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The Requirement of the Glutamic Acid Residue at the Third Position from the Carboxyl Termini of the Laminin γ Chains in Integrin Binding by Laminins*

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Laminins are the major cell-adhesive proteins in the basement membrane, consisting of three subunits termed α, β, and γ. The putative binding site for integrins has been mapped to the G domain of the α chain, although trimerization with β and γ chains is necessary for the G domain to exert its integrin binding activity. The mechanism underlying the requirement of β and γ chains in integrin binding by laminins remains poorly understood. Here, we show that the C-terminal region of the γ chain is involved in modulation of the integrin binding activity of laminins. We found that deletion of the C-terminal three but not two amino acids within the γ1 chain completely abrogated the integrin binding activity of laminin-511. Furthermore, substitution of Gln for Glu-1607, the amino acid residue at the third position from the C terminus of the γ1 chain, also abolished the integrin binding activity, underscoring the role of Glu-1607 in integrin binding by the laminin. We also found that the conserved Glu residue of the γ2 chain is necessary for integrin binding by laminin-332, suggesting that the same mechanism operates in the modulation of the integrin binding activity of laminins containing either γ1 or γ2 chains. However, the peptide segment modeled after the C-terminal region of γ1 chain was incapable of either binding to integrin or inhibiting integrin binding by laminin-511, making it unlikely that the Glu residue is directly recognized by integrin. These results, together, indicate a novel mechanism operating in ligand recognition by laminin binding integrins.

Laminins are a family of glycoproteins present in the basement membrane (1–3). All laminins are large heterotrimeric glycoproteins composed of α, β, and γ chains that assemble into a cross-shaped structure. To date, five α chains (α1–α5), three β chains (β1–β3), and three γ chains (γ1–γ3) have been identified, combinations of which yield at least 15 isoforms with distinct subunit compositions (4). Laminins contribute to basement membrane architecture and influence cell adhesion, spreading, and migration through binding to their cell surface receptors, particularly the integrin family of cell adhesion receptors (5–9).

Integrins play important roles in cell-matrix adhesion and signaling events regulating proliferation and differentiation of cells. Among the various integrin family members, α6β1, α6β4, α3β1, and α7β1 have been shown to be the major laminin receptors expressed in many cell types (10). Binding sites for these integrins have been mapped to the C-terminal globular (G)[3] domain of the laminin α chains (6, 11–15). The G domain consists of five tandemly repeated LG modules of ~200 amino acid residues, designated LG1 through LG5. By analogy with the identification of the Arg-Gly-Asp (RGD) cell-adhesive motif in fibronectin, many attempts have been made to identify specific sequences mimicking the integrin binding activity of laminins. However, neither recombinant fragments of the G domain nor synthetic peptides modeled after the sequences in the G domain have shown any significant cell-adhesive activity comparable with that of intact laminins (15–18). Previously, we found that deletion of the LG4-5 modules did not compromise the ability of laminin-511[4] (α5β1γ1) to bind α3β1/α6β1 integrins, but further deletion up to the LG3 module resulted in the loss of its integrin binding ability (19). These results indicate that the LG3 module is required for integrin binding by laminin-511, although LG4-5 modules are dispensable. Nevertheless, LG3 or other LG modules of the α5 chain did not exhibit any significant activity to bind the α6β1 integrin when recombinantly expressed alone or in tandem with adjacent modules (20), suggesting that G domain *per se* is not sufficient to recapitulate the integrin binding activity of laminins.

Despite the importance of the G domain in the α chain, several lines of evidence indicate that heterotrimerization with β and γ chains is required for laminins to exert their integrin binding activities. Thus, the "E8 fragment" produced by brief elastase digestion of mouse laminin-111 retained almost the full activity of the parental molecule to mediate integrin-dependent cell-substratum adhesion but lost its activity when its α1 chain

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[3] The abbreviations used are: G domain, globular domain; mAb, monoclonal antibody; BSA, bovine serum albumin; TBS, Tris-buffered saline; LG, laminin G-like; GST, glutathione *S*-transferase; LN, laminin.

[4] A new nomenclature of laminin isoforms (51) has been used throughout this paper: laminin-311, laminin-α3β1γ1 (also designated laminin-6); laminin-332, laminin-α3β3γ2 (laminin-5); laminin-511, laminin-α5β1γ1 (laminin-10).

fragment containing LG1–3 modules was separated from the disulfide-linked β1-γ1 dimer fragment (21). Cell adhesive activity was restored to the α1 chain fragment upon *in vitro* recombination and refolding with the β1-γ1 dimer (21, 22). The importance of heterotrimerization with the β and γ chains in the integrin binding activity of laminins has also been demonstrated with the recombinant E8 fragment of laminin-332, which was reconstructed by combining the α3 chain fragment containing LG1–3 modules with the disulfide-bonded β3-γ2 dimer segment (23). These results indicate that not only the G domain of the α chain but also heterotrimerization of the α chain with the β and γ chains within their coiled-coil domains is required for integrin binding by laminins, although the molecular basis of the requirement for the β and γ chains remains to be elucidated.

To address the role of β and γ chains in integrin binding by laminins, we focused on the putative interface between the G domain of the α chain and the heterotrimerized coiled-coil domains. The coiled-coil domain of mouse laminin-111 has been visualized by electron microscopy as cylinders (24–26); thus, it is conceivable that the C-terminal regions of the β and γ chains are in close proximity to and possibly interact with the G domain, thereby regulating the integrin binding activity of the G domain. Given the extension of nine amino acids in the γ1 chain against only one amino acid in the β1 chain, both of which are C-terminal to the conserved disulfide bond between the β1 and γ1 chains (Fig. 1*A*), we focused our attention on the role of the C-terminal 9-amino acid extension of the γ1 chain in integrin binding by laminin-511, a potent ligand for α6β1 and α3β1 integrins. In the present study we examined the integrin binding activities of a series of recombinant laminin-511 mutants with deletions or substitutions within the C-terminal region of the γ1 chain. Our results show that Glu-1607 at the third position from the C terminus is a prerequisite for laminin-511 recognition by integrins.

EXPERIMENTAL PROCEDURES

Antibodies, Proteins, and Peptides—mAbs against the human laminin α5 chain (5D6; Ref. 27), the human laminin γ1 chain (C12SW), and the human laminin α3 chain (2B10) were produced in our laboratory. The function-blocking mAb against integrin α6 subunit (AMCI 7-4) was kindly provided by Dr. Masahiko Katayama (Eisai Co., Ltd, Tsukuba, Japan) (28). Hybridoma cells secreting the function-blocking mAb against the integrin β1 subunit (AIIB2), developed by Dr. Caroline Damsky (University of California, San Francisco, CA), were obtained from the Developmental Studies Hybridoma Bank (University of Iowa, IA). The function-blocking mAb specific for integrin α5 subunit (8F1) was produced in our laboratory (29). Horseradish peroxidase-conjugated anti-His_6 and anti-FLAG mAbs were purchased from Qiagen and Sigma, respectively. A rabbit horseradish peroxidase-conjugated anti-hemagglutinin tag polyclonal antibody was purchased from QED Bioscience (San Diego, CA). A polyclonal antibody against the ACID/BASE coiled-coil peptides was generously provided by Dr. Junichi Takagi (Institute for Protein Research, Osaka University). Anti-GST mAb and horseradish peroxidase-conjugated streptavidin were purchased from Zymed Laboratories Inc. (San Francisco, CA). Human vitronectin was purified from human serum according to Yatohgo *et al.* (30). Human laminin-332 was purified from the conditioned medium of MKN45 human gastric carcinoma cells. The synthetic peptides modeled after the γ1 C-terminal sequence (NTPSIEKP; designated as γ1C peptide) and its mutant form (NTPSIQKP; designated as γ1C(EQ) peptide) were purchased from Biologica (Nagoya, Japan).

