $\alpha6\beta1$—Integrin $\alpha6\beta1$ was purified from human placenta by immunoaffinity chromatography as follows. Human placenta (approximately 100 g) was extracted with TBS(+) (25 mM Tris-HCl, pH 7.5, 130 mM NaCl, 1 mM $CaCl_2$, 1 mM $MgCl_2$) containing 1 mM PMSF and 100 mM *n*-octyl-β-D-glucoside. The extract was clarified by centrifugation at $100,000\times g$ for 2 h and then passed through an immunoaffinity column of the anti-peptide 6C antibody immobilized on CNBr-activated Sepharose 4B (bed volume, 2 ml). The column was washed with TBS(+) containing 1 mM PMSF and 50 mM *n*-octyl-β-D-glucoside, and then the bound integrin $\alpha6\beta1$ was eluted with the same buffer containing 250 μg/ml of peptide 6C. Integrin $\alpha5\beta1$ was purified from the same placental extract as described previously (*29*). Both $\alpha6\beta1$ and $\alpha5\beta1$ were further purified on WGA-Sepharose as described (*30*). The purity of the integrin $\alpha6\beta1$ was verified by SDS-PAGE, followed by immunoblot analysis with antibodies specific to each integrin subunit.

Selection of $\alpha6\beta1$-Binding Phage—Phage binding to integrin $\alpha6\beta1$ was screened essentially as described (*31*). Wells of 96-well microtiter plates (Greiner, Frickenhausen, Germany) that had been precoated with protein A (5 μg/ml) and blocked with 3.5% BSA were incubated with the antibody RM22 against the cytoplasmic domain of the human $\beta1$ subunit (10 μg/ml) in TBS(+) for 2 h, followed by incubation with the purified human $\alpha6\beta1$ (approximately 50 μg/ml) at 4°C for 1 h to immobilize the $\alpha6\beta1$ on the wells. The ligand-binding activity of the immobilized $\alpha6\beta1$ was verified by the specific binding of ^{125}I-laminin-1 (data not shown).

An aliquot of the amplified phage library expressing degenerate pentadecapeptides ($1\times10^{10-11}$ transducing units) was diluted in TBS(Mn) (25 mM Tris-HCl, pH 7.5, 130 mM NaCl, 1 mM $MnCl_2$) containing 0.1% BSA and precleared in wells coated with protein A and RM22, but not with integrin $\alpha6\beta1$, for 1 h. The unadsorbed phage were then transferred to the wells loaded with integrin $\alpha6\beta1$. After 1 h incubation, the wells were washed three times with TBS(Mn) containing 0.5% Tween 20. The bound phage were eluted with 10 mM EDTA in TBS containing 0.1% BSA. The eluted phage were amplified in strain K91kan and recovered by precipitation with polyethylene glycol. Panning was repeated six more times, *i.e.* for a total seven times. After the final round of panning, phage DNA was sequenced with Taq DNA polymerase (Takara Shuzo) and the primer, 5′-CAGGACCCTCATAGTTAGCGTAACG-3′, using an Applied Biosystems DNA sequencer, Model 373A.

Solid-Phase Integrin Binding Assay—Integrin $\alpha6\beta1$ was diluted with TBS(+) to approximately 10 μg/ml. The wells of 96-well microtiter plates were coated directly with $\alpha6\beta1$ at 4°C overnight, and then blocked with 3.5% BSA. After repeated washing with TBS(+), ^{125}I-labeled laminin (1 μg/ml) was incubated in the presence or absence of 10 mM EDTA, anti-integrin antibodies, or synthetic peptides in TBS(Mn) containing 0.1% BSA at room temperature for 3 h. The wells were washed three times with TBS containing 0.5% Tween 20. The bound integrin $\alpha6\beta1$ was solubilized with 1% SDS and then quantified with an Aloka model ARC-600 gammacounter. The radioactivity bound to BSA-coated wells was subtracted as a control for nonspecific binding. The ligand binding activity of the purified integrin $\alpha5\beta1$ was quantified as described above using ^{125}I-fibronectin.

Cells—RD human rhabdomyosarcoma cells were obtained from the Japanese Cancer Research Resources Bank. RD cells were maintained in DMEM containing 10% fetal bovine serum.

Cell Spreading Assay—Synthetic peptides P3 (VSWFSRHRYSPFAVS), P3S (RFSVAVSSHYPFWSR), and CP1 (YDELPQLVTLPHPNL) were each conjugated to BSA with glutaraldehyde (*32*), and the resultant peptide-BSA conjugates (50 μg/ml) were coated onto 96-well microtiter plates (NUNC, Roskilde, Denmark) for 2 h at 37°C, and then blocked with 1% BSA in PBS (8.1 mM Na_2HPO_4, 1.5 mM KH_2PO_4, 137 mM NaCl, 2.7 mM KCl, pH 7.4) for 1 h

at 37°C. RD cells were detached with 0.05% trypsin/1 mM EDTA and resuspended in the assay medium [a 1:1 mixture of DMEM and HEPES-buffered saline (20 mM HEPES, 137 mM NaCl, 3 mM KCl, pH 7.1)]. Cells were seeded at 4×10^4 cells/well on the 96-well plates coated with the peptide-BSA conjugates and incubated for 1 h under a 5% CO_2 atmosphere at 37°C. Unattached cells were removed by gentle washing with PBS. The attached cells were fixed with 4% formaldehyde and then stained with 0.5% toluidine blue at room temperature for 30 min. Cells with a well spread morphology were counted in six independent fields per well.

