

The C-terminal Region of Laminin β Chains Modulates the Integrin Binding Affinities of Laminins^{*[5]}

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Yukimasa Taniguchi, Hiroyuki Ido, Noriko Sanzen, Maria Hayashi, Ryoko Sato-Nishiuchi, Sugiko Futaki, and Kiyotoshi Sekiguchi¹

From the Laboratory of Extracellular Matrix Biochemistry, Institute for Protein Research, Osaka University, 3-2 Yamadaoka, Suita, Osaka 565-0871, Japan

Laminins are major cell-adhesive proteins in basement membranes that are capable of binding to integrins. Laminins consist of three chains (α , β , and γ), in which three laminin globular modules in the α chain and the Glu residue in the C-terminal tail of the γ chain have been shown to be prerequisites for binding to integrins. However, it remains unknown whether any part of the β chain is involved in laminin-integrin interactions. We compared the binding affinities of pairs of laminin isoforms containing the $\beta 1$ or $\beta 2$ chain toward a panel of laminin-binding integrins, and we found that $\beta 2$ chain-containing laminins ($\beta 2$ -laminins) bound more avidly to $\alpha 3\beta 1$ and $\alpha 7X2\beta 1$ integrins than $\beta 1$ chain-containing laminins ($\beta 1$ -laminins), whereas $\alpha 6\beta 1$, $\alpha 6\beta 4$, and $\alpha 7X1\beta 1$ integrins did not show any preference toward $\beta 2$ -laminins. Because $\alpha 3\beta 1$ contains the “X2-type” variable region in the $\alpha 3$ subunit and $\alpha 6\beta 1$ and $\alpha 6\beta 4$ contain the “X1-type” region in the $\alpha 6$ subunit, we hypothesized that only integrins containing the X2-type region were capable of discriminating between $\beta 1$ -laminins and $\beta 2$ -laminins. In support of this possibility, a putative X2-type variant of $\alpha 6\beta 1$ was produced and found to bind preferentially to $\beta 2$ -laminins. Production of a series of swap mutants between the $\beta 1$ and $\beta 2$ chains revealed that the C-terminal 20 amino acids in the coiled-coil domain were responsible for the enhanced integrin binding by $\beta 2$ -laminins. Taken together, the results provide evidence that the C-terminal region of β chains is involved in laminin recognition by integrins and modulates the binding affinities of laminins toward X2-type integrins.

Laminins are large glycoproteins exclusively localized in basement membranes, which represent thin sheets of extracellular matrix bound by a variety of cell types, including epithelial, endothelial, muscle, and glial cells. Laminins are composed of three polypeptide chains (α , β , and γ), which assemble into a disulfide-bonded heterotrimer with a cross-shaped structure.

There are five α chains ($\alpha 1$ – $\alpha 5$), three β chains ($\beta 1$ – $\beta 3$), and three γ chains ($\gamma 1$ – $\gamma 3$) in mammals (1, 2), combinations of which give rise to at least 12 distinct isoforms expressed in tissue-specific and developmentally regulated manners (1, 3).

Laminins play pivotal roles in embryonic development. Mice deficient in expression of the $\gamma 1$ chain, which is present in most laminin isoforms except for laminin-332² and some $\gamma 3$ chain-containing isoforms, fail to deposit basement membranes and die at the peri-implantation stage of embryonic development (4). Gene knockouts of other laminin chains also result in severe phenotypes. Mice deficient in the $\alpha 5$ chain die around embryonic day 17 because of multiple developmental abnormalities, including failure of neural tube closure and digit separation and abnormal placental, kidney, and lung morphogenesis (5–7). Mice lacking the $\alpha 2$ chain show adult lethality because of severe and progressive skeletal muscle degeneration (8, 9). These phenotypes can be accounted for by defects in the physical strength of basement membranes and/or the adhesive interactions of cells with basement membranes and subsequent signaling events involving the integrin family of cell adhesion receptors (6, 10).

