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Characterization of the Ligand-Binding Specificities of Integrin α3β1 and α6β1 Using a Panel of Purified Laminin Isoforms Containing Distinct α Chains

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Integrins α3β1 and α6β1 are two major laminin receptors expressed on the surface of mammalian cells. Interactions of cells with laminins through these integrins play important roles in cell adhesion, differentiation, motility, and matrix assembly. To determine the binding specificity and affinity of these integrins toward various types of laminins at the level of direct protein-protein interactions, we purified integrins α3β1 and α6β1 from human placenta, and examined their binding to a panel of laminin isoforms, each containing distinct α chains (*i.e.*, laminin-1, laminin-2/4, laminin-5, laminin-8, and laminin-10/11). Integrin α3β1 showed clear specificity for laminin-5 and laminin-10/11, with no significant binding to laminin-1, laminin-2/4, and laminin-8. In contrast, integrin α6β1 showed a broad spectrum of specificity, with apparent binding affinity in the following order: laminin-10/11 > laminin-5 > laminin-1 > laminin-2/4 ≅ laminin-8. Integrin titration assays demonstrated that laminin-10/11 was the most preferred ligand among the five distinct laminin isoforms for both α3β1 and α6β1 integrins. Given that laminin-10/11 is the major basement membrane component of many adult tissues, the interaction of laminin-10/11 with these integrins should play a central role in the adhesive interactions of epithelial cells with underlying basement membranes.

Key words: basement membrane, cell adhesion, integrin, laminin.

Adhesive interactions of epithelial cells with their underlying basement membrane are instrumental in regulating the development and maintenance of epithelial tissues (*1*). The basement membrane contains type IV collagens, laminins, nidogens, and heparan sulfate proteoglycans such as perlecan and agrin; and among these laminins play an important role in cell adhesion to the basement membrane. Laminins are a family of glycoproteins consisting of α, β, and γ chains. To date, five α, three β, and three γ chains have been identified, and combinations of these have been shown to give rise to at least 12 different laminin isoforms (*2*). These laminin isoforms are expressed in a tissue-specific and developmentally regulated manner, suggesting that they are functionally distinct. Cell adhesion to laminins is mediated by a variety of cell-surface receptors including a range of integrins, syndecans, and α-dystroglycan, among which integrins play central roles in laminin-mediated cell adhesion and subsequent signal transduction across the plasma membrane (*3–5*).

Integrins are heterodimeric adhesion receptors consisting of an α subunit non-covalently associated with a β chain. Ten integrin types, including α1β1, α2β1, α3β1, α6β1, α7β1, and α6β4, have been shown to mediate cell adhesion to laminins with distinct ligand-binding specificities (*3*). Among these laminin-binding integrins, α3β1 and α6β1 are known to be the major laminin receptors, since function-blocking antibodies against these integrins strongly inhibited the laminin-mediated adhesion of various cell types. Mapping of the integrin binding sites of laminin-1 and laminin-5 using their proteolytic fragments, a panel of function-blocking anti-laminin antibodies, and various recombinant laminins and their mutant forms with deletions and amino acid substitutions has shown that the integrin-binding sites are localized within the G domain of laminin α chains (*6*, *7*). Thus, the α chain containing the G domain appears to be the major determinant of the integrin-binding specificity of laminins.

The integrin-binding specificities of various extracellular matrix ligands have mainly been studied by inhibition assays of cell adhesion by anti-integrin antibodies. Integrin α3β1, once regarded as a promiscuous receptor for laminin-1, fibronectin, and collagens, has been shown to specifically mediate cell adhesion to laminin-5 (α3β3γ2) and laminin-10/11 (α5β1γ1/α5β2γ1) (*8–10*). On the other hand, integrin α6β1 is considered to be a major receptor for laminin-1 (α1β1γ1) (*11*, *12*), although it could also bind to laminin-5 (*13*) and laminin-10/11 (*14*). Laminin-2/4 (α2β1γ1/α2β2γ1) has been shown to be recognized by α3β1, α6β1, and α7β1 (*13*, *15*), while laminin-8 (α4β1γ1) was reported to be recognized by only α6β1 (*16*), or both α3β1 and α6β1 (*17*). It should be noted, however, that cells express multiple laminin receptors with dis-

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tinct binding specificities, *e.g.*, different types of integrins, syndecans, and α-dystroglycan, making it difficult to pinpoint the specificity of individual receptors by antibody inhibition of overall cell adhesion onto a particular type of laminin. To overcome this difficulty in defining the ligand-binding specificity and affinity of laminin-binding integrins, we purified α3β1 and α6β1 integrins from human placenta under non-denaturing conditions, and developed solid phase binding assays using purified integrins either reconstituted into ^{3}H-labeled liposomes or solubilized in detergent. Our data clearly show that both α3β1 and α6β1 share a preference for laminin-5 and laminin-10/11, but differ in their binding capability for laminin-1, laminin-2/4, and laminin-8, as well as in their binding affinity for laminin-5 and laminin-10/11.