RESULTS

Purification of Integrin α6β1—Integrin α6β1 was purified from human placenta by immunoaffinity chromatography using a rabbit polyclonal antibody against the cytoplasmic domain of the α6 subunit, followed by WGA-Sepharose chromatography. The integrin α6β1 thus purified migrated on SDS-PAGE as two bands corresponding to ~140 and 115-120 kDa, consistent with the reported masses of the α6 and β1 subunits, respectively (Fig. 1A). The authenticity of the purified α6β1 was further confirmed by immunoblot analysis with antibodies specific for the α6 and β1 subunits (Fig. 1B). No detectable integrin α6β4 copurified with α6β1, since the characteristic 190 kDa band of the β4 subunit was not detectable upon SDS-PAGE (Fig. 1A) or immunoprecipitation with a monoclonal anti-β4 antibody (data not shown).

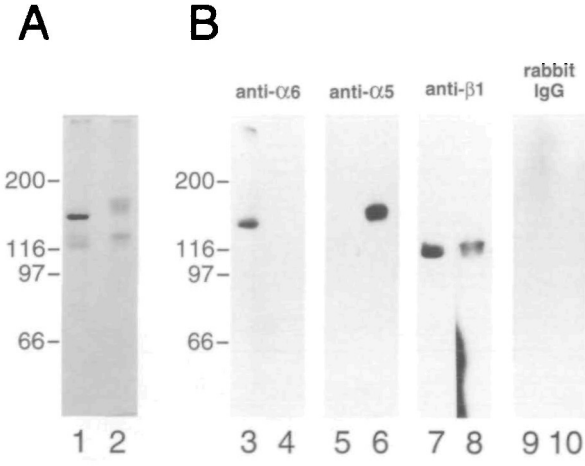

Fig. 1. **SDS-PAGE and immunoblot analyses of purified integrins α6β1 and α5β1.** Affinity-purified integrins α6β1 (lanes 1, 3, 5, 7, and 9) and α5β1 (lanes 2, 4, 6, 8, and 10) were analyzed on 10% SDS-polyacrylamide gels under nonreducing conditions. Proteins were visualized with Coomassie Brilliant Blue (A) or transferred to nitrocellulose membranes followed by immunostaining with rabbit antibodies specific for the cytoplasmic domain of the α6 subunit (lanes 3 and 4), the α5 subunit (RM66; lanes 5 and 6), and the β1 subunit (RM22; lanes 7 and 8), and with control rabbit immunoglobulin G (lanes 9 and 10). Note that the purified α6β1 was specifically stained with the anti-α6 and anti-β1 antibodies.

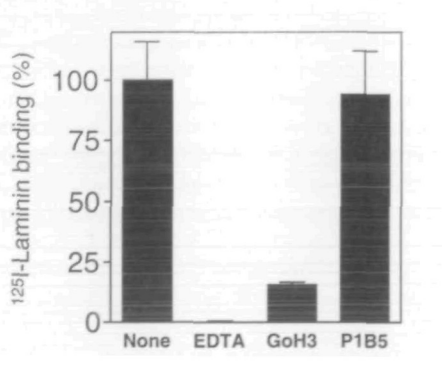

Fig. 2. **Binding of laminin-1 to purified integrin α6β1.** The binding of laminin-1 to the purified α6β1 was assayed in the absence or presence of the following inhibitors: 10 mM EDTA, anti-α6 antibody GoH3 (5 μg/ml), and anti-α3 antibody P1B5 (ascites at 1:100 dilution). Assays were carried out in quadruplicate and the values shown are means. Error bars represent standard deviation.

TABLE I. **Deduced amino acid sequences of peptides binding to integrin α6β1 selected from a 15-mer phage display library.**

Peptide[a]	Amino acid sequence[b]	
P1	FGRIPSPLAYTYSFR	(15)
P2	HRWMPHVFAVRQGAS	(12)
P3	VSWFSRHRYSPFAVS	(11)
	QLQSYRFFFPSYMGG	(5)
	WSNRMPPLFTPWYPP	(3)
	YWALHHSGWPFSRGS	(2)
	GYPWRIRPWASGPFL	(2)
	RRLVFWHGFETTGPR	(2)
	DRWRPALPVVLFPLH	(2)
	GPWYCTLGLCHFRSS	(2)
	GWPSFSNHPFLYPRW	(2)
	LNPFRSLFFPALDNL	(2)
	LSWPLHAGRGFRWVS	(2)
	VGFLGLKRGPPGVDA	(2)
	GRWAFAPSSWHLYSR	(2)
	GAGMLRWFGYPALYG	(2)
	SRVRFPAWGLPFSPV	
	WFPGPITFIPRPWSS	
	WWMSRPSRLLYYEYG	
	HRVQFAGWGFPGFRL	
	WHWRLPRSTWHPTSV	

[a]Notation of peptides. [b]The amino acid sequence designation is based on a single letter code. The numbers of phage displaying the same peptide sequence are indicated in parentheses.