Integrins are heterodimeric membrane proteins composed of noncovalently associated α and β subunits. To date, 24 integrin types consisting of distinct α and β subunits have been identified in mammals (11). Among these integrins, $\alpha 3\beta 1$, $\alpha 6\beta 1$, $\alpha 6\beta 4$, and $\alpha 7\beta 1$ integrins have been shown to serve as the major laminin receptors in various cell types (12, 13). Further diversity has been introduced to $\alpha 7\beta 1$ integrin by the presence of two $\alpha 7$ subunits, $\alpha 7X1$ and $\alpha 7X2$, which differ in their extracellular β -propeller domains (12). The $\alpha 7$ subunit contains alternatively spliced X1 or X2 regions that are located at the surface-exposed loop connecting blades III and IV of the β -propeller domain. The X1 and X2 regions have been shown to modulate the ligand-binding specificities and affinities of $\alpha 7\beta 1$ integrins. Specifically, $\alpha 7X1\beta 1$ integrin binds to laminin-511 with higher affinities than to laminin-111 and -211, whereas $\alpha 7X2\beta 1$ integrin binds more avidly to laminin-111 and -211 than to laminin-511 (14, 15).

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[5] The on-line version of this article (available at <http://www.jbc.org>) contains supplemental Fig. 1.

¹ To whom correspondence should be addressed. Tel.: 81-6-6879-8617; Fax: 81-6-6879-8619; E-mail: sekiguch@protein.osaka-u.ac.jp.

² A new nomenclature for laminin isoforms (56) has been used throughout this paper as follows: laminin-111, laminin- $\alpha 1\beta 1\gamma 1$ (also designated laminin-1); laminin-211, laminin- $\alpha 2\beta 1\gamma 1$ (also designated laminin-2); laminin-121, laminin- $\alpha 1\beta 2\gamma 1$ (also designated laminin-3); laminin-221, laminin- $\alpha 2\beta 2\gamma 1$ (also designated laminin-4); laminin-332, laminin- $\alpha 3\beta 3\gamma 2$ (also designated laminin-5); laminin-421, laminin- $\alpha 4\beta 2\gamma 1$ (also designated laminin-9); laminin-511, laminin- $\alpha 5\beta 1\gamma 1$ (also designated laminin-10); laminin-521, laminin- $\alpha 5\beta 2\gamma 1$ (also designated laminin-11).

Accumulating evidence indicates that three laminin globular (LG)³ domains, LG1–3, in the α chains are prerequisites for integrin binding by laminins (16, 17). However, laminin α chain monomers do not show any significant activities for binding to integrins and require heterotrimerization with β and γ chains to fully exert their activities (18, 19). Recently, we found that the C-terminal region of laminin γ chains, particularly the glutamic acid residue at the third position from the C terminus, is critically involved in laminin recognition by integrins (20). Deletion or substitution of this glutamic acid residue, which is conserved between the γ 1 and γ 2 chains, abrogates the integrin binding activities of laminin isoforms containing these γ chains, whereas isoforms containing the γ 3 chain, whose C-terminal tail is shorter than those of the γ 1 and γ 2 chains and lacks the glutamic acid residue, are unable to bind to integrins (20, 21). Despite the emerging evidence for roles of the α and γ chains in integrin binding by laminins, it remains unknown whether the β chains contribute to laminin recognition by integrins.

Among the three β chains, the β 1 and β 2 chains exhibit ~50% sequence homology and share the same domain structure (2, 22). Both the β 1 and β 2 chains form disulfide-bonded dimers with the γ 1 chain and assemble with all types of α chains to yield α X β 1 γ 1 or α X β 2 γ 1 heterotrimers (23–26). Immunohistochemical analyses revealed that although the β 1 and β 2 chains are both ubiquitously distributed in various tissues, the β 2 chain dominates at sites with specialized functions and/or unique structures, such as neuromuscular junctions and glomerular basement membranes of the kidney (27–29). Consistent with the enriched expression of the β 2 chain at these sites, deficiency in the β 2 chain leads to abnormal structures of neuromuscular junctions and dysfunction of glomerular basement membranes (27, 30, 31). Although little is known about the functional distinctions between laminin isoforms containing the β 1 chain (referred to as “ β 1-laminins”) and those containing the β 2 chain (referred to as “ β 2-laminins”), the β 2-containing laminin-421 has been shown to bind to voltage-gated calcium channels, components of neuromuscular junctions, through the C-terminal 20-kDa region of the β 2 chain containing the tripeptide sequence Leu-Arg-Glu, which is absent from the corresponding region of the β 1 chain (32). However, it remains unexplored whether β 1-laminins and β 2-laminins differ in their integrin-binding specificities or affinities.