MATERIALS AND METHODS

Reagents and Antibodies—Peptides were synthesized with an Applied Biosystems peptide synthesizer, Model 431A, followed by purification by reverse-phase high-performance liquid chromatography. CNBr-activated Sepharose 4B, wheat germ agglutinin (WGA)-Sepharose, Protein-G Sepharose 4B, and glutathione-Sepharose were purchased from Pharmacia (Uppsala, Sweden). Polyclonal antibodies against the cytoplasmic domains of integrins α3 and α6 were prepared by immunizing rabbits with the synthetic peptides KSQPSETERLTDDY (α3A) and IHAQPSDKERLTSDA (α6A) as conjugates with keyhole limpet hemocyanin. The anti-peptide α3A and α6A antibodies were purified by affinity chromatography on columns of the antigenic peptides immobilized on CNBr-activated Sepharose 4B. Other antibodies used were: anti-human integrin α3 mAb (P1B5) from Chemicon (Tamecula, CA); anti-human integrin α6 (GoH3) from Immunotech (Marseille, France); anti-human integrin α5 from Transduction Laboratories (Lexington, KY); anti-human integrin β1 (TS2/16) purified on a Protein-G Sepharose 4B column from the conditioned medium of hybridoma cells purchased from American Type Culture Collection (Manassas, VA); anti-human integrin β4 (C-20) from Santa Cruz Biotechnology (Santa Cruz, CA); goat anti-rabbit IgG antibody coupled to horseradish peroxidase from Cappel (Aurora, OH); and anti-human integrin α5 (*8*F*1*) and human laminin β1 chain (*4*F*5*) mAbs produced in our laboratory as described previously (*17*, *18*). The mAb specific for the human laminin α4 chain (2–11H) was produced by fusion of Sp2/O mouse myeloma cells with spleen cells from mice immunized with a glutathione S-transferase (GST) fusion protein containing the I/II domain of the human laminin α4 chain.

Purification of Integrins—Integrins α3β1 and α6β1 were purified from human placenta by immunoaffinity chromatography as described previously (*19*). Human placenta (approximately 100 g) was extracted with TBS (+) (20 mM Tris-HCl, pH 7.5, 0.13 M 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 × *g* for 2 h, then passed over immunoaffinity columns of the anti-α3A or α6A peptide antibodies. The columns were washed with TBS (+) containing 1 mM PMSF and 50 mM n-octyl-β-D-glucoside, and the bound integrins were eluted with the same buffer containing 200 μg/ml of the antigenic α3A or α6A peptides. Integrin α5β1 was purified from the same placental extract as described previously (*18*). All integrins were further purified on a WGA-Sepharose column as described (*18*). The purity of the integrins was verified by SDS-PAGE, followed by immunoblot analysis with antibodies specific to each integrin subunit.

Adhesive Proteins—Mouse laminin-1 was purified from mouse Engelbreth-Holm-Swarm tumor tissues by the method of Paulsson (*20*). Human laminin-2/4 (also referred to as merosin) was purchased from Chemicon. Human laminin-5 was purified from the conditioned medium of MKN45 human gastric cancer cells by immunoaffinity chromatography using polyclonal antibodies against the human laminin γ2 chain (*21*). Human laminin-8 was purified from the conditioned medium of T98G human glioma cells as described (*17*), except that the mAb 2–11H was used as the immunoaffinity ligand. Human laminin-10/11 was purified from the conditioned medium of A549 human lung adenocarcinoma cells (*10*). Human plasma fibronectin and vitronectin were purified from out-dated human plasma by gelatin- and heparin-affinity chromatography, respectively, as described previously (*22*, *23*). The GST-invasin fusion protein was constructed as follows. The plasmid pJL309 encoding invasin fused with maltose binding protein (MBP-INV497) was kindly provided by Dr. Ralph R. Isberg (Tufts University, Boston, MA) (*6*). A cDNA fragment encoding the C-terminal 497 amino acid residues of invasin was amplified by polymerase chain reaction (PCR) and inserted into pGEX4T-1 vector (Pharmacia) for expression as the GST fusion protein. The GST-invasin fusion protein was expressed in *Escherichia coli* strain DH5α and purified on a glutathione-Sepharose column.

Integrin-Liposome Binding Assay—Purified integrins (20 μg/ml) were reconstituted into liposomes as described previously (*18*). Microtiter plates were coated with adhesive proteins or purified integrins overnight at 4°C, then blocked with PBS (–) containing 2% bovine serum albumin (BSA) for 30 min at 37°C. Integrin-liposomes were added to the microtiter plates and incubated for 6 h at room temperature in the presence of 1 mM $MnCl_2$ or 10 mM EDTA. The plates were washed with TBS containing 1 mM $MnCl_2$ [TBS (Mn)], and bound liposomes were recovered in 1% SDS. The radioactivity of the bound liposomes was quantified using a Packard TRI-CARB 1500 liquid scintillation analyzer (Research Parkway Meriden, CT).

Integrin Binding Assay and Scatchard Plot Analysis—Integrin titration assays were carried out by the method of Eble *et al.* (*24*). Microtiter plates were coated with laminin-5 or laminin-10/11 (10 nM) overnight at 4°C, then blocked with 2% BSA for 2 h at room temperature. Plates were washed with TBS (Mn) containing 0.1% BSA and 0.02% Tween-20 (Buffer A). Serially diluted α3β1 or α6β1 was added to the plates and allowed to bind to the substrate-adsorbed ligand protein for 3 h in the presence of 1 mM $MnCl_2$ or 10 mM EDTA. The plates were then washed with 25 mM HEPES (pH 7.6) containing 1 mM $MnCl_2$ or 10 mM EDTA, and bound integrins were fixed to them by incubation with 2.5% glutaraldehyde for 10 min. The wells were washed with TBS (Mn), and the

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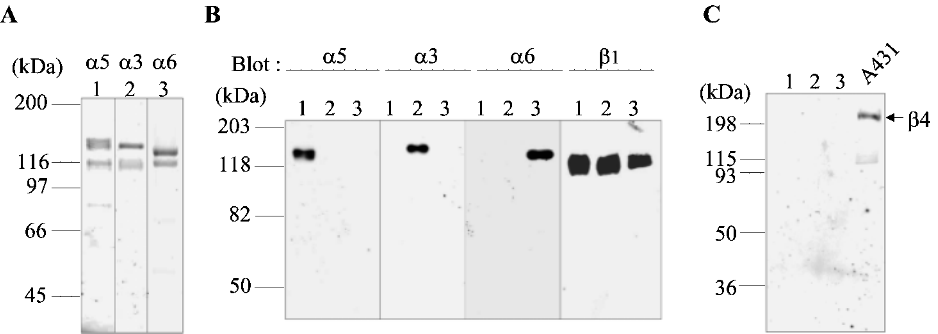