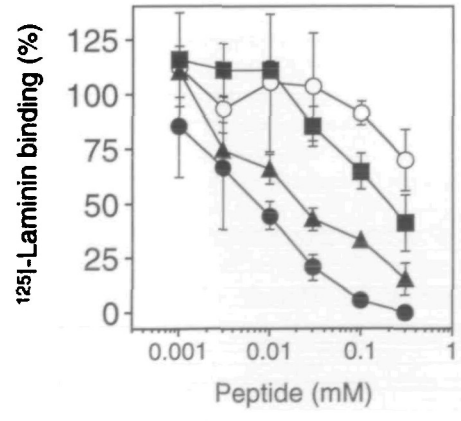

Fig. 3. **Inhibition of laminin-1 binding to α6β1 by synthetic peptides.** The binding of ^{125}I-laminin-1 to purified α6β1 adsorbed on microtiter plates was assayed in the absence or presence of increasing concentrations of the following synthetic peptides, as described under "EXPERIMENTAL PROCEDURES": (■) P1; (▲) P2; (●) P3; (○) CP1. Assays were carried out in triplicate and the values shown are means. Error bars represent standard deviation.

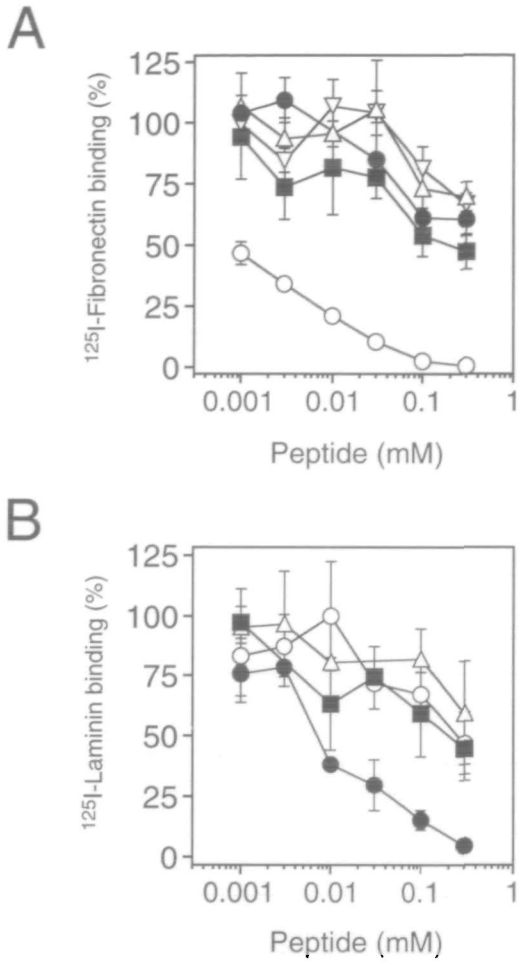

Fig. 4. **Inhibition of ligand binding to $\alpha5\beta1$ and $\alpha6\beta1$ by synthetic peptides.** The binding of ^{125}I-fibronectin to purified $\alpha5\beta1$ (A) and that of ^{125}I-laminin-1 to $\alpha6\beta1$ (B) were assayed in the absence or presence of increasing concentrations of the following synthetic peptides, as described under "EXPERIMENTAL PROCEDURES": (●) P3; (■) P3S; (△) CP1; ( ) GRGDSP; (▽) GRGESP. Assays were carried out in triplicate and the values shown are means. Error bars represent standard deviation.