Construction of Expression Vectors—Soluble, clasped α6, α3, and β1 integrin expression vectors were prepared as described previously (10, 20). αV and β3 integrin expression vectors were generously provided by Dr. Junichi Takagi (31, 32). Expression vectors for human laminin α5 chain lacking LG4-5 modules (pcDNA-α5ΔLG4-5), LG3–5 modules (pcDNA-α5ΔLG3–5), β1 chain (pCEP-β1), and γ1 chain (pcDNA3.1-γ1) were constructed as described (19, 33). Expression vectors for the laminin γ1 chain lacking the C-terminal 8 amino acids (γ1Δ8AA), 3 amino acids (γ1Δ3AA), 2 amino acids (γ1Δ2AA), or 1 amino acid (γ1Δ1AA) and that encoding the mutant γ1 chain with substitution of Gln for Glu-1607 (γ1EQ) were prepared as follows. cDNAs encoding γ1Δ8AA, γ1Δ3AA, γ1Δ2AA, γ1Δ1AA, and γ1EQ were amplified by PCR using pcDNA3.1-γ1 as a template. PCR products were digested with BbvCI and XbaI and inserted into the corresponding restriction sites of pcDNA3.1-γ1. The PCR primers used were 5′-ACAGGCTGCTCAAGAAGCCG-3′ (forward primer of γ1Δ8AA, γ1Δ3AA, γ1Δ2AA, γ1Δ1AA, and γ1EQ), 5′-AGCTTCTAGACTAGAAGCAGCCAGATGG-3′ (reverse primer of γ1Δ8AA), 5′-AGCTTCTAGACTAAATGGACGGGGTGTTG-3′ (reverse primer of γ1Δ3AA), 5′-AGCTTCTAGACTATTCAATGGACGGGGTG-3′ (reverse primer of γ1Δ2AA), 5′-AGCTTCTAGACTACTTTTCAATGGACGG-3′ (reverse primer of γ1Δ1AA), and 5′-AGCTTCTAGACTAGGGCTTCTGAATGGAC-3′ (reverse primer of γ1EQ).

Expression vectors for the E8 fragment of laminin-511, a heterotrimer of the truncated α5, β1, and γ1 chains (designated α5E8, β1E8, and γ1E8, respectively), and its mutant forms were prepared as follows. cDNAs encoding α5E8 (Ala^{2534}–Ala^{3322}), β1E8 (Leu^{1561}–Leu^{1786}), and γ1E8 (Asn^{1364}–Pro^{1609}) were amplified by PCR using pcDNA-α5, pCEP-β1, and pcDNA3.1-γ1 as templates. His_6 tag (for α5E8), hemagglutinin tag (for β1E8), and FLAG tag (for γ1E8) sequences were added by extension PCR with a HindIII site at the 5′ end and an EcoRI site at the 3′ end. PCR products were digested with HindIII/EcoRI and inserted into the corresponding restriction sites of the expression vector pSecTag2B (Invitrogen). cDNAs encoding γ1E8 mutants were amplified by PCR, and PCR products were inserted into the γ1E8 expression vector using the following pairs of primers; 5′-AATGACATTCTCAACAACCTGAAAG-3′ (forward primer of E8-γ1Δ3AA and E8-γ1EQ), 5′-CTAAATGGACGGGGTGTTGAAG-3′ (reverse primer of E8-γ1Δ3AA), and 5′-CTAGGGCTTCTGAATGGACGGGGTG-3′ (reverse primer of E8-γ1EQ).

Expression vectors for human laminin α3 chain lacking LG4-5 modules (nucleotides 1–4128), β3 chain, γ2 chain, and γ2 chain mutants were prepared as follows. A full-length cDNA encoding the α3 subunit lacking LG4-5 modules (GenBank™ accession number NM_000227) was amplified by reverse tran-

scription-PCR as a series of ~1-kilobase fragments. After sequence verification, error-free cDNA fragments were ligated in tandem, and the resulting cDNA of the α3 chain lacking LG4-5 modules was inserted into the expression vector pcDNA3.1 (Invitrogen). A full-length cDNA encoding the β3 subunit (GenBank™ accession number NM_001017402) was purchased from Open Biosystems (Huntsville, AL) and inserted into the expression vector pcDNA3.1. A partial cDNA encoding the γ2 subunit (GenBank™ accession number NM_005562) was purchased from Open Biosystems, and the remaining portion of the cDNA was amplified by reverse transcription-PCR. After sequence verification, error-free cDNA fragments were ligated in tandem to construct a cDNA encompassing the whole open reading frame, and the resulting cDNA was inserted into the expression vector pcDNA3.1. cDNAs encoding γ2 mutants were amplified by PCR, and PCR products were inserted into the γ2 expression vector. The list of primer sequences is available upon request.

Expression vectors for GST fusion proteins containing FNTPSIEKP (GST-γ1(8AA)), QVTRGDVFT (sequence derived from vitronectin; GST-RGD), or QVTRGEVFT (GST-RGE) were constructed by inserting cDNA fragments encoding the individual peptide sequences into the EcoRI/NotI restriction sites of pGEX4T-1 (Amersham Biosciences). The insert cDNAs were amplified by overlap extension PCR. The PCR primers used were 5′-AATTGAATTCTTCAACACCCCGTCCATTG-3′ and 5′-ATATATATGCGGCCGCCTAGGGCTTTTCAATGGACGG-3′ (for GST-γ1(8AA)), 5′-AATTGAATTCCAAGTGACTCGCGGGGATG-3′ and 5′-ATATATATGCGGCCGCCTAAGTGAACACATCCCCGCG-3′ (for GST-RGD), and 5′-AATTGAATTCCAAGTGACTCGCGGGGAAG-3′ and 5′-ATATATATGCGGCCGCCTAAGTGAACACTTCCCCGCG-3′ (for GST-RGE).

Expression and Purification of Recombinant Proteins—Recombinant laminin-511 mutants were produced using the Free-Style™ 293 Expression system (Invitrogen) and purified from conditioned media as described previously (19). Recombinant laminin-311 and laminin-332, both lacking LG4-5 modules, and their mutants were produced using the same expression system. The resultant conditioned media were applied to immunoaffinity columns conjugated with an anti-human laminin α3 mAb 2B10. The columns were washed with TBS (50 mM Tris-HCl, pH 7.4, 150 mM NaCl), and bound laminins were eluted with 0.1 M triethylamine, neutralized, and dialyzed against TBS. Recombinant α6β1, α3β1, and αVβ3 integrins were produced using the same expression system and purified from the conditioned media using anti-FLAG columns (Sigma) described previously (20).

The recombinant E8 fragment of laminin-511 and its mutants were produced using the Free-Style™ 293 Expression system. The conditioned media were applied to nickel nitrilotriacetic acid affinity columns (Qiagen), and bound proteins were eluted with 200 mM imidazole. The eluted proteins were further purified on anti-FLAG columns (Sigma). After dialysis against TBS, the purity of these recombinant proteins was verified by SDS-PAGE followed by Coomassie Brilliant Blue staining or immunoblotting with anti-His tag (for α5E8), anti-hemagglutinin tag (for β1E8), or anti-FLAG tag (for γ1E8) mAbs.

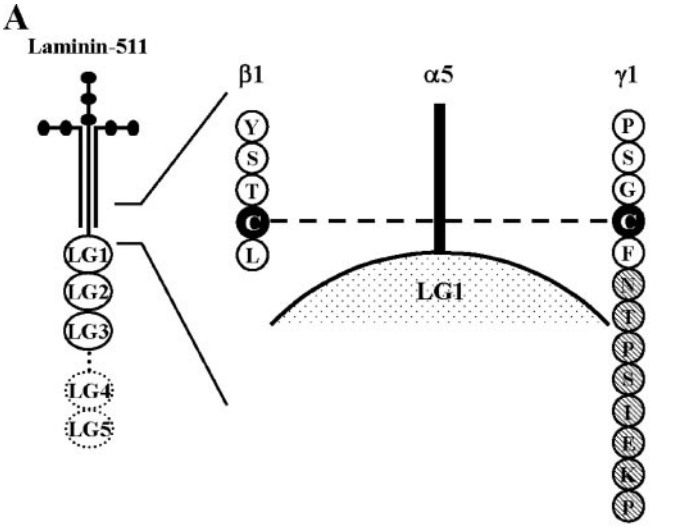
GST fusion proteins (GST-γ1(8AA), GST-RGD, and GST-RGE) were induced in *Escherichia coli* with 0.1 mM isopropyl-β-D-thiogalactopyranoside and purified on glutathione-Sepharose 4B columns (Amersham Biosciences) after lysis of the cells by sonication. Bound proteins were eluted with 50 mM Tris-HCl, pH 8.0, containing 10 mM glutathione. After dialysis against TBS, the purity of these recombinant proteins were verified by SDS-PAGE followed by Coomassie Brilliant Blue staining or immunoblotting.