Fig. 1. **SDS-PAGE and immunoblot analyses of purified integrins.** Purified integrins α5β1 (lane 1), α3β1 (lane 2), and α6β1 (lane 3) were analyzed on 8% SDS-polyacrylamide gels under non-reducing conditions, except that immunoblotting with anti-α5 and anti-β4 antibodies were carried out under reducing conditions. Proteins were visualized with Coomassie Brilliant Blue (A) or transferred to nitrocellulose membranes followed by immunostaining with antibodies specific for integrin α5, α3, α6, or β1 subunits (B), or for β4 subunits (C). A431 cells were lysed by 1% Nonidet P-40, and used as a positive control for immunoblotting of integrin β4 (C).

bound integrins were quantified by an enzyme linked immunosorbent assay (ELISA). Briefly, the wells were incubated with rabbit polyclonal anti-α3A or anti-α6A peptide antiserum (diluted 1:1,000) for 1 h at room temperature in the Buffer A, washed three times with Buffer A, then incubated with secondary goat anti-rabbit IgG antibody coupled to horseradish peroxidase (diluted 1: 3,000) for 1 h. After washes with Buffer A, the bound antibodies were quantified by the absorbance at 490 nm after incubation with *o*-phenylenediamine. The absorbance obtained in the presence of 10 mM EDTA was subtracted as background from each readout. The dissociation constants (K_d) were determined by Scatchard plot analysis. We defined ν, which represents the moles of bound ligand per moles of total ligand, by

$$\nu = \frac{A([R]) - A([R]_b)}{A(\max) - A([R]_b)}$$

where $A([R])$ represents the absorbance at wavelength 490 nm (A_{490}) at the given integrin concentration R. The A_{490} obtained in the presence of 10 mM EDTA was used as the background $A([R]_b)$ as described above. $A(\max)$ is the maximum $A490$ obtained at saturating concentrations of the integrins. The $\nu/[R]$ was plotted against ν, and the K_d values were determined from the slopes by linear regression analysis.

RESULTS

Purification of Integrins—Integrins α3β1 and α6β1 were purified from human placenta by immunoaffinity chromatography. We used rabbit polyclonal antibodies raised against synthetic peptides derived from the cytoplasmic domains of the α3A and α6A subunits as immunoaffinity ligands and eluted the bound integrins with a large excess of antigenic peptides without acids or urea, to avoid protein denaturation during elution (*19*). Integrin α5β1 was also purified on an affinity column containing the cell-binding domain of fibronectin. Lectin chromatography on a WGA-Sepharose column was included to concentrate the integrins in the eluates and to remove the antigenic peptides (or GRGDSP peptide for the purification of α5β1 integrin). The integrin α3β1 thus purified migrated on SDS-PAGE as two bands in the regions of 150 kDa and 115 kDa (Fig. 1A), consistent with the masses reported for the α3 and β1 subunits, respectively. The purified integrin α6β1 gave two bands in the regions of 140 kDa and 115 kDa (Fig. 1A), corresponding to the α6 and β1 subunits, respectively. The authenticity of these purified integrins was further confirmed by immunoblot analysis with antibodies specific for the integrin α5, α3, α6, and β1 subunits (Fig. 1B). The typical yields of integrins α3β1 and α6β1 were approximately 100 μg and 60 μg, respectively, from 100 grams of placenta. Although the α6 chain can associate with both β1 and β4 chains, no 190-kDa band corresponding to the β4 chain was detected upon SDS-PAGE (Fig. 1A) or immunoblot analysis with polyclonal anti-β4 antibodies (Fig. 1C). Since the polyclonal antibody against the α6A cytoplasmic domain was used as the immunoaffinity ligand for the purification of α6-containing integrins, the α6 chain associated with the β4 chain may not be bound by the immunoaffinity column due to steric hindrance imposed by the bulky cytoplasmic domain of β4.

Ligand Binding Specificities of Purified Integrins α3β1 and α6β1—The binding specificities of integrins α3β1 and α6β1 toward a panel of laminin isoforms were studied by integrin-liposome binding assay. Laminin-1, laminin-2/4, laminin-5, laminin-8, and laminin-10/11, each having different laminin α chains, were coated on 96-well plates and incubated with α3β1 and α6β1 integrins reconstituted into ^{3}H-labeled liposomes to determine their integrin binding activities. Assays were carried out in the presence of 1 mM $MnCl_2$ to fully activate the integrins. Integrin α3β1 preferentially bound to laminin-5 and laminin-10/11, although only a marginal binding was observed with laminin-2/4, and there was no significant binding with laminin-1 and laminin-8 (Fig. 2A). Despite the restricted specificity of integrin α3β1, integrin α6β1 showed relatively broad specificities toward all the laminin isoforms examined. Integrin α6β1 strongly bound to laminin-1, laminin-5, and laminin-10/11, and to a lesser extent, laminin-2/4 and laminin-8 (Fig. 2B). No significant binding of either integrin α3β1 or α6β1 was observed on the surfaces coated with fibronectin or vitronectin, although both integrins displayed a high affinity for the recombinant invasin fragment included as a positive control. The specificity of the integrin-liposome binding assays was further confirmed by the complete inhibition of integrin binding in the presence of 10 mM EDTA. Integrin α5β1, a well-known fibronectin receptor, bound strongly to fibronectin, and to a lesser extent to vitronectin; but it failed to bind to the laminin isoforms, except that it weakly bound to laminin-