In this study, we produced pairs of β 1-laminins and β 2-laminins sharing the same α chains, and we compared their binding activities toward a panel of laminin-binding integrins to investigate their functional distinctions. We found that β 1-laminins and β 2-laminins differ in their binding affinities toward a subset of laminin-binding integrins that contain the X2 region within their α subunits. We mapped the region modulating the integrin-binding affinities to the C-terminal 20 amino acid residues of the β chains and found that the C-terminal region of the laminin β 2 chain up-regulates the binding affinities toward X2 region-containing integrins.

EXPERIMENTAL PROCEDURES

Antibodies and Reagents—Mouse monoclonal antibodies (mAbs) against the human laminin α 1 (1F11 and 5A3), α 2 (3F12 and 22W2), α 5 (5D6 and 15H5), and γ 1 (C12SW) chains were produced as described previously (16, 33). A rat mAb against the human laminin β 2 chain (6A3) was produced by fusion of Sp2/0 mouse myeloma cells with spleen cells from rats immunized with recombinant laminin-221 as an immunogen. The specificity of 6A3 was determined using laminin β 1 and β 2 swap mutants as detailed below. A mouse mAb against the human laminin β 1 chain (DG10) was purchased from Biomol (Plymouth Meeting, PA). A mouse anti-FLAG[®] M2 mAb was purchased from Sigma. Horseradish peroxidase-conjugated mouse anti-His₆ mAb and rabbit anti-hemagglutinin (HA) tag polyclonal antibody (pAb) were obtained from Qiagen (Valencia, CA) and QED Bioscience (San Diego), respectively. A rabbit pAb against ACID/BASE coiled-coil peptides was kindly provided by Dr. Junichi Takagi (Institute for Protein Research, Osaka University, Osaka, Japan). Horseradish peroxidase-conjugated donkey anti-mouse IgG was purchased from Jackson ImmunoResearch (West Grove, PA). Horseradish peroxidase-conjugated goat anti-rat IgG was obtained from American Qualex Antibodies (San Clemente, CA). A streptavidin-horseradish peroxidase conjugate was purchased from Zymed Laboratories Inc.

Construction of Expression Vectors—Expression vectors for the human laminin α 1 (pcDNA3.1- α 1), α 2 (pcDNA3.1- α 2), α 5 (pcDNA3.1- α 5), β 1 (pCEP4- β 1), and β 2 (pcDNA3.1- β 2) chains were prepared as described previously (16, 21, 34). An expression vector for a FLAG-tagged laminin β 2 chain was constructed by inserting the following nucleic acid sequence encoding a FLAG tag between positions 96 and 97 from the starting codon of the β 2 mRNA: 5'-CAA GCA CCA ATC GAT GAT TAC AAG GAC GAC GAT GAC AAG-3' (the FLAG tag sequence is shown with underlines). An expression vector for the human laminin γ 1 chain (pSecTag2A- γ 1) was generated as follows. A cDNA encoding the laminin γ 1 chain (pcDNA3.1- γ 1) (34) was digested with XbaI, followed by digestion with BamHI. The resulting cDNA fragment was ligated into the EcoRV-BamHI sites of pSecTag2A (Invitrogen) from which the endogenous signal peptide had been deleted.

Expression vectors for individual E8 fragments of the human laminin α 1, α 2, α 5, β 1, and γ 1 chains (designated α 1E8, α 2E8, α 5E8, β 1E8, and γ 1E8, respectively) were prepared as described (20, 21). His₆, HA, and FLAG tags were added to the recombinant α E8 (α 1E8, α 2E8, and α 5E8), β 1E8, and γ 1E8 chains, respectively. An expression vector for a truncated laminin β 2 chain (designated β 2E8) was prepared as follows. A cDNA encoding laminin β 2E8 (Leu¹⁵⁷³-Gln¹⁷⁹⁸) was amplified by PCR using pcDNA3.1- β 2 as a template, with an EcoRI site at the 3' end, and then inserted to the EcoRV and EcoRI sites of pBluescript[®] KS(+) (Stratagene, La Jolla, CA), which had been modified to include a cDNA segment encoding an HA tag by extension PCR. The resulting cDNA encoding HA-tagged β 2E8 was recloned into pSecTag2B (Invitrogen) cleaved with KpnI and EcoRI. Expression vectors for the following swap mutants of the laminin β 1 and β 2 chains were prepared by overlap

³ The abbreviations used are: LG, laminin globular; BSA, bovine serum albumin; HA, hemagglutinin; mAb, monoclonal antibody; pAb, polyclonal antibody; TBS, Tris-buffered saline.