Binding Assays for α6β1, α3β1, and αVβ3 Integrins—Solid-phase integrin binding assays of recombinant laminins, their mutants, or GST fusion proteins were performed using purified recombinant α6β1, α3β1, and αVβ3 integrins. 96-well microtiter plates were coated with recombinant proteins for testing at the indicated concentrations. The amounts of the recombinant proteins adsorbed on the plates were quantified with mAb 5D6 (for laminin-511, E8, and their mutants), mAb 2B10 (for laminin-311, laminin-332, and their mutants), or anti-GST antibody (for GST fusion proteins) to confirm the equality of the amounts of adsorbed proteins. After blocking with 3% BSA, plates were incubated with the 20 nM recombinant α6β1, α3β1, or αVβ3 integrin in the presence of 5 mM Mn^{2+} at 37 °C for 1 h. α6β1, α3β1, and αVβ3 integrins were used without proteolytic unclasping, since the binding of Mn^{2+} to the integrin can overcome the structural constraint imposed by an artificially introduced interchain disulfide bond (32). After washing with TBS containing 5 mM Mn^{2+} and 0.05% Tween 20, bound proteins were quantified after sequential incubations with the biotinylated anti-ACID/BASE antibody and horseradish peroxidase-conjugated streptavidin (20). For integrin binding inhibition assays using function-blocking mAbs against integrin subunits or synthetic peptides, adhesive proteins coated on the microtiter plates were incubated with α6β1 integrin in the presence of mAbs (100 μg/ml) or γ1C or γ1C(EQ) peptide at 37 °C for 1 h before incubation with biotinylated anti-ACID/BASE antibody. Bound integrins were detected as described above. For determination of the apparent dissociation constants by saturation binding assays, plates were coated with laminins (5 nM). After blocking with BSA, serially diluted α6β1 integrin was added to the plates, and bound integrin was quantified. The apparent dissociation constants were determined as described previously (34).

Cell Adhesion Assay—Cell adhesion assays were performed as described previously (19) using K562 human leukemia cells transfected with cDNA encoding an α6 integrin subunit (kindly provided by Dr. Arnoud Sonnenberg, The Netherland Cancer Institute, Amsterdam). The cells were maintained in RPMI1640 supplemented with 10% fetal calf serum and used without pretreatment with Mn^{2+} to fully activate integrins. After fixation with formaldehyde and subsequent staining with Diff-Quik (International Reagents Corp., Kobe, Japan), attached cells were counted in three independent wells using Scion Image software (Scion Corp., Frederick, MD).

RESULTS

Deletion of the C-terminal Region of the γ1 Chain Abrogates Integrin Binding Activity—To address the potential involvement of the C-terminal region of the γ1 chain in integrin bind-



C-terminal sequence of γ1 chain	
LN511-γ1	P S G C F N T P S I E K P
LN511-γ1Δ8AA	P S G C F
LN511-γ1Δ3AA	P S G C F N T P S I
LN511-γ1Δ2AA	P S G C F N T P S I E
LN511-γ1Δ1AA	P S G C F N T P S I E K
LN511-γ1EQ	P S G C F N T P S I Q K P

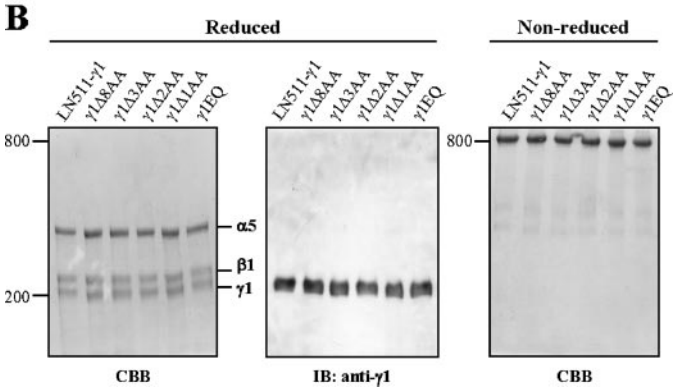


FIGURE 1. **Recombinant laminin-511 and its mutant proteins with deletions or amino acid substitution in the γ1 chain.** *A*, schematic representation of recombinant laminin-511 and the C-terminal amino acid sequences of its γ1 chain with deletions or amino acid substitution. Cysteines are *circled* in *black*, and the disulfide bond is depicted by a *broken line* in the *upper scheme*. The C-terminal eight amino acid residues of the γ1 chain are *shaded*. C-terminal amino acid sequences of intact γ1 chain and its mutants are shown in the *box*. The cysteine residues are *underlined*. *LN511-γ1*, recombinant laminin-511 lacking LG4-5 modules but containing intact γ1 chain; *LN511-γ1Δ8AA*, LN511-γ1 lacking the γ1 C-terminal 8 amino acids; *LN511-γ1Δ3AA*, LN511-γ1 lacking the γ1 C-terminal 3 amino acids; *LN511-γ1Δ2AA*, LN511-γ1 lacking the γ1 C-terminal 2 amino acids; *LN511-γ1Δ1AA*, LN511-γ1 lacking only the most C-terminal amino acid of the γ1 chain; *LN511-γ1EQ*, LN511-γ1 in which the glutamic acid residue (*E*) was replaced by glutamine (*Q*, *closed box*). *B*, purified LN511-γ1 and its mutants were analyzed by SDS-PAGE on 4% gels under reducing (*left* and *center panels*) and non-reducing conditions (*right panel*) followed by Coomassie Brilliant Blue (*CBB*) staining (*left* and *right panels*) or immunoblotting (*IB*) with a mAb against the laminin γ1 chain (*center panel*). Under reducing conditions, each recombinant protein gave three bands, one corresponding to the α5 chain and two lower bands corresponding to the β1 and γ1 chains; the latter was detected by an mAb against the γ1 chain. Under nonreducing conditions, they gave a single band migrating at ~800 kDa, confirming that they were purified as trimers of the α5, β1, and γ1 chains. The positions of molecular size markers are shown in the *left margin*.

ing by laminin, we produced a mutant laminin-511 in which the C-terminal eight amino acids were deleted from the γ1 chain (designated LN511-γ1Δ8AA; see Fig. 1*A*). The mutant laminin-511 also lacked the LG4-5 modules that are required for α-dystroglycan binding but not for integrin binding (19). A recombinant laminin-511 containing an intact γ1 chain and the α5 chain deleted of LG4-5 (designated LN511-γ1) was used as a control laminin-511 throughout this study. The authenticity of the recombinant proteins as well as other mutant laminin-511 proteins with deletions or amino acid substitutions (see below) was verified by SDS-PAGE under reducing and non-reducing conditions (Fig. 1*B*). Under reducing conditions, the recombinant proteins gave three bands upon Coomassie Brilliant Blue staining, one corresponding to the α5 chain lacking LG4-5 modules and two lower bands corresponding to the β1 chain and intact or mutant γ1 chains, the latter specifically detected by immunoblotting with a mAb against the γ1 chain (Fig. 1*B*). Under nonreducing conditions, LN511-γ1 and its deletion mutants gave a slowly migrating band near the top of the gel, confirming that they were purified as trimers of the α5, β1, and γ1 chains. Solid-phase binding assays with the α6β1 integrin showed that the control laminin-511 (LN511-γ1) was fully active in binding to the α6β1 integrin, whereas the mutant laminin-511 lacking the C-terminal eight amino acids of the γ1 chain (LN511-γ1Δ8AA) exhibited only marginal activity comparable with that of another mutant laminin-511 lacking the LG3–5 modules of the α5 chain (designated ΔLG3–5; Fig. 2*A*), which have been shown to be devoid of integrin binding activity (19). Because LN511-γ1 and LN511-γ1Δ8AA have common α5 and β1 subunits, the significant reduction in integrin binding activity would be ascribable to the deletion of C-terminal eight amino acids in the γ1 chain, indicating its involvement in integrin binding by laminin-511.