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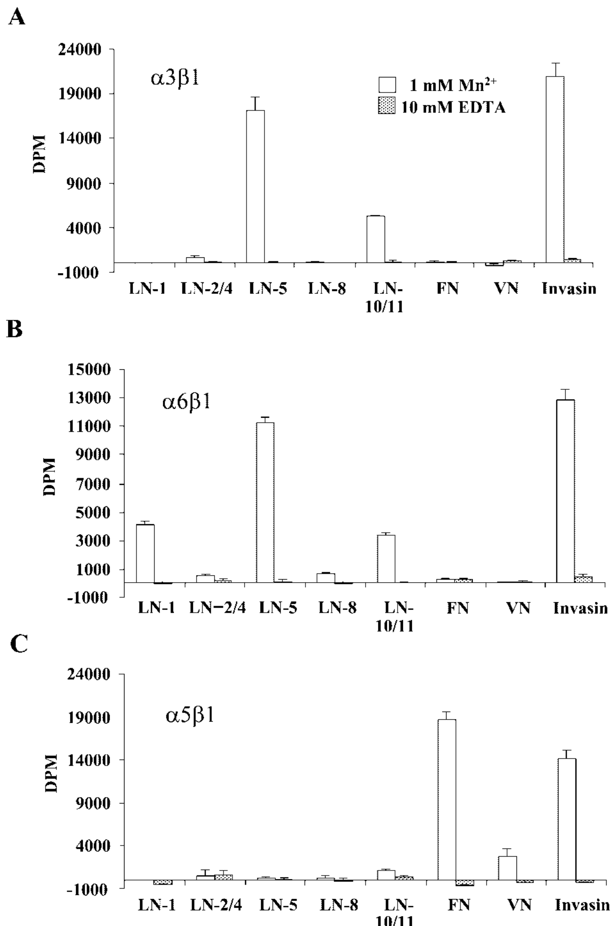

Fig. 2. **Ligand binding specificity of purified integrins.** Integrins α3β1 (A), α6β1 (B), and α5β1 (C) reconstituted into ^{3}H-labeled liposomes were allowed to bind to plates coated with the following adhesive ligands: laminin-1, laminin-2/4, laminin-5, laminin-8, laminin-10/11, fibronectin, vitronectin, and GST-invasin, in the presence of 1 mM $MnCl_2$ (open bars) or 10 mM EDTA (shaded bars). The concentration of these ligands used was 25 nM, except for fibronectin and vitronectin, which were coated at 100 nM. The binding of integrin-liposomes to uncoated plates blocked with BSA was regarded as nonspecific binding and subtracted as the background. The specific binding of integrin-liposomes is expressed as the mean DPM in triplicate determinations. Error bars represent standard deviations.

Table 1. **Dissociation constants of integrins α3β1 and α6β1 toward laminin-5 and laminin-10/11.**

	K_d (nM)*	
	Laminin-5	Laminin-10/11
α3β1	4.0 ± 0.7	1.5 ± 0.6
α6β1	15 ± 2	5.8 ± 1.2

*Mean ± SD calculated from three independent experiments.

10/11 (Fig. 2C). The binding of α5β1 integrin to laminin-10/11 was inhibited by exogenously added GRGDSP peptide (data not shown), indicating that integrin α5β1 bound to laminin-10/11 in an RGD-dependent manner. The specificity of the binding of integrin-liposomes was further verified by inhibition assays using function-blocking anti-integrin mAbs. The binding of integrin α3β1 to laminin-10/11 was inhibited by the anti-α3 mAb P1B5, but not by the anti-α6 or anti-α5 mAbs. Similarly, the binding of α6β1 to laminin-1 was completely inhibited by the anti-α6 mAb GoH3, but not by the anti-α3 or anti-α5 mAbs (Fig. 3). The binding specificity of integrin α5β1 was also confirmed by the anti-α5 mAb 8F1 (*18*).

Laminin-10/11 Is the Most Preferred Ligand for Both α3β1 and α6β1 Integrins—For a more quantitative analysis of the ligand-binding affinity of α3β1 and α6β1 integrins, we developed an ELISA to quantify the binding of detergent-solubilized integrins to substrate-adsorbed laminin isoforms. Upon titration of the ligand binding with increasing concentrations of integrins α3β1 and α6β1, both bound more readily to laminin-10/11 than to laminin-5 (Figs. 4A and 5A). Integrin α3β1 did not show any significant binding to other laminin isoforms, consistent with the results obtained by the integrin-liposome binding assays. In contrast, integrin α6β1 exhibited a broad spectrum of specificity with apparent binding affinities in the following order: laminin-10 > laminin-5 > laminin-1 > laminin-2/4 ≅ laminin-8. The dissociation constants of laminin-5 and laminin-10/11 for integrins α3β1 and α6β1 were determined from Scatchard plots (Figs. 4B and 5B). The dissociation constants of laminin-10/11 for α3β1 and α6β1 integrins were 1.5 ± 0.6 nM and 5.8 ± 1.2 nM (Table 1), respectively, significantly lower than those of laminin-5 for α3β1 and α6β1 integrins (4.0 ± 0.7 nM and 15 ± 2 nM, respectively). The dissociation constants for other laminin isoforms could not be determined due to the only partial saturation of integrin binding at the highest concentrations available. It was note-

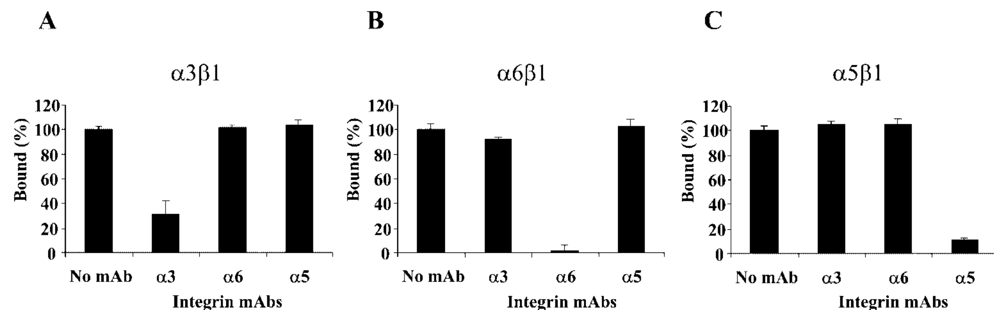

Fig. 3. **Inhibition of integrin binding by function-blocking anti-integrin antibodies.** ^{3}H-labeled liposomes containing integrins α3β1 (A), α6β1 (B), or α5β1 (C), were preincubated with function-blocking antibodies (50 μg/ml) against integrin α3 (P1B5), α6 (GoH3), and α5 (8F1) at room temperature for 10 min, then allowed to bind to substrates coated with their ligands, *i.e.*, 2 nM laminin-10/11 (A), 10 nM laminin-1 (B), and 10 nM fibronectin (C). The amounts of bound liposomes are expressed as the percentages of those obtained in the absence of the function-blocking mAbs. The results shown are the means of triplicate determinations with standard deviations.