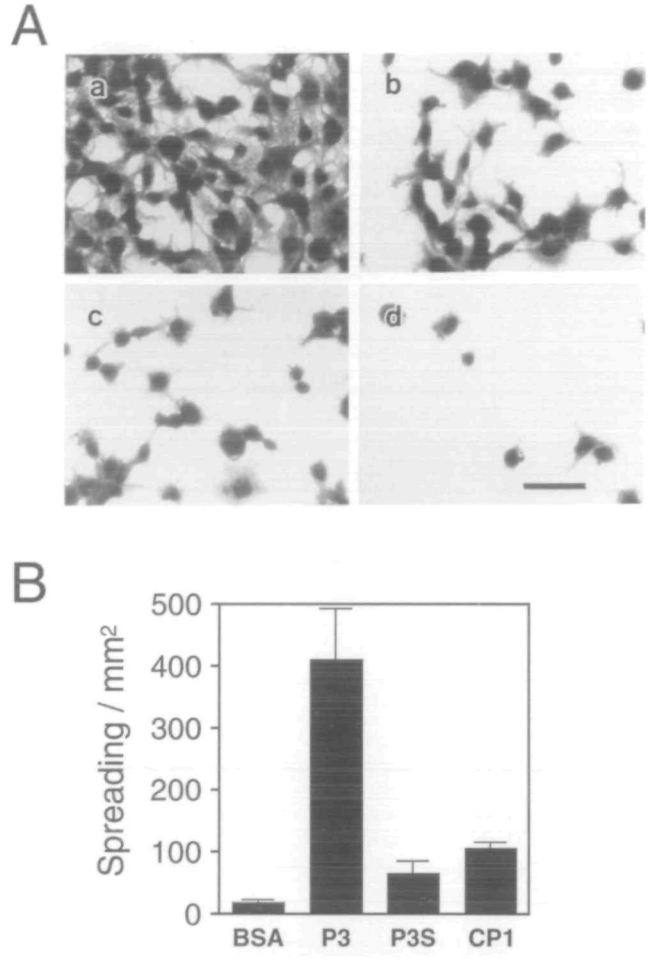

Fig. 5. **Cell spreading activity of synthetic peptides.** (A) Spreading of RD cells on microtiter plates coated with BSA conjugates of P3 (a), P3S (b), or CP1 (c), or with BSA alone (d), as described under "EXPERIMENTAL PROCEDURES." Bar, 50 μm. (B) The numbers of cells with a well-spread morphology per unit area (1 mm^2) were determined. The values shown are means of six separate determinations and error bars represent standard deviation.

The ligand binding activity and specificity of the purified $\alpha6\beta1$ were verified by means of a solid-phase binding assay with ^{125}I-laminin-1 (Fig. 2). Binding of ^{125}I-laminin-1 was completely abolished on the removal of divalent cations with 10 mM EDTA, and strongly inhibited by the function-blocking monoclonal anti-$\alpha6$ antibody, GoH3, but not by the monoclonal antibody, P1B5, that blocks the adhesive function of another laminin-binding integrin, $\alpha3\beta1$.

Screening of the Phage Display Library—To identify peptide ligands for integrin $\alpha6\beta1$, we screened a phage library expressing degenerate pentadecapeptides by panning with the purified $\alpha6\beta1$. Phage bound to the substrate-adsorbed $\alpha6\beta1$ were eluted with 10 mM EDTA, based on the divalent cation dependency of the integrin function. The percentage of phage recovered was $\sim10^{-4}$% after the first round of panning and it gradually increased to 10^{-1}% after the fifth round of panning. After the seventh round, 73 phage clones were randomly selected and the nucleotide sequences encoding degenerate pentadecapeptides were determined. Amino acid sequences deduced from the nucleotide sequences are summarized in Table I. A total of 21 peptide sequences were identified, among which three distinct ones, FGRIPSPLAYTYSFR (P1), HRWMPHVFA-VRQGAS (P2), and VSWFSRHRYSPFAVS (P3), represented more than 50% of the phage sequenced. All of the 21 sequences contained more than one arginine residue, although no unique consensus sequences containing arginine residue(s) emerged from these sequences. None of the peptide sequences exhibited significant homology to the $\alpha1$, $\beta1$, or $\gamma1$ subunit of laminin-1.

Inhibition of Laminin-1 Binding to $\alpha6\beta1$ by Synthetic Peptides—To examine the binding affinity and specificity of the selected peptides toward integrin $\alpha6\beta1$, synthetic peptides modeled after the dominantly selected sequences, *i.e.* P1, P2, and P3, were examined as to their ability to inhibit binding of laminin-1 to purified $\alpha6\beta1$ (Fig. 3). All three peptides inhibited laminin-1 binding to $\alpha6\beta1$ in a dose-dependent manner, with IC_{50} in the order of P3 < P2 < P1. P3 was most potent ($IC_{50} = \sim8 \times 10^{-6}$ M) and inhibited laminin-1 binding to $\alpha6\beta1$ almost completely at 0.1 mM, whereas the control peptide, CP1, modeled after a biologi-