To narrow down the amino acid residues involved in integrin binding, we produced a series of mutant laminin-511 with shorter deletions within the C-terminal region of the γ1 chain and examined their integrin binding activities (Fig. 1*A*). Mutant laminin-511 lacking the C-terminal 1 or 2 amino acids (designated LN511-γ1Δ1AA and LN511-γ1Δ2AA, respectively) retained the integrin binding activity but were less active than control laminin-511 (Fig. 2*A*). Reduced integrin binding affinity of these deletion mutants was confirmed by determination of apparent dissociation constants by saturation integrin binding assays; the estimated dissociation constants of LN511-γ1Δ1AA and LN511-γ1Δ2AA for α6β1 integrin were 2.4 ± 0.41 and 2.2 ± 1.4 nM, respectively, ~5-fold lower than that of control laminin-511 (K_d, 0.43 ± 0.1 nM) (Fig. 3). Further deletion up to the third amino acid residue, *i.e.* Glu-1607, resulted in a significant abrogation of the integrin binding activity of laminin-511. A dramatic loss of the integrin binding activity upon deletion of the C-terminal three but not two amino acids from the γ1 chain indicated that Glu-1607 at the third position from the C terminus played a critical role in the potent integrin binding by laminin-511.

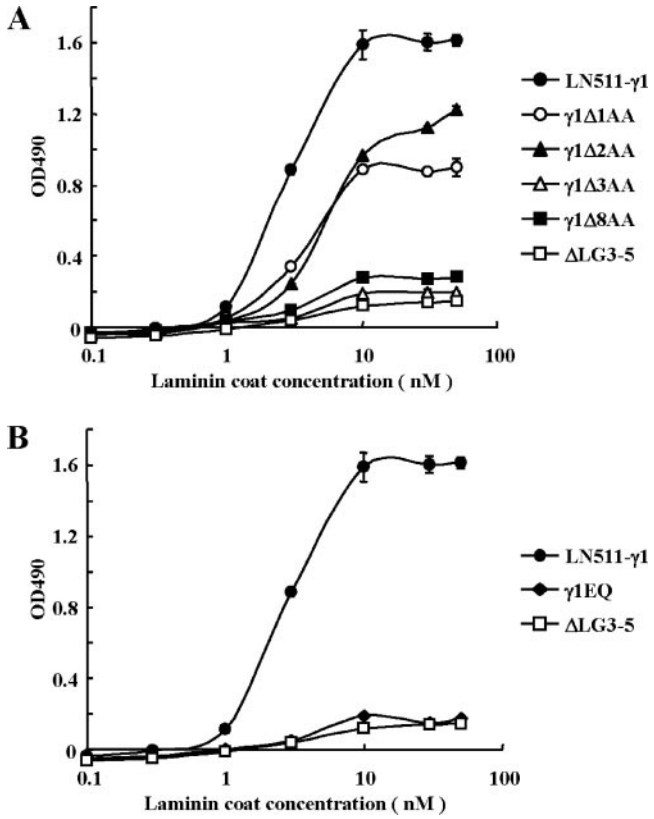
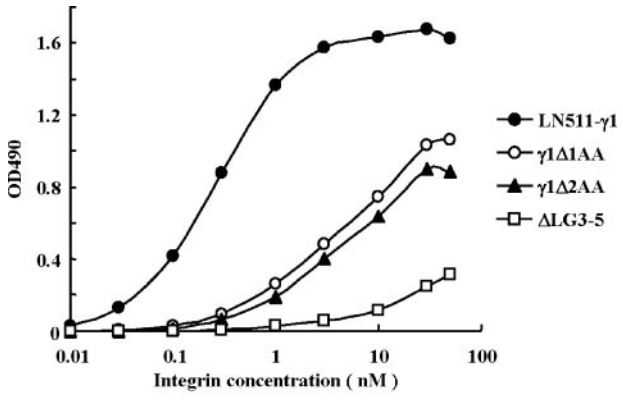


FIGURE 2. **Binding of α6β1 integrin to laminin-511 mutants.** 96-well microtiter plates were coated with increasing concentrations of the following laminin-511 mutants, blocked with BSA, and then incubated with 20 nM integrin α6β1. *A*, LN511-γ1Δ1AA, LN511-γ1Δ2AA, LN511-γ1Δ3AA, LN511-γ1Δ8AA, and ΔLG3–5. *B*, LN511-γ1EQ and ΔLG3–5. LN511-γ1 was used as a positive control. The amounts of bound α6β1 integrin were quantified as described under "Experimental Procedures." Each point represents the mean of triplicate assays and the S.D., respectively.



	LN511-γ1	γ1Δ1AA	γ1Δ2AA
K_d (nM)	0.43±0.1	2.4±0.41	2.2±1.4

FIGURE 3. **Titration curves of recombinant α6β1 integrin bound to laminin-511 mutants.** The amounts of the integrins bound in the presence of 5 mM EDTA were taken as nonspecific binding and subtracted as background. The results are the means of duplicate determinations. Dissociation constants of recombinant α6β1 integrin toward laminin-511 mutants are shown in the *box*.

To address the role of the Glu-1607 in integrin binding by laminin-511, we produced a mutant laminin-511 in which Glu-1607 of the γ1 chain was replaced with glutamine (designated LN511-γ1EQ; see Fig. 1*A*). As expected, the laminin-511 with E1607Q substitution was almost devoid of integrin binding activity (Fig. 2*B*), confirming the importance of Glu-1607 in integrin binding by laminin-511.

Integrin Binding Activity of the E8 Fragment of Laminin-511 and Its Mutant Forms—The failure in the disulfide bond formation between the β1 and γ1 chains near the C terminus has been shown to destabilize the conformation of the coiled-coil domain of laminin (26). Because the deletions and amino acid substitution introduced to the γ1 chain were very close to the conserved cysteine residue near the C terminus, these mutations could compromise the disulfide bond formation between β1 and γ1 chains, resulting in the destabilization of the coiled-coil domain of the mutant proteins and, hence, the abrogation of their integrin binding activities.

To examine this possibility, we produced E8 fragments of control and mutant laminin-511 consisting of three truncated subunits modeled after the E8 fragment of laminin-111 (Fig. 4), since the truncated fragments of β1 and γ1 chains contain only one Cys residue, allowing us to assess the effect of the mutations introduced to the C-terminal region of the γ1 chain on the disulfide bond formation between β1 and γ1 chains. The authenticity of the resulting recombinant E8 fragments of laminin-511 was verified by SDS-PAGE and immunoblotting with a mAb against the FLAG tag, which was added to the N terminus of the truncated γ1 chain. Under reducing conditions, each recombinant protein gave three bands upon Coomassie Brilliant Blue staining, one corresponding to the truncated α5 chain and two lower bands corresponding to the truncated β1 and γ1 chains (Fig. 4*B*). Control and mutant E8 fragments gave two bands under nonreducing conditions, one corresponding to the truncated α5 chain and another corresponding to the heterodimer of the truncated β1 and γ1 chains. Because the truncated β1 and γ1 chains contain only one Cys residue near the C terminus, these results confirmed that the disulfide bond formation between these two chains was not compromised by the mutations introduced near the C terminus of the γ1 chain.

The E8 fragment of control laminin-511, designated E8-γ1, exhibited potent integrin binding activity (Fig. 4*C*). Saturation integrin binding assays demonstrated that the K_d of E8-γ1 for the α6β1 integrin was 1.5 ± 0.02 nM, roughly comparable with that of the parental protein (*i.e.* LN511-γ1). However, two E8 mutants with either deletion of the C-terminal three amino acids (E8-γ1Δ3AA) or substitution of Gln for Glu-1607 (E8-γ1EQ) were almost devoid of integrin binding activity, further confirming the importance of Glu-1607 in integrin binding by laminin-511. These results also demonstrated that the loss of integrin binding activity by deletion of the C-terminal three amino acids or E1607Q substitution was not due to a defect in disulfide bond formation between the β1 and γ1 chains at their C termini, but rather, it was due to the critical role of the region containing Glu-1607 in sustaining the active conformation of the G domain or to its involvement in integrin binding by comprising a part of the integrin recognition surface. The integrin binding to E8-γ1 was strongly inhibited by function-blocking mAbs against integrin α6 and β1 subunits. It was completely blocked by a combination of anti-α6 and anti-β1 mAbs (Fig. 4*D*), confirming the integrin binding specificity of E8-γ1.