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extension PCR using pSecTag2B- β 1E8 and pSecTag2B- β 2E8 as templates: Swap1, Leu¹⁵⁷³-Gln¹⁶²² of β 2 were substituted with Leu¹⁵⁶¹-Glu¹⁶¹⁰ of β 1; Swap2, Leu¹⁵⁷³-Ser¹⁶⁷⁸ of β 2 were substituted with Leu¹⁵⁶¹-Ser¹⁶⁶⁶ of β 1; Swap3, Leu¹⁵⁷³-Ser¹⁷⁵⁵ of β 2 were substituted with Leu¹⁵⁶¹-Leu¹⁷⁴³ of β 1; Swap4, Leu¹⁵⁷³-Leu¹⁷⁷⁶ of β 2 were substituted with Leu¹⁵⁶¹-Leu¹⁷⁶⁴ of β 1; Swap5, Leu¹⁵⁷³-Thr¹⁷⁹⁶ of β 2 were substituted with Leu¹⁵⁶¹-Thr¹⁷⁸⁴ of β 1; Swap6, Glu¹⁷⁶⁵-Thr¹⁷⁸⁴ of β 1 were substituted with Glu¹⁷⁷⁷-Thr¹⁷⁹⁶ of β 2. Schematic structures of the chimeric mutants are depicted in Fig. 5.

Expression vectors for truncated forms of integrin α subunits (α 3, α 6, α 7X1, and α 7X2) with an ACID peptide and a FLAG tag sequence at their C termini were prepared as described (14, 36). Expression vectors for the extracellular domains of the integrin β 1 and β 4 subunits with a BASE peptide and a His₆ tag sequence at their C termini were kindly provided by Dr. Junichi Takagi (Institute for Protein Research, Osaka University) (37).

An expression vector for the putative X2-type variant of the human integrin α 6 subunit (designated α 6X2) was constructed as follows. A cDNA encoding the putative human integrin α 6X2 subunit was assembled from the genomic sequence information for the exon/intron boundaries (Ensembl Transcript ID, ENST00000375221) (supplemental Fig. S1) (12, 38). The predicted amino acid sequence of integrin α 6X2 is available from the UniProt Knowledgebase data bank (Swiss-Prot accession number P23229). Human genomic DNA was isolated from 293-F cells using a DNeasy[®] tissue kit (Qiagen). Exon 6 encoding the X2 region was amplified by PCR using the genomic DNA as a template, and the resulting cDNA was ligated to a cDNA encompassing exons 1–4 by overlap extension PCR, followed by ligation to a cDNA containing exons 7 and 8. The resulting cDNA segment encompassing nucleotides –13 to 1041 (supplemental Fig. S1) was cloned into the KpnI/AccI sites of pCR[®]-Blunt II-TOPO[®] (Invitrogen), excised with the same pair of restriction enzymes, and then ligated to KpnI/AccI-cleaved pBluescript KS(+) (Stratagene) in which an extracellular domain of the α 6 integrin subunit was ligated, yielding a cDNA encoding the putative X2 variant of the integrin α 6 subunit. The α 6X2 cDNA was excised from pBluescript KS(+) with KpnI/NotI and ligated to KpnI/NotI-cleaved pcDNA3.1(+) that had been engineered to express the cloned cDNA as a fusion protein with a C-terminal peptide segment containing ACID and FLAG peptides in tandem. A list of the primer sequences used to construct the expression vectors in this study is available upon request.

Expression and Purification of Recombinant Proteins—Recombinant laminin-111, -211, and -511 were produced using a FreeStyle[™] 293 Expression System (Invitrogen) and purified from conditioned media using immunoaffinity columns conjugated with the following mAbs (16, 21): 1F11 for laminin α 1; 3F12 for laminin α 2; and 5D6 for laminin α 5. Recombinant laminin-121, -221, and -521 were produced using the same expression system and purified from conditioned media using anti-FLAG[®] M2-agarose columns (Sigma