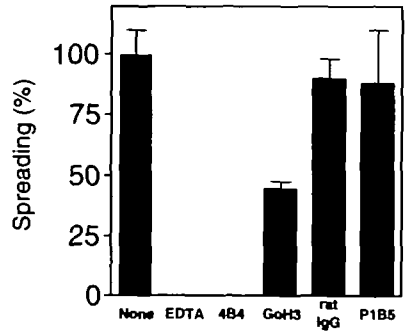

Fig. 6. **Inhibition by integrin antagonists of cell spreading onto the P3-BSA conjugate.** Microtiter plates were coated with 50 μg/ml of P3-BSA conjugate and blocked with 1% BSA. Trypsinized RD cells were preincubated in assay medium containing 10 mM EDTA, rat monoclonal anti-α6 antibody GoH3 (100 μg/ml), control rat immunoglobulin G_{2a} (100 μg/ml), mouse monoclonal anti-β1 antibody 4B4 (ascites at 1:20 dilution), or mouse monoclonal anti-α3 antibody P1B5 (ascites at 1:20 dilution) on ice for 10 min, and then transferred to microtiter plates precoated with the P3-BSA conjugate. The cells were attached to the plates by incubation for 1 h at 37°C in the presence of integrin antagonists, as described above. The numbers of cells with a well-spread morphology per unit area (1 mm^2) were determined and expressed as percentages of the control value obtained with no added antagonist.

cally inactive region of human fibronectin did not inhibit the binding at the same concentration. P2 was less inhibitory than P3, with IC_{50} of $\sim 2 \times 10^{-5}$ M, while P1 showed only a weak inhibitory effect ($IC_{50} > \sim 2 \times 10^{-4}$ M). The concentration of the P3 peptide required for 50% inhibition of laminin-1 binding to α6β1 was approximately 10,000 times higher than that of ^{125}I-laminin-1 used in the assay, indicating that the affinity of the P3 peptide to α6β1 was four orders of magnitude lower than that of intact laminin-1.

Since the P3 peptide showed the highest affinity to α6β1, the binding specificity of this peptide was further examined. Figure 4 shows the effects of various synthetic peptides on ligand binding by α6β1 and α5β1. P3 inhibited the binding of laminin-1 to α6β1 but not that of fibronectin to α5β1, although the GRGDSP peptide was inhibitory to α5β1 but not to α6β1. The control peptide CP1 interfered with the ligand binding of neither α6β1 nor α5β1. The binding specificity of the P3 peptide was further examined by scrambling the amino acid sequence. The scrambled version of P3 (RFSVAVSSHYPFWSR; designated P3S) barely had an inhibitory effect on either α6β1 or α5β1 (Fig. 4), indicating that the binding of P3 to α6β1 was dependent on its amino acid sequence.

Cell Spreading Activity of Synthetic Peptides—Specific binding of the P3 peptide to integrin α6β1 was further examined by means of a cell adhesion assay involving human RD rhabdomyosarcoma cells. The P3 peptide conjugated to BSA and adsorbed onto 96-well culture plates induced extensive cell attachment and subsequent cell spreading, although the scrambled version of P3 and the control CP1 peptide induced cell attachment to lesser extents and were barely active in promoting cell spreading (Fig. 5). The spreading of RD cells on P3 peptide-coated plates was inhibited by EDTA, and by function-blocking anti-α6 monoclonal antibody GoH3 and anti-β1 monoclonal antibody 4B4, but not by monoclonal anti-α3 antibody P1B5 (Fig. 6). Inhibition by GoH3 was less prominent than that by 4B4, suggesting that other β1-containing integrins may also be involved in cell adhesion to P3-coated substrates.

DISCUSSION

We identified a novel cell-adhesive peptide by screening a random phage display library with purified integrin α6β1. The peptide, P3, thus selected was considered to bind specifically to α6β1 from the following observations: (1) The phage displaying the P3 peptide was significantly enriched upon repeated panning with substrate-adsorbed α6β1; (2) The P3 peptide specifically inhibited the binding of α6β1 to laminin-1, a major extracellular matrix ligand for α6β1, but not the binding of α5β1 to fibronectin; (3) The binding affinity of the P3 peptide to α6β1 was dependent on the amino acid sequence of P3, since the scrambled peptide, P3S, was barely inhibitory to laminin-1 binding to α6β1; (4) The P3 peptide promoted cell attachment and subsequent cell spreading when used to coat the substratum after conjugation to BSA; (5) P3-mediated cell spreading was inhibited by function-blocking antibodies against the α6 and β1 subunits. The binding site on integrin α6β1 for the P3 peptide was considered to be close if not identical to that for laminin-1, since the same antibodies against the α6 and β1 subunits inhibited the cell adhesion to both laminin-1 and the P3 peptide. It is likely, therefore, that the P3 peptide inhibited laminin-1 binding to α6β1 competitively but not allosterically.

Despite the well-established binding specificity of α6β1 for laminin-1, the P3 peptide did not show significant sequence homology to laminin-1 subunits. This could have been due to the incomplete coverage of the entire pentadecapeptide repertoire by the phage library used in this study. Given that the maximum number of independent phage clones in the library was of the order of 10^8 and probably much less after multiple rounds of phage amplification, the library could only encompass the entire repertoire of random hexapeptides. The α6β1 recognition sequence within laminin-1 may not be reproduced in an active conformation by hexamer or shorter peptides displayed on the phage protein pIII.