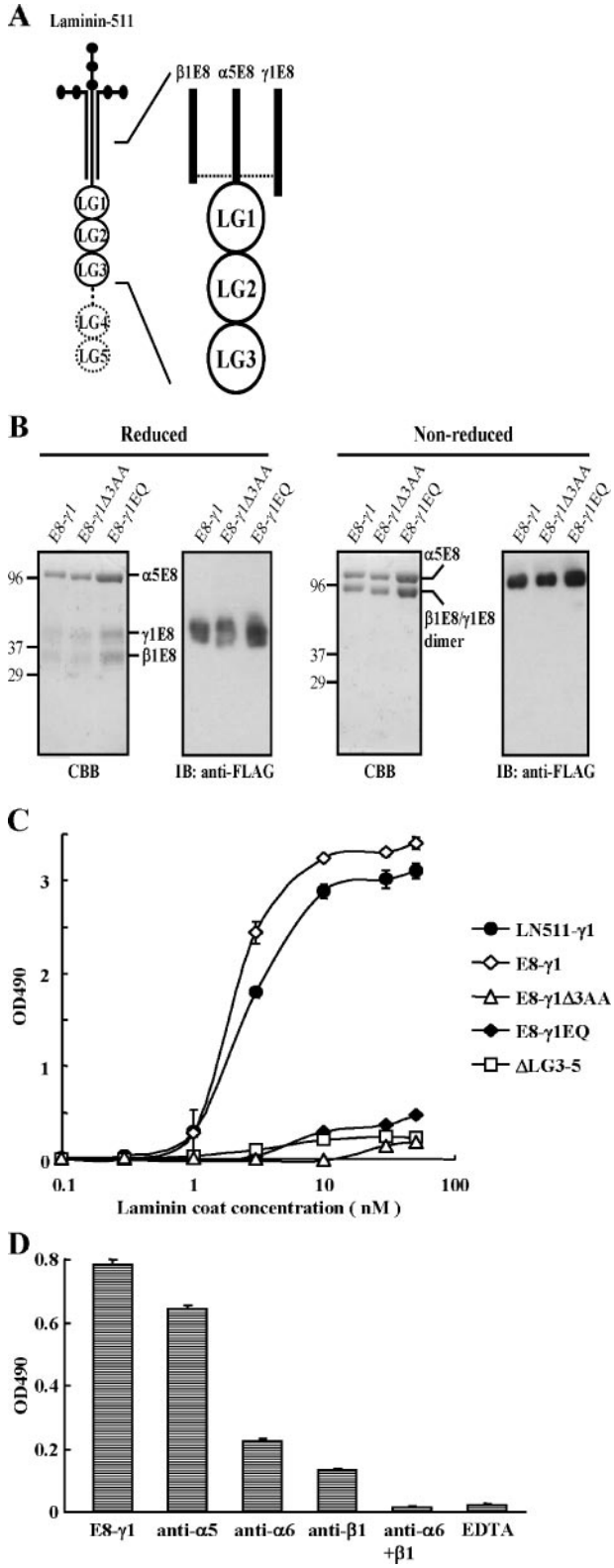


FIGURE 4. **Production of recombinant E8 fragments of control and mutant laminin-511.** *A*, schematic representations of recombinant laminin-511 and its E8 fragment. *B*, control E8 fragment (E8-γ1) and its mutant proteins (E8-γ1Δ3AA and E8-γ1EQ) were analyzed by SDS-PAGE on 12% gels under reducing (*left panels*) and non-reducing (*right panels*) conditions followed by Coomassie Brilliant Blue (*CBB*) staining or immunoblotting (*IB*) with a mAb against the FLAG tag. The positions of molecular size markers are shown in the *left margin*. *C*, binding of α6β1 integrin to E8 fragments of laminin-511 and its mutant proteins. 96-well microtiter plates were coated with increasing concentrations of LN511-γ1 and E8 fragments of laminin-511 (E8-γ1) and its mutant proteins (E8-γ1Δ3AA and E8-γ1EQ), blocked with BSA, and then incubated with 20 nM α6β1 integrin. *D*, inhibition of integrin binding to E8-γ1 using anti-integrin mAbs. 96-well microtiter plates were coated with E8-γ1 (5 nM). α6β1 integrin (20 nM) was preincubated with function-blocking mAbs against integrin subunits and then added to the precoated wells. The amounts of bound α6β1 integrin were quantified as described under "Experimental Procedures." Each *column* and *bar* represents the mean of triplicate assays and the S.D., respectively.

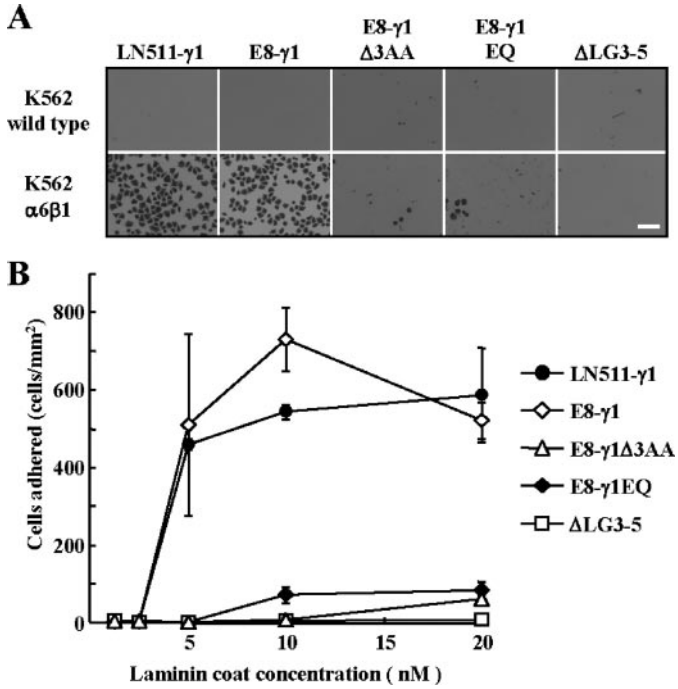


FIGURE 5. **Cell-adhesive activity of E8 fragments of laminin-511 and its mutant proteins.** K562 cells were incubated at 37 °C for 30 min on 96-well microtiter plates coated with LN511-γ1 and E8 fragments of laminin-511 (E8-γ1) and its mutant proteins (E8-γ1Δ3AA and E8-γ1EQ). Adherent cells were fixed and stained as described under "Experimental Procedures." *A*, representative images of wild type K562 and K562 cells expressing α6β1 integrin adhering to the substrates. *Bar* = 60 μm. *B*, cells adhering to the substrates were counted as described under "Experimental Procedures." Each point represents the mean of triplicate assays and the S.D., respectively.

Reduced Cell-adhesive Activity of Laminin-511 with E1607Q Substitution—The importance of Glu-1607 in integrin binding was further examined by cell adhesion assays using K562 cells stably transfected with α6 integrin (35, 36). To avoid the auxiliary interactions of laminin-511 with αVβ3 and other integrins recognizing the Arg-Gly-Asp sequences within domain IVa (also designated domain L4b) of the α5 chain (37), we examined the cell-adhesive activities of the E8 fragments of control and mutant laminin-511 proteins. Mutant E8 fragments either lacking the C-terminal three amino acids or having E1607Q substitution were almost devoid of cell-adhesive activity, whereas the control E8 fragment was fully active in mediating cell adhesion of K562 cells expressing the α6β1 integrin (Fig. 5). Control K562 cells untransfected with the α6 integrin did not adhere to any of the recombinant E8 fragments, confirming the specificity of the α6β1 integrin-dependent cell adhesion. These results further support our conclusion that the C-terminal region of the γ1 chain, in particular Glu-1607 at the third position from the C terminus, played an important role in integrin binding by laminin-511.