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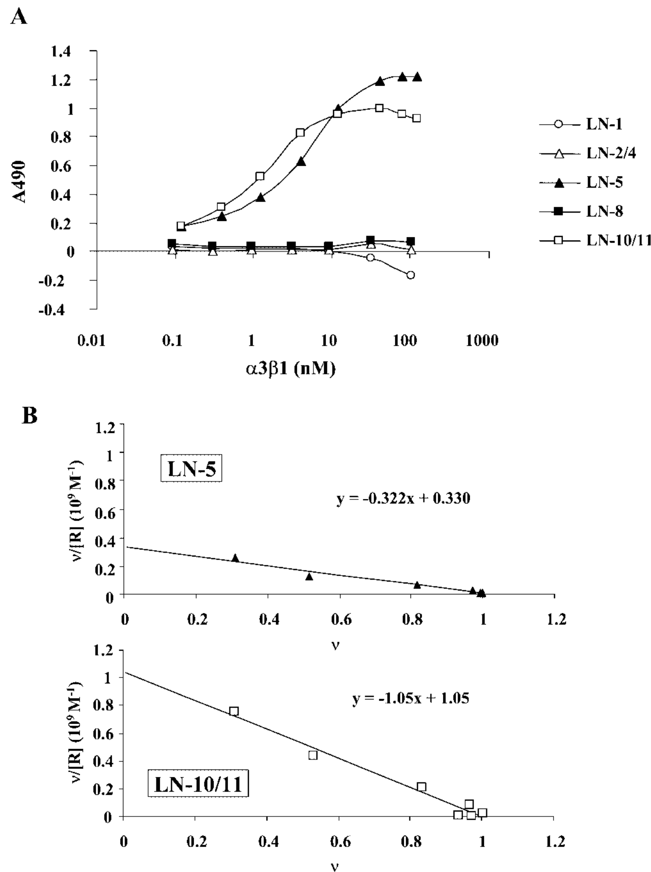

Fig. 4. **Titration curves of integrin α3β1 to laminin-5 and laminin-10/11.** (A) Increasing concentrations of integrin α3β1 were allowed to bind to microtiter plates coated with laminin-1 (open circles), laminin-2/4 (open triangles), laminin-5 (closed triangles), laminin-8 (closed squares), or laminin-10/11 (open squares) in the presence of 1 mM $MnCl_2$. Bound integrin α3β1 was quantified by ELISA as described in the "MATERIALS AND METHODS." The amounts of integrin α3β1 bound in the presence of 10 mM EDTA was taken as nonspecific binding and subtracted as the background. The results shown are the means of triplicate (for laminin-5 and laminin-10/11) or duplicate (for other isoforms) determinations. (B) Scatchard plots of the data of laminin-5 and laminin-10/11 shown in (A). The $\nu/[R]$ were plotted against the ν, and analyzed by linear regression analysis. The dissociation constants determined from the slopes of three independent experiments are shown in Table 1.

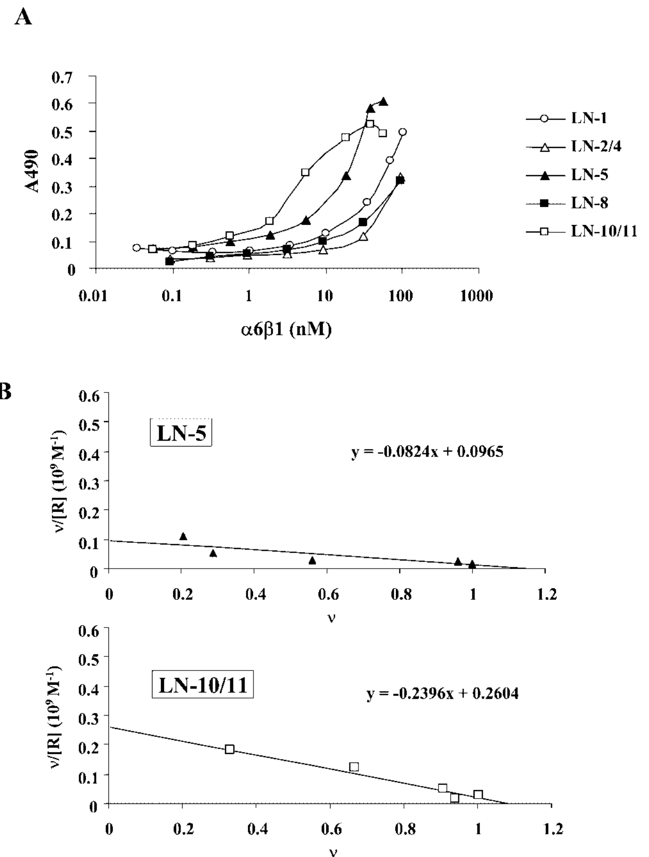

Fig. 5. **Titration curves of integrin α6β1 to laminin-5 and laminin-10/11.** (A) Binding of integrin α6β1 to laminin isoforms containing distinct α chains were determined as described in the legend for Fig. 4. (B) Scatchard plots of the data for laminin-5 and laminin-10/11 shown in (A). The dissociation constants determined from the slopes of three independent experiments are shown in Table 1.

worthy that the levels of maximal integrin binding for laminin-5 were higher than those for laminin-10/11 (Figs. 4A and 5A). This could be due to the difference in the densities of laminin isoforms immobilized on the solid surface, since laminin-5 is significantly smaller in molecular size than laminin-10/11 due to truncation in the N-terminal region of all three subunit chains, yielding a higher density of immobilized ligands than laminin-10/11. Similarly, laminin-5 showed an apparent higher integrin binding than laminin-10/11 in integrin-liposome binding assays (Fig. 2). This could also reflect the difference in the maximal levels of integrin binding, but not a difference in the binding affinity per se, since the substrates were coated with laminin isoforms at a relatively high concentration (i.e., 25 nM) in the integrin-liposome binding assays. These results together indicate that among the known laminin isoforms containing α1-α5 chains, laminin-10/11 is the most preferred ligand with the highest affinity toward both α3β1 and α6β1 integrins.