Another and more likely possibility is that the α6β1 recognition site within laminin-1 can be generated only after assembly of three subunit chains. The α6β1 recognition site has been mapped to the distal half of the long arm of laminin-1, represented by the elastase-released E8 fragment (*10, 11*). Dissociation of the E8 fragment into an α1 chain-derived 140 kDa fragment and a β1-γ1 dimer-derived 80 kDa fragment has been shown to abolish the ability of the E8 fragment to promote α6β1-dependent cell adhesion (*15*). Conversely, a functionally active ternary complex can be regenerated upon association of an α1 chain-derived recombinant fragment with the 80 kDa β1-γ1 dimer-derived fragment (*33*). It is likely, therefore, that the binding of α6β1 to the E8 fragment requires either a defined conformation of the binding site effected upon assembly of three subunits or simultaneous contact at multiple sites on distinct laminin-1 subunits.

Besides laminin-1, integrin α6β1 has been shown to bind to invasin (*34*), a bacterial protein involved in the bacterial invasion of mammalian tissues, and fertilin/PH30, a sperm

protein involved in the binding of sperm to an egg (*35*). The $\alpha 6\beta 1$ binding sites of these proteins have been mapped to small proteolytic or synthetic peptides which exhibit no sequence homology to any of the laminin-1 subunits (*36, 37*). The P3 peptide exhibits no sequence homology to either invasin or fertilin/PH30. It remains possible, however, that the P3 peptide mimics a novel $\alpha 6\beta 1$ recognition site of an unknown $\alpha 6\beta 1$ ligand protein.

Many synthetic peptides modeled after laminin-1 chains have been shown to promote cell-substrate adhesion in an integrin-dependent (*18-20*) or -independent manner (*16, 17, 38, 39*). Gehlsen *et al.* (*18*) reported that a synthetic peptide modeled after the G domain of the $\alpha 1$ chain of laminin-1, designated GD6, was capable of promoting cell spreading through interaction with integrin $\alpha 3\beta 1$. Recently, Nomizu *et al.* (*20*) synthesized a total of 113 overlapping peptides that altogether cover the entire 950 amino acid G domain sequence, and found that two peptides, NRWHSIYITRFG (AG-10) and TWYKIAFQRNRK (AG-32), were capable of promoting cell attachment and spreading in an $\alpha 6\beta 1$-dependent manner, although the abilities of these peptides to mediate cell spreading were four orders of magnitude lower than that of intact laminin-1 on a molar basis. Our failure to select these sequences from the phage library could be due to either the absence of these peptides in the repertoire of the library or the low affinity of these peptides for $\alpha 6\beta 1$, that could not support the stable binding of the phage displaying these sequences to purified $\alpha 6\beta 1$. Another G domain-derived peptide, SINNNR (*19*), was reported to inhibit lung alveolar formation and to promote cell adhesion *in vitro* in an $\alpha 6\beta 1$-dependent manner (*40*). The synthetic SINNNR peptide, however, did not inhibit the binding of laminin-1 to purified $\alpha 6\beta 1$ (O. Murayama, unpublished observation).

Phage display libraries have been successfully employed to identify peptide sequences capable of binding to RGD-directed integrins (*22-25*). Thus, most of the phage selected by panning with αIIb$\beta 3$ (*24*), $\alpha 5\beta 1$ (*22, 23*), and $\alpha v\beta 3$ (*25*) displayed RGD-containing sequences, including those in cyclic structures in which the RGD tripeptide is flanked by two Cys residues. The cyclic forms of RGD-containing peptides were shown to have higher affinities to RGD-directed integrins than those of linear forms, consistent with the observation that the functionally active RGD motifs in the tenth type III repeat of fibronectin (*41*) and in snake venom proteins (*42, 43*) are located within a flexible loop structure. Utilizing a phage library displaying cyclic random heptapeptides constrained by disulfides, Koivunen *et al.* (*23*) successfully isolated a novel non-RGD peptide, CRRETAWAC, that specifically bound to integrin $\alpha 5\beta 1$ with high affinity comparable to that of a cyclic RGD peptide. Given the importance of the conformation of the $\alpha 6\beta 1$ recognition site within laminin-1, phage libraries displaying cyclic random peptides should be useful in identifying novel ligand peptides for $\alpha 6\beta 1$ with higher affinity and specificity than the P3 peptide.