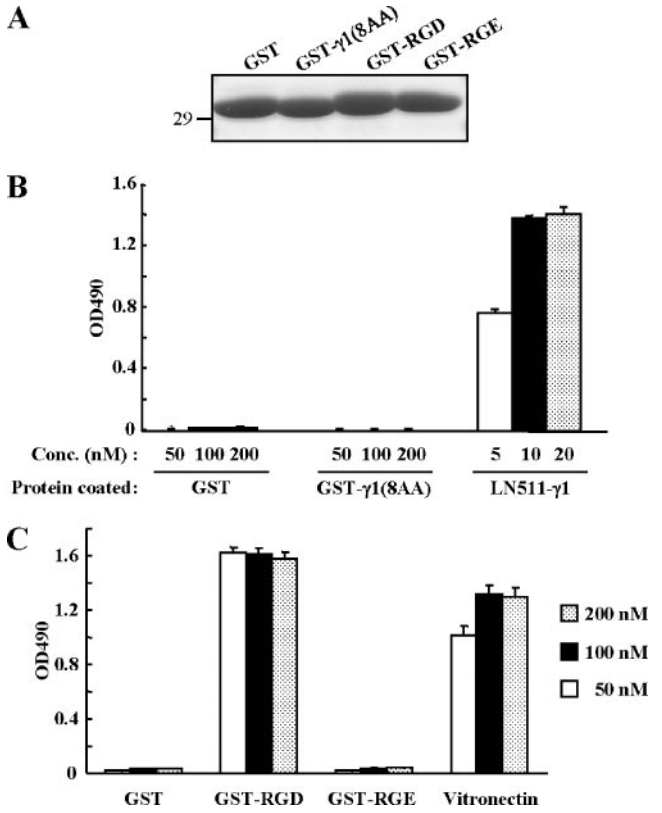


FIGURE 6. **Integrin binding activities of the C-terminal peptide segment of the γ1 chain.** *A*, SDS-PAGE profiles of GST fusion proteins containing the γ1 C-terminal 8 amino acids (GST-γ1(8AA)), QVTRGDVFT (GST-RGD), or QVTRGEVFT (GST-RGE) peptides. The RGD-containing peptide was modeled after vitronectin. Proteins were stained with Coomassie Brilliant Blue. The position of the 29-kDa molecular size marker is shown in the left margin. *B*, α6β1 integrin binding activities of GST, GST-γ1(8AA), and LN511-γ1. 96-well microtiter plates were coated with recombinant proteins at the indicated concentrations and then incubated with 20 nM α6β1 integrin. The amounts of bound integrin were quantified as described in under "Experimental Procedures." *C*, αVβ3 integrin binding activities of GST, GST-RGD, GST-RGE, and purified vitronectin. Each *column* and *bar* represents the mean of triplicate assays and the S.D., respectively. The RGD-containing peptide fused to GST was equally active in binding to α6β1 integrin as vitronectin.

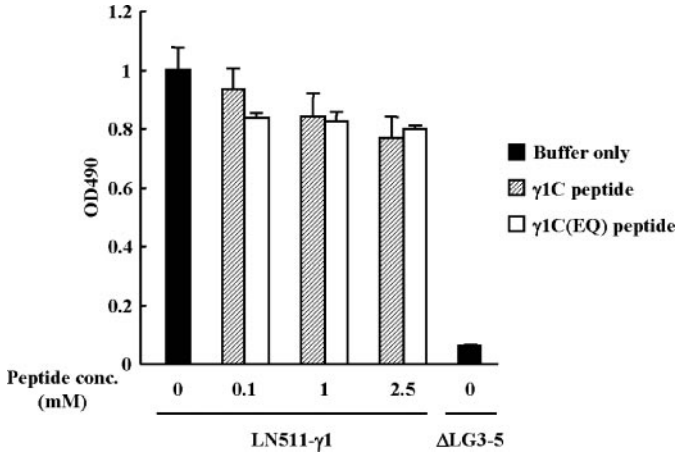


FIGURE 7. **Inhibition of α6β1 integrin binding to laminin-511 by synthetic γ1 peptides.** α6β1 integrin (20 nM) was incubated on microtiter plates coated with 10 nM LN511-γ1 in the presence of increasing concentrations of γ1C (NTPSIEKP) and γ1C(EQ) (NTPSIQKP) peptides for 1 h. Bound integrins were quantified as described under "Experimental Procedures." Each *column* and *bar* represents the mean of triplicate assays and S.D., respectively.

The C-terminal Region of the γ1 Chain Does Not Function as an Integrin-binding Site—The binding profiles of the γ1Δ3AA and γ1EQ mutants of laminin-511 toward α6β1 integrin raised the possibility that the C-terminal region of the γ1 chain could comprise part of the binding site for α6β1 integrin. To explore this possibility, we expressed the C-terminal eight amino acid residues of the γ1 chain in bacteria as a GST fusion protein (GST-γ1(8AA): Fig. 6*A*) and examined whether it could bind to the α6β1 integrin. Neither GST alone nor GST-γ1(8AA) showed any significant integrin binding activity, even at the coating concentrations as high as 200 nM, although control LN511-γ1 bound a significant amount of the integrin even at 5 nM (Fig. 6*B*), making it unlikely that the C-terminal peptide segment of the γ1 chain is directly recognized by the α6β1 integrin. The failure of GST-γ1(8AA) to bind to the α6β1 integrin could arise from the bulky GST domain, blockading direct interaction of the γ1 peptide with the integrin, although the RGD- but not RGE-containing peptide segment fused to GST exhibited significant binding activity toward the αVβ3 integrin (Fig. 6*C*), making this possibility unlikely.

To further explore the possibility that the γ1 C-terminal peptide directly binds to the integrin, we examined whether a synthetic peptide modeled after the γ1 C-terminal sequence was capable of inhibiting integrin binding. Thus, binding of the soluble α6β1 integrin to immobilized LN511-γ1 was determined in the presence of increasing concentrations of the synthetic 8 amino acid peptide derived from the C-terminal region of the γ1 chain (designated γ1C peptide) or its mutant form, in which Glu-1607 was replaced by glutamine (γ1C(EQ) peptide; Fig. 7) The γ1C peptide did not exert any significant inhibitory effect on integrin binding to LN511-γ1 even at 2.5 mM (125,000-fold molar excess to the integrin added) as compared with the control peptide with the E1607Q substitution. These results argue against the direct recognition of the C-terminal region of the γ1 chain by the integrin and support the possibility that the γ1 chain modulates the integrin binding activity of laminin-511 through interaction with the G domain of the α5 chain, possibly stabilizing the conformation of the G domain active in integrin binding.

Glu-1607 Is Required for Laminin Binding by α3β1 Integrin—Laminin-511 has been shown to serve as a potent ligand for both α3β1 and α6β1 integrins (10, 34). Therefore, we next examined whether Glu-1607 was required for laminin-511 binding to the α3β1 and α6β1 integrins. Solid-phase binding assays using the α3β1 integrin demonstrated that γ1Δ3AA and γ1EQ mutant proteins were barely active in binding to the α3β1 integrin, exhibiting a >80% decrease when compared with control laminin-511 (Fig. 8*A*). Thus, Glu-1607 is required for high affinity binding of laminin-511 to both α3β1 and α6β1 integrins, underscoring its importance in laminin-511 recognition by cognitive integrins.

To extend the role of Glu-1607 in integrin binding to other laminin isoforms containing the γ1 chain, we examined integrin binding to laminin-311 (α3β1γ1), which is also a ligand for the α3β1 integrin (38). We expressed and purified laminin-311 lacking LG4-5 modules of the α3 chain (designated LN311-γ1) and its γ1 mutants lacking the C-terminal three amino acids (LN311-γ1Δ3AA) or having an E1607Q substitution (LN311-