DISCUSSION

In previous studies, integrin α3β1 was purified on affinity columns containing either human laminin with undefined α subunits or the GD-6 peptide modeled after the LG5 domain of the laminin α1 chain (*25*, *26*). However, the homogeneity of these α3β1 integrins was not carefully verified by immunoblot analysis using a panel of anti-integrin antibodies. The mouse laminin α1 chain, from which the GD-6 peptide was derived, was reported to be recognized by integrin α6β1 but not α3β1 (*13*), although cell adhesion to GD-6-coated substrates was reported to be strongly inhibited by the function-blocking mAb against integrin α3β1, but not the mAb against α6β1 (*25*). More recently, recombinant integrin α3β1 has been expressed in insect cells as a truncated, soluble form (*24*), although its ligand-binding specificity toward the various laminin isoforms has not been thoroughly explored. Furthermore, we could not exclude the possibility that truncation of the C-terminal transmembrane and cytoplasmic domains might influence ligand-binding spe-

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cificity as well as affinity of integrins. Among other laminin-binding integrins, integrin α7β1 was recently expressed as a truncated form in mammalian cells, and its binding specificity toward various laminin isoforms was determined (*27*). However, the α6 chain-containing integrins, α6β1 and α6β4, have not been characterized with respect to ligand-binding specificity using purified proteins in intact forms or as recombinant, truncated forms. We previously purified integrin α6β1 from human placenta, but its ligand binding specificity was not explored due to the unavailability of purified laminin isoforms containing different α chains. The recent establishment of protocols for the purification of laminin isoforms containing the α3, α4, and α5 chains in our laboratory (*10*, *17*, *21*) has enabled us to determine the binding specificity and affinity of both integrins α3β1 and α6β1 after purification under non-denaturing conditions. Our data showed that integrin α3β1 has a more restricted specificity than integrin α6β1 and is capable of binding to laminin-5 and laminin-10/11 with high affinity, although it does not show any significant affinity to other isoforms. In contrast, integrin α6β1 has a broad spectrum of specificity and is capable of binding to all laminin isoforms, regardless of the type of the α chain, with binding affinity in the following order: laminin-10/11 > laminin-5 > laminin-1 > laminin-2/4 ≅ laminin-8. Although we could not determine the dissociation constants for laminin-1, laminin-2/4, and laminin-8 due to their partial or null saturation of integrin binding, our results clearly show that among all the laminin isoforms examined in this study, laminin-10/11 is the most preferred ligand for both integrins α3β1 and α6β1.

There is ample evidence that cell adhesion to substrates coated with laminin-5 or laminin-10/11 is strongly inhibited by a function-blocking antibody against integrin α3β1, but not by other antibodies, including those against integrin α6β1 (*8*, *10*, *28*). Nevertheless, there is also conflicting evidence that both α3β1 and α6β1 integrins serve as major receptors for laminin-5 and laminin-10/11 (*13*, *14*). Elucidation of the receptors involved in cell adhesion to substrates coated with a given adhesive ligand depends on the repertoire of integrins and other adhesion receptors expressed on the cell surface as well as the expression levels of these receptors. Since the repertoire and expression levels of integrins vary among different cell types, it is difficult to elucidate the integrin-binding specificities of individual laminin isoforms by cell adhesion inhibition assays using function-blocking anti-integrin antibodies. Thus, adhesion of A549 human lung carcinoma cells that express a high level of integrin α3β1 but a very low level of α6β1 was strongly inhibited by the mAb against integrin α3β1, although HT1080 human fibrosarcoma cells expressing not only α3β1 but also α6β1 at relatively high levels adhered to laminin-10/11 through both α3β1 and α6β1 integrins (*14*). Our data clearly show that both integrins α3β1 and α6β1 can bind to laminin-5 and laminin-10/11 with high affinity, although the dissociation constants of integrin α3β1 for these laminin isoforms are slightly lower than those of integrin α6β1. Consistent with our results, K562 cells transfected with the integrin α6 subunit have been shown to adhere to both laminin-5 and laminin-10/11 without integrin stimulation (*14*). It should be also noted that the K562 cells expressing α6β1 integrin could adhere to laminin-1 only after stimulation (*13*, *14*). Consistent with these observations, the binding activity of integrin α6β1 to laminin-1 was significantly lower than those to laminin-5 and laminin-10/11.

Despite strong affinities toward laminin-5 and laminin-10/11, integrin α3β1 did not show any significant binding to laminin-8 in our liposome binding assays. Instead, integrin α6β1 was found to bind laminin-8 with a low affinity. These results are different to our previous report that cell adhesion to laminin-8 was mediated by both α6β1 and α3β1 integrins with a slight preference for the former integrin (*17*). This discrepancy could be due to a difference in the protocol used for laminin-8 purification. In this study, we used an anti-laminin α4 mAb for immunoaffinity purification of laminin-8, while an anti-laminin β1 mAb was used as the immunoaffinity ligand in the previous study (*17*). Although our data show that only α6β1 integrin can bind to the laminin-8 purified on an anti-laminin α4 chain column, both α6β1 and α3β1 integrins can bind to the laminin-8 purified on an anti-laminin β1 chain column (Nishiuchi R., Fujiwara H., unpublished observations). It is likely, therefore, that the laminin-8 purified on the anti-laminin β1 column contains an unidentified ligand for the α3β1 integrin. Consistent with this possibility, strict dependency on integrin α6β1 was recently reported for the cell adhesion to recombinant laminin-8 produced in human 293 cells (*16*).