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REFERENCES

1. Roskelley, C.D., Srebrow, A., and Bissell, M.J. (1995) A hierarchy of ECM-mediated signalling regulates tissue-specific gene expression. *Curr. Opin. Cell Biol.* **7**, 736-747
2. Beck, K., Hunter, I., and Engel, J. (1990) Structure and function of laminin: Anatomy of a multidomain glycoprotein. *FASEB J.* **4**, 148-160
3. Tryggvason, K. (1993) The laminin family. *Curr. Opin. Cell Biol.* **5**, 877-882
4. Mecham, R.P. (1991) Receptors for laminin on mammalian cells. *FASEB J.* **5**, 2538-2546
5. Kibbey, M.C., Jucker, M., Weeks, B.S., Neve, R.L., Van Nostrand, W.E., and Kleinman, H.K. (1993) β-Amyloid precursor protein binds to the neurite-promoting IKVAV site of laminin. *Proc. Natl. Acad. Sci. USA* **90**, 10150-10153
6. Mercurio, A.M. (1995) Laminin receptors: Achieving specificity through cooperation. *Trends Cell Biol.* **5**, 419-423
7. Sonnenberg, A., Modderman, P.W., and Hogervorst, F. (1988) Laminin receptor on platelets is the integrin VLA-6. *Nature* **336**, 487-489
8. Shaw, L.M., Messier, J.M., and Mercurio, A.M. (1990) The activation dependent adhesion of macrophages to laminin involves cytoskeletal anchoring and phosphorylation of the $\alpha 6\beta 1$ integrin. *J. Cell Biol.* **110**, 2167-2174
9. Cooper, H.M., Tamura, R.N., and Quaranta, V. (1991) The major laminin receptor of mouse embryonic stem cells is a novel isoform of the $\alpha 6\beta 1$ integrin. *J. Cell Biol.* **115**, 843-850
10. Aumailley, M., Timpl, R., and Sonnenberg, A. (1990) Antibody to integrin $\alpha 6$ subunit specifically inhibits cell-binding to laminin fragment 8. *Exp. Cell Res.* **188**, 55-60
11. Sonnenberg, A., Linders, C.J.T., Modderman, P.W., Damsky, C.H., Aumailley, M., and Timpl, R. (1990) 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. *J. Cell Biol.* **110**, 2145-2155
12. Edgar, D., Timpl, R., and Thoenen, H. (1984) The heparin binding domain of laminin is responsible for its effects on neurite outgrowth and neuronal survival. *EMBO J.* **3**, 1463-1468
13. Goodman, S.L., Deutzmann, R., and von der Mark, K. (1987) Two distinct cell-binding domains in laminin can independently promote nonneuronal cell adhesion and spreading. *J. Cell Biol.* **105**, 589-598
14. Aumailley, M., Nurcombe, V., Edgar, D., Paulsson, M., and Timpl, R. (1987) The cellular interactions of laminin fragments: Cell adhesion correlates with two fragment-specific high affinity binding sites. *J. Biol. Chem.* **262**, 11532-11538
15. Deutzmann, R., Aumailley, M., Wiedemann, H., Pysny, W., Timpl, R., and Edgar, D. (1990) Cell adhesion, spreading and neurite stimulation by laminin fragment E8 depends on maintenance of secondary and tertiary structure in its rod and globular domain. *Eur. J. Biochem.* **191**, 513-522
16. Tashiro, K., Sephel, G.C., Weeks, B., Sasaki, M., Martin, G.R., Kleinman, H.K., and Yamada, Y. (1989) A synthetic peptide containing the IKVAV sequence from the A chain of laminin mediates cell attachment, migration and neurite outgrowth. *J. Biol. Chem.* **264**, 16174-16182
17. Skubitz, A.P.N., Letourneau, P.C., Wayner, E., and Furcht, L.T. (1991) Synthetic peptides from the carboxy-terminal globular domain of the A chain of laminin: Their ability to promote cell adhesion and neurite outgrowth, and interact with heparin and the $\beta 1$ integrin subunit. *J. Cell Biol.* **115**, 1137-1148
18. Gehlsen, K.R., Sriramarao, P., Furcht, L.T., and Skubitz, A.P.N. (1992) A synthetic peptide derived from the carboxy terminus of the laminin A chain represents a binding site for the $\alpha 3\beta 1$ integrin. *J. Cell Biol.* **117**, 449-459
19. Matter, M.L. and Laurie, G.W. (1994) A novel laminin E8 cell adhesion site required for lung alveolar formation in vitro. *J. Cell Biol.* **124**, 1083-1090
20. Nomizu, M., Kim, W.H., Yamamura, K., Utani, A., Song, S.-Y., Otaka, A., Roller, P.P., Kleinman, H.K., and Yamada, Y. (1995)