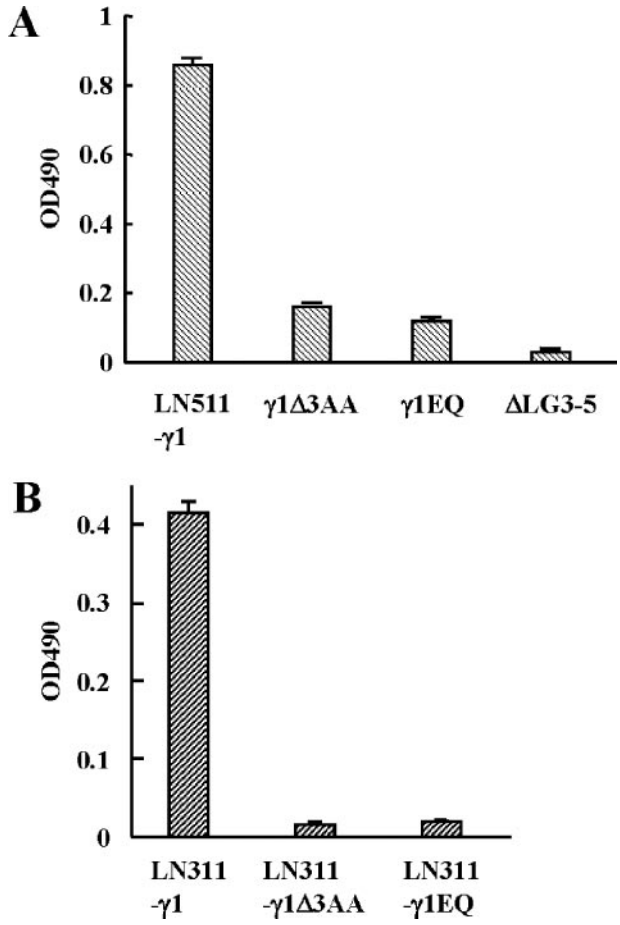


FIGURE 8. **Binding of the α3β1 integrin to laminin-511, laminin-311, and their mutant proteins.** *A*, 96-well microtiter plates were coated with 10 nM LN511-γ1 and its mutant proteins, blocked with BSA, and then incubated with 20 nM α3β1 integrin. The amounts of bound α3β1 integrin were quantified as described under "Experimental Procedures." *B*, binding of the α3β1 integrin to laminin-311 mutants. *LN311-γ1*, recombinant laminin-311 lacking the LG4-5 modules but containing intact γ1 chain; *LN311-γ1Δ3AA*, LN311-γ1 lacking the γ1 C-terminal 3 amino acids; *LN311-γ1EQ*, LN311-γ1 in which glutamic acid residue was replaced by glutamine.

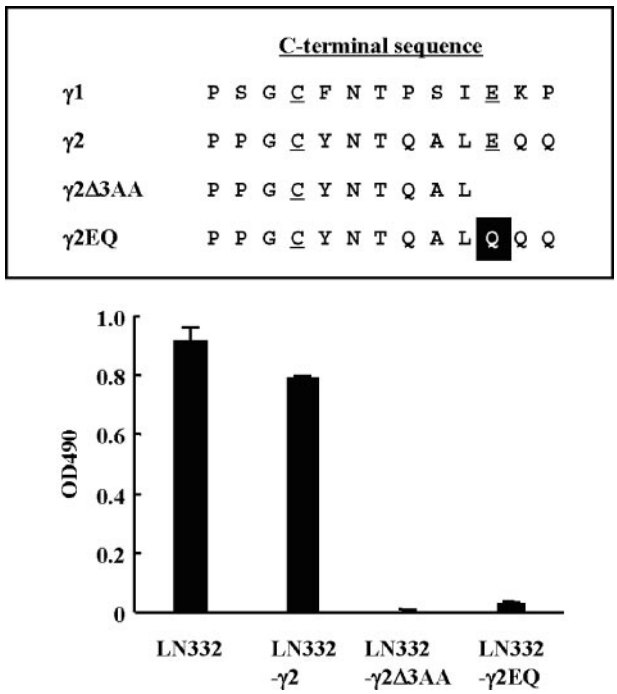


FIGURE 9. **Binding of the α3β1 integrin to laminin-332 mutant proteins.** C-terminal amino acid sequences of intact γ1 and γ2 chains and the mutant forms of γ2 chain are shown in the *box*. Cysteine and glutamic acid residues conserved between γ1 and γ2 chains are *underlined*. The *closed box* represents the substituted glutamine residue. *LN332*, human laminin-332 purified from the conditioned medium of MKN45 human carcinoma cells; *LN332-γ2*, recombinant laminin-332 lacking the LG4-5 modules but containing intact γ2 chain; *LN332-γ2Δ3AA*, LN332-γ2 lacking the C-terminal 3 amino acids of γ2 chain; *LN332-γ2EQ*, LN332-γ2 in which glutamic acid residue (*E*) was replaced by glutamine (*Q*). Each *column* and *bar* represents the mean of triplicate assays and the S.D., respectively.

γ1EQ) and examined their α3β1 integrin binding activities. As expected, LN311-γ1 exhibited potent α3β1 integrin binding activity, whereas LN311-γ1Δ3AA and LN311-γ1EQ were almost devoid it (Fig. 8*B*). These results are consistent with the conclusion that Glu-1607 is indispensable for γ1-containing laminins to exert their integrin binding activities.

Glutamic Acid Residue at the Third Position from the C Terminus of the γ2 Chain Is Required for Integrin Binding by Laminin-332—The glutamic acid residue at the third position from the C terminus is conserved between laminin γ1 and γ2 chains (Fig. 9). The presence of the conserved glutamic acid residue (Glu-1191) in the γ2 chain raised the possibility that this Glu residue is also required for integrin binding of the γ2 chain-containing laminin, *i.e.* laminin-332 (α3β3γ2). To address this possibility, we expressed and purified laminin-332 lacking LG4-5 modules (designated LN332-γ2) and its mutant proteins either lacking the C-terminal three amino acids of the γ2 chain (LN332-γ2Δ3AA) or having E1191Q substitution within the γ2 chain (LN332-γ2EQ) and examined their α3β1 integrin binding activities. Recombinant LN332-γ2 was found to possess potent α3β1 integrin binding activity, comparable with that of laminin-332 purified from a conditioned medium of MKN45 human carcinoma cells (Fig. 9). However, neither γ2 chain mutants, LN332-γ2Δ3AA and LN332-γ2EQ, showed any significant binding to the α3β1 integrin. These results indicate that Glu-1191 within the γ2 chain is involved in integrin binding by laminin-332 and provide evidence that the C-terminal region of the γ chains, particularly the Glu residue at the third position from the C terminus, has a critical role in integrin binding by laminins containing either γ1 or γ2 chains.

DISCUSSION

Despite accumulating evidence of the requirement of heterotrimerization of the α chain with a disulfide-bonded dimer of the β and γ chains in integrin binding by laminins (21–23), no studies have before directly addressed the roles of β and γ chains in integrin binding through production of mutant laminin isoforms with deletions and/or amino acid substitutions in the β and/or γ chains. In the present study we provide evidence that three amino acids within the γ1 C-terminal region, particularly Glu-1607 at the third position from the C terminus, are required for the integrin binding activity of laminin-511. This conclusion is based on the following observations. First, deletion of the C-terminal three but not two amino acids within the γ1 chain completely abrogated the integrin binding activity of laminin-511, as was the case with substitution of Gln for Glu-1607 at the third position from the γ1 C terminus. Second, the

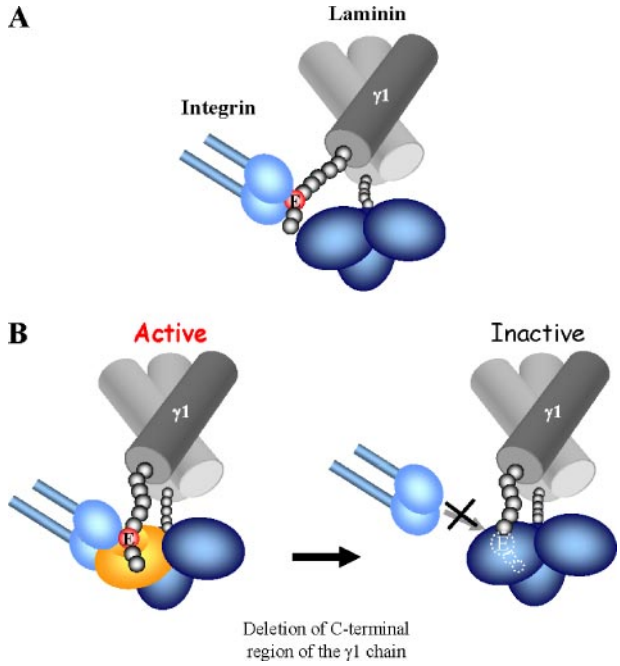


FIGURE 10. **Schematic models for γ chain-dependent recognition of laminin by integrin.** In *model A*, the Glu residue within the C-terminal region of the γ chain is directly involved in integrin binding by laminin as a critical amino acid residue recognized by integrin. In *model B*, the C-terminal Glu residue of the γ chain interacts with the G domain and induces (or exposes) the putative integrin-binding site within the G domain through conformational modulation. Alternatively, the C-terminal Glu residue may also comprise part of the integrin recognition site, whose contribution to integrin binding by laminins is rather auxiliary and not sufficient to be detected in direct integrin binding assays or integrin binding inhibition assays.

C-terminal region of the γ1 chain is required for not only LN-511 but also LN-311 to bind to integrins. Third, the Glu residue at the third position from the C terminus is conserved for both γ1 and γ2 chains, and deletion of the C-terminal three amino acids within the γ2 chain or substitution of Gln for Glu-1191 in the γ2 chain completely abrogated the integrin binding activity of laminin-332.

One of the likely explanations for the requirement of the γ1 C-terminal region, particularly the Glu residue at the third position from the C terminus, in integrin binding by laminins could be that Glu-1607 comprises part of the integrin recognition site of γ1 chain-containing laminins (Fig. 10*A*). Because the metal ion bound to the MIDAS (metal ion-dependent adhesion site) motif within integrins directly coordinates the side chain of acidic residues in ligands (39), acidic residues such as Asp or Glu are considered to act as key residues in ligands to interact with integrins (40, 41). However, despite the importance of the Glu-1607 in integrin binding by γ1 chain-containing laminins, the C-terminal peptide segment of the γ1 chain fused to GST and did not show any significant activity to bind the α6β1 integrin, and the synthetic peptide modeled after the C-terminal peptide sequence of the γ1 chain did not exert any significant inhibitory effect on integrin binding by laminin-511 even at concentrations as high as 2.5 mM. These results argue strongly against the possibility that the C-terminal segment of the γ1 chain including Glu-1607 functions as an integrin-binding site, although we cannot exclude the possibility that the γ1 C-terminal segment comprises part of the integrin recognition site, whose contribution to integrin binding by laminins is rather auxiliary and not sufficient to be detected in direct integrin binding or integrin binding competition assays. Another potential explanation for the requirement of the C-terminal region of the γ1 chain in integrin binding by laminins could be that deletion or replacement of Glu-1607 may result in instability in laminin chain assembly and interferes with disulfide bond formation between β and γ chains near their C termini. Because the conformation of the coiled-coil domain of laminin has been shown to be destabilized by disruption of the C-terminal disulfide bonds between β and γ chains (26), deletion of the C-terminal three amino acids from the γ1 chain or E1607Q substitution could impair β-γ dimer formation through disulfide bonding, thereby leading to destabilization of the conformation of laminin-511 and, hence, inactivation of its integrin binding activity. However, SDS-PAGE analysis of purified E8-γ1Δ3AA and E8-γ1EQ under non-reducing conditions showed that a disulfide bond was spontaneously formed between truncated β1 and γ1 chains (Fig. 4*B*), making this possibility unlikely.

The third explanation for the requirement of the C-terminal region of the γ1 chain in integrin binding is that the Glu residue at the third position from the C terminus of γ1 chain directly interacts with the G domain and alters its local or global conformation, thereby exposing the putative integrin-binding site within the G domain that otherwise remains cryptic. A hypothetical model for the structure of the entire G domain has been proposed (14) based on the three-dimensional structure of LG4-5 modules of the α2 chain and the known length of interdomain linkers (42). The model predicts that LG1–3 modules have the shape of a cloverleaf and that the coiled-coil domain adjacent to the LG1–3 modules seems to make direct contact with the cloverleaf. This model together with the results obtained in this study raises the possibility that the Glu residue in the C-terminal region of γ chains is in direct contact with one of the LG modules and alters either the local conformation of individual LG1–3 modules or the global configuration of the tandem array of LG1–3 modules, thereby exposing the putative integrin-binding site within LG1–3 modules (Fig. 10*B*). Previously, we demonstrated that the LG3 module is indispensable for binding to the α6β1 integrin (19), although neither the LG3 module alone nor a tandem array of LG1–3 modules was capable of binding to the α6β1 integrin (20), suggesting that a tandem array of the LG1–3 modules *per se* is not sufficient to recapitulate the integrin binding activity of laminins. It is tempting, therefore, to speculate that the integrin binding activity depends on both strictly regulated conformation of LG1–3 modules and Glu-1607 of the γ1 chain, the latter interacting with LG1–3 modules and thereby stabilizing their active conformation, so that deletion or replacement of the Glu-1607 results in abrogation of the integrin binding activity of LG1–3 modules through destabilization of their active conformation. Further investigation is needed to confirm this possibility through defining the putative site within the G domain that interacts with Glu-1607.

We also showed that Glu-1191 within the γ2 chain, which is located at the third position from the C terminus, is also required for the integrin binding activity of laminin-332, extending the importance of the Glu residue at the third posi-

tion from the C terminus to the γ2 chain. Previously, a novel variant γ2 chain arising from alternative splicing of the 3′ most exon was identified and designated γ2 transcript variant 2 (GenBank™ accession number NM_018891; Refs. 43 and 44). This variant γ2 chain is shorter than the normal γ2 chain and lacks Glu-1191. It seems likely, therefore, that the laminin isoform containing the variant γ2 chain is defective in binding to the α3β1 integrin due to the absence of Glu-1191 that is required for the integrin binding by laminin-332. The transcript for the variant γ2 chain was detected in cerebral cortex, lung, and distal tubules of the kidney (43). Generation of two splice variants of laminin γ2 chains with and without integrin binding activity may be a novel mechanism modulating the interaction of cells with the basement membrane containing laminin-332 in these tissues.

It is interesting to note that a novel γ chain isoform, γ3 (GenBank™ accession number NM_006059), also lacks the Glu residue that is conserved between the γ1 and γ2 chains (45). Furthermore, the C-terminal region of the γ3 chain consists of only four amino acid residues after the conserved Cys residue, significantly shorter than the corresponding regions of other γ chains. These differences between γ3 and other γ chains raise the possibility that the laminin isoform containing the γ3 chain is defective in binding to integrins. Recently, Yan and Cheng (46) reported, however, that the γ3 chain forms a heterotrimer with α3 and β3 chains and serves as a ligand for the α6β1 integrin at the apical ectoplasmic specialization in rat testes. Thus, the γ3 chain-containing laminin may well retain integrin binding activity despite the absence of the conserved C-terminal Glu residue in the γ3 chain. Heterotrimerization of the γ3 chain with α3 and β3 chains suggests that the laminin containing the γ3 chain may circumvent the loss of the Glu residue, so that the G domain of laminin-333 can maintain active conformation in the absence of the conserved Glu residue in the γ3 chain. However, laminin-333 has been neither purified nor examined directly for its cell-adhesive and integrin binding activities. It remains to be determined whether laminin-333 is capable of binding to α6β1 and other laminin-binding integrins and, if so, how potent the binding is as compared with the binding of laminin-332 to α6β1 and other laminin binding integrins.

Little is known about the role of the laminin β chain in integrin binding by laminins. Although the YIGSR sequence within the short arm of the β1 chain has been proposed as an active site that mediates cell attachment, migration, and receptor binding (47–49), the biological significance of this sequence remains to be confirmed. In contrast to the nine amino acid extension C-terminal to the conserved Cys residue in γ1 and γ2 chains, the C-terminal region of the β1, β2, and β3 chains contains only one amino acid extension C-terminal to the conserved Cys residue, making it less likely that the C-terminal region of β chains is directly involved in modulation of the integrin binding activity of laminins, although we cannot yet exclude the possibility. Recently, Nishimune *et al.* (50) demonstrated that the laminin β2 chain, a component of the synaptic cleft at the neuromuscular junction, binds directly to calcium channels that are required for neurotransmitter release from motor nerve terminals. Furthermore, they showed that a C-terminal 20-kDa fragment of the β2 chain, in particular leucine-arginine-glutamate tripeptide within this fragment, is necessary and sufficient for laminin interactions with voltage-gated calcium channels, suggesting that the C-terminal region of the β chain functions as the binding site for protein(s) other than integrins.

In conclusion, our data showed that the Glu residue within the C terminus of the γ1 chain is necessary for binding to α6β1/α3β1 integrins and is potentially involved in maintenance of the active conformation of the G domain. It is interesting to note that this Glu residue is highly conserved from nematodes to mammals, suggesting that molecular mechanisms underlying integrin binding to laminins are evolutionarily conserved throughout metazoans. Our results provide new insights into the modulatory role of the γ chain in integrin binding by laminins.

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The Requirement of the Glutamic Acid Residue at the Third Position from the Carboxyl Termini of the Laminin γ Chains in Integrin Binding by Laminins

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