Our data show that integrin α5β1, a specific receptor for fibronectin, is capable of binding to laminin-10/11 with a low, but significant, affinity. Binding of integrin α5β1 to laminin-10/11 seems specific, since the binding was inhibited in the presence of 10 mM EDTA (Fig. 2C) or 100 μM GRGDSP peptide. This binding should not be due to any contamination of fibronectin in the laminin-10/11 used, since the binding activity remained unaffected after laminin-10/11 was passed over a gelatin-Sepharose column to remove any trace amount of contaminating fibronectin (data not shown). Recently, Sasaki *et al.* (*29*) reported that domain IVa of the laminin α5 chain contains two RGD motifs, which are capable of binding to integrins αvβ3 and α5β1 in an RGD-dependent manner. Therefore, it is likely that laminin-10/11 can bind to integrin α5β1 through the RGD motifs in domain IVa. However, the physiological relevance of the RGD-dependent binding of integrin α5β1 to laminin-10/11 remains undefined, since K562 cells known to express integrin α5β1 could not adhere to laminin-10/11, even after integrin activation by 12-*O*-tetradecanoylphorbol 13-acetate or Mn^{2+} (Nishiuchi R., unpublished observations).

The dissociation constants of integrin α3β1 for laminin-5 and laminin-10/11 were determined to be ~4 nM and ~1.5 nM, respectively, based on the titration curve and Scatchard plot analysis. Unlike the integrin-liposome binding assays, purified integrins were incubated directly on laminin-coated substrates without reconstitution into liposomes in these titration assays. The dissociation constant of integrin α3β1 for binding to laminin-5 thus determined was apparently seven-fold lower than that determined with recombinant integrin α3β1 lacking the transmembrane and cytoplasmic regions of both the α3 and β1 subunits (*24*). This apparent discrepancy in the K_d values for laminin-5 could be due to a difference in

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the avidity between intact and recombinant α3β1. In our titration assays, integrin α3β1 was dispersed in a buffer containing 50 mM n-octyl-β-D-glucoside solution to form integrin-detergent comicelles, thereby facilitating multivalent interaction between integrin α3β1 and the substrate-adsorbed laminins, although the truncated recombinant integrin binds to the substrate-immobilized laminins through monovalent interaction. Furthermore, possible differences in glycosylation patterns might also contribute to the apparent difference in the estimated dissociation constants, since recombinant α3β1 was produced in insect cells, but the α3β1 used in this study was from human placenta. Differences in glycosylation may modify the ligand binding affinity of integrin α3β1. It should also be noted that the association of truncated α3 and β1 chains was stabilized by the leucine zipper segments attached to their C-termini. Forced association of the α3 and β1 subunits through the leucine zipper segments may impose serious constraints on recombinant α3β1, preventing it from assuming a fully active conformation. In support of this possibility, Takagi *et al.* (*30*) showed that recombinant integrin αvβ3, in which association of the truncated αv and β3 subunits was stabilized by an artificial clasp attached to their C-termini, had a significantly lower affinity for its ligand than an unclasped form of recombinant integrin αvβ3.

Our results clearly show that among the five known laminin isoforms having distinct α chains, laminin-10/11 is the most preferred ligand for both integrins α3β1 and α6β1. Given that laminin-10/11 is the laminin isoform most abundantly expressed in the basement membrane of adult epithelial tissues, and that integrin α3β1 is the most abundantly expressed integrin in epithelial cells, it is conceivable that the high affinity interaction of laminin-10/11 with integrin α3β1 plays a central role in the assembly and maintenance of epithelial tissues, as well as in the regulation of motility and survival of epithelial cells through activation of integrin-mediated signaling events (*31*, *32*). Our approach for dissecting the complex interactions of cells with basement membrane using purified laminin-binding integrins and laminin isoforms should provide an excellent model for better understanding the mechanisms governing the interaction of laminin-binding integrins with their specific ligands.