Identification of cell binding sites in the laminin α1 chain carboxyl-terminal globular domain by systematic screening of synthetic peptides. *J. Biol. Chem.* **270**, 20583-20590
21. Scott, J.K. and Smith, G.P. (1990) Searching for peptide ligands with an epitope library. *Science* **249**, 386-390
22. Koivunen, E., Gay, D.A., and Ruoslahti, E. (1993) Selection of peptides binding to the α5β1 integrin from phage display library. *J. Biol. Chem.* **268**, 20205-20210
23. Koivunen, E., Wang, B., and Ruoslahti, E. (1994) Isolation of a highly specific ligand for the α5β1 integrin from a phage display library. *J. Cell Biol.* **124**, 373-380
24. O'Neil, K.T., Hoess, R.H., Jackson, S.A., Ramachandran, N.S., Mousa, S.A., and DeGrado, W.F. (1992) Identification of novel peptide antagonists for GPIIb/IIIa from a conformationally constrained phage peptide library. *Proteins* **14**, 509-515
25. Healy, J.M., Murayama, O., Maeda, T., Yoshino, K., Sekiguchi, K., and Kikuchi, M. (1995) Peptide ligands for integrin αvβ3 selected from random phage display libraries. *Biochemistry* **34**, 3948-3955
26. Paulsson, M., Aumailley, M., Deutzmann, R., Timpl, R., Beck, K., and Engel, J. (1987) Laminin-nidogen complex: Extraction with chelating agents and structural characterization. *Eur.J. Biochem.* **166**, 11-19
27. Timpl, R., Rohde, H., Risteli, L., Ott, U., Robey, P.G., and Martin, G.R. (1982) Laminin. *Methods Enzymol.* **82**, 831-838
28. Sekiguchi, K. and Hakomori, S. (1983) Domain structure of human plasma fibronectin: Differences and similarities between human and hamster fibronectins. *J. Biol. Chem.* **258**, 3967-3973
29. Pytela, R., Pierschbacher, M.D., and Ruoslahti, E. (1985) Identification and isolation of a 140 kd cell surface glycoprotein with properties expected of a fibronectin receptor. *Cell* **40**, 191-198
30. Pytela, R., Pierschbacher, M.D., Argraves, S., Suzuki, S., and Ruoslahti, E. (1987) Arginine-glycine-aspartic acid adhesion receptors. *Methods Enzymol.* **144**, 475-489
31. Parmley, S.F. and Smith, G.P. (1988) Antibody-selectable filamentous fd phage vectors: Affinity purification of target genes. *Gene* **73**, 305-318
32. Harlow, E. and Lane, D. (1988) *Antibodies: A Laboratory Manual*, pp. 78-81, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY
33. Sung, U., O'Rear, J.J., and Yurchenco, P.D. (1993) Cell and heparin binding in the distal long arm of laminin: Identification of active and cryptic sites with recombinant and hybrid glycoprotein. *J. Cell Biol.* **123**, 1255-1268
34. Isberg, R.R. and Leong, J.M. (1990) Multiple β1 chain integrins are receptors for invasin, a protein that promotes bacterial penetration into mammalian cells. *Cell* **60**, 861-871
35. Almeida, E.A.C., Huovila, A.-P.J., Sutherland, A.E., Stephens, L.E., Calarco, P.G., Shaw, L.M., Mercurio, A.M., Sonnenberg, A., Primakoff, P., Myles, D.G., and White, J.M. (1995) Mouse egg integrin α6β1 functions as a sperm receptor. *Cell* **81**, 1095-1104
36. Leong, J.M., Morrissey, P.E., and Isberg, R.R. (1993) A 76-amino acid disulfide loop in the Yersinia pseudotuberculosis invasin protein is required for integrin receptor recognition. *J. Biol. Chem.* **268**, 20524-20532
37. Myles, D.G., Kimmel, L.H., Blobel, C.P., White, J.M., and Primakoff, P. (1994) Identification of a binding site in the disintegrin domain of fertilin required for sperm-egg fusion. *Proc. Natl. Acad. Sci. USA* **91**, 4195-4198
38. Graf, J., Iwamoto, Y., Sasaki, M., Martin, G.R., Kleinman, H.K., Robey, F.A., and Yamada, Y. (1987) Identification of an amino acid sequence in laminin mediating cell attachment, chemotaxis, and receptor binding. *Cell* **48**, 989-996
39. Charonis, A.S., Skubitz, A.P.N., Koliakos, G.G., Reger, L.A., Dege, J., Vogel, A.M., Wohlhueter, R., and Furcht, L.T. (1988) A novel synthetic peptide from the B1 chain of laminin with heparin-binding and cell adhesion-promoting activities. *J. Cell Biol.* **107**, 1253-1260
40. Chen, L., Laurie, S.M., and Laurie, G.W. (1994) α6β1 integrin binds to SN-peptide within the first loop of the laminin-1 G domain. *Mol. Biol. Cell* (*Supplement to Volume* **5**), Abstracts for the Thirty-fourth Annual Meeting of the American Society for Cell Biology, abstract #H56
41. Main, A.L., Harvey, T.S., Baron, M., Boyd, J., and Campbell, I.D. (1992) The three-dimensional structure of the tenth type III module of fibronectin: An insight into RGD-mediated interactions. *Cell* **71**, 671-678
42. Adler, M., Lazarus, R.A., Dennis, M.S., and Wagner, G. (1991) Solution structure of kistrin, a potent platelet aggregation inhibitor and GPIIb-IIIa antagonist. *Science* **253**, 445-448
43. Saudek, V., Atkinson, R.A., and Pelton, J.T. (1991) Three-dimensional structure of echistatin, the smallest active RGD protein. *Biochemistry* **30**, 7369-7372