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REFERENCES

1. Roskelley, C.C., Srebrow, A., and Bissell, M.M. (1995) A hierarchy of ECM-mediated signalling regulates tissue-specific gene expression. *Curr. Opin. Cell Biol.* **7**, 736–747
2. Colognato, H. and Yurchenco, P.P. (2000) Form and function: the laminin family of heterotrimers. *Dev. Dyn.* **218**, 213–234
3. Belkin, A.M. and Stepp, M.M. (2000) Integrins as receptors for laminins. *Microsc. Res. Tech.* **51**, 280–301
4. Woods, A. (2001) Syndecans: transmembrane modulators of adhesion and matrix assembly. *J. Clin. Invest.* **107**, 935–941
5. Pall, E.E., Bolton, K.K., and Ervasti, J.J. (1996) Differential heparin inhibition of skeletal muscle alpha-dystroglycan binding to laminins. *J. Biol. Chem.* **271**, 3817–3821
6. 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
7. Hirosaki, T., Mizushima, H., Tsubota, Y., Moriyama, K., and Miyazaki, K. (2000) Structural requirement of carboxyl-terminal globular domains of laminin alpha 3 chain for promotion of rapid cell adhesion and migration by laminin-5. *J. Biol. Chem.* **275**, 22495–22502
8. Carter, W.W., Ryan, M.M., and Gahr, P.P. (1991) Epiligrin, a new cell adhesion ligand for integrin alpha 3 beta 1 in epithelial basement membranes. *Cell* **65**, 599–610
9. Kikkawa, Y., Umeda, M., and Miyazaki, K. (1994) Marked stimulation of cell adhesion and motility by ladsin, a laminin-like scatter factor. *J. Biochem. (Tokyo)* **116**, 862–869
10. Kikkawa, Y., Sanzen, N., and Sekiguchi, K. (1998) Isolation and characterization of laminin-10/11 secreted by human lung carcinoma cells. laminin-10/11 mediates cell adhesion through integrin alpha3 beta1. *J. Biol. Chem.* **273**, 15854–15859
11. 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
12. Sonnenberg, A., Linders, C.C., Modderman, P.P., Damsky, C.C., 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
13. Delwel, G.G., de Melker, A.A., Hogervorst, F., Jaspars, L.L., Fles, D.D., Kuikman, I., Lindblom, A., Paulsson, M., Timpl, R., and Sonnenberg, A. (1994) Distinct and overlapping ligand specificities of the alpha 3A beta 1 and alpha 6A beta 1 integrins: recognition of laminin isoforms. *Mol. Biol. Cell* **5**, 203–215
14. Kikkawa, Y., Sanzen, N., Fujiwara, H., Sonnenberg, A., and Sekiguchi, K. (2000) Integrin binding specificity of laminin-10/11: laminin-10/11 are recognized by alpha 3 beta 1, alpha 6 beta 1 and alpha 6 beta 4 integrins. *J. Cell Sci.* **113**, 869–876
15. Yao, C.C., Ziober, B.B., Squillace, R.R., and Kramer, R.R. (1996) Alpha7 integrin mediates cell adhesion and migration on specific laminin isoforms. *J. Biol. Chem.* **271**, 25598–255603
16. Kortesmaa, J., Yurchenco, P., and Tryggvason, K. (2000) Recombinant laminin-8 (alpha(4)beta(1)gamma(1)). Production, purification, and interactions with integrins. *J. Biol. Chem.* **275**, 14853–14859
17. Fujiwara, H., Kikkawa, Y., Sanzen, N., and Sekiguchi, K. (2001) Purification and characterization of human laminin-8. Laminin-8 stimulates cell adhesion and migration through alpha3beta1 and alpha6beta1 integrins. *J. Biol. Chem.* **276**, 17550–17558
18. Manabe, R., Ohe, N., Maeda, T., Fukuda, T., and Sekiguchi, K. (1997) Modulation of cell-adhesive activity of fibronectin by the alternatively spliced EDA segment. *J. Cell Biol.* **139**, 295–307
19. Murayama, O., Nishida, H., and Sekiguchi, K. (1996) Novel peptide ligands for integrin alpha 6 beta 1 selected from a phage display library. *J. Biochem. (Tokyo)* **120**, 445–451
20. 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
21. Fukushima, Y., Ohnishi, T., Arita, N., Hayakawa, T., and Sekiguchi, K. (1998) Integrin alpha3beta1-mediated interaction with laminin-5 stimulates adhesion, migration and invasion of malignant glioma cells. *Int. J. Cancer* **76**, 63–72
22. Sekiguchi, K., Hakomori, S., Funahashi, M., Matsumoto, I., and Seno, N. (1983) Binding of fibronectin and its proteolytic fragments to glycosaminoglycans. Exposure of cryptic glycosaminoglycan-binding domains upon limited proteolysis. *J. Biol. Chem.* **258**, 14359–14365
23. Yatohgo, T., Izumi, M., Kashiwagi, H., and Hayashi, M. (1988) Novel purification of vitronectin from human plasma by

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heparin affinity chromatography. *Cell Struct. Funct.* **13**, 281–292

24. Eble, J.J., Wucherpfennig, K.K., Gauthier, L., Dersch, P., Krukonis, E., Isberg, R.R., and Hemler, M.M. (1998) Recombinant soluble human alpha 3 beta 1 integrin: purification, processing, regulation, and specific binding to laminin-5 and invasin in a mutually exclusive manner. *Biochemistry* **37**, 10945–51095
25. Gehlsen, K.K., Sriramarao, P., Furcht, L.L., and Skubitz, A.A. (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
26. Sriramarao, P., Steffner, P., and Gehlsen, K.K. (1993) Biochemical evidence for a homophilic interaction of the alpha 3 beta 1 integrin. *J. Biol. Chem.* **268**, 22036–22041
27. von der Mark, H., Williams, I., Wendler, O., Sorokin, L., von der Mark, K., and Poschl, E. (2001) Alternative splice variants of alpha7beta1 integrin selectively recognize different laminin isoforms. *J. Biol. Chem.* **277**, 6012–6016
28. Miyazaki, K., Kikkawa, Y., Nakamura, A., Yasumitsu, H., and Umeda, M. (1993) A large cell-adhesive scatter factor secreted by human gastric carcinoma cells. *Proc. Natl Acad. Sci. USA* **90**, 11767–11771
29. Sasaki, T. and Timpl, R. (2001) Domain IVa of laminin alpha5 chain is cell-adhesive and binds beta1 and alphaVbeta3 integrins through Arg-Gly-Asp. *FEBS Lett.* **509**, 181–185
30. Takagi, J., Petre, B., Walz, T., and Springer, T. (2002) Global conformational rearrangements in integrin extracellular domains in outside-in and inside-out signaling. *Cell* **110**, 599–611
31. Gu, J., Sumida, Y., Sanzen, N., and Sekiguchi, K. (2001) Laminin-10/11 and fibronectin differentially regulate integrin-dependent Rho and Rac activation via p130(Cas)-CrkII-DOCK180 pathway. *J. Biol. Chem.* **276**, 27090–27097
32. Gu, J., Fujibayashi, A., Yamada, K.K., and Sekiguchi, K. (2002) Laminin-10/11 and fibronectin differentially prevent apoptosis induced by serum removal via phosphatidylinositol 3-kinase/Akt- and MEK1/ERK-dependent pathways. *J. Biol. Chem.* **277**, 19922–19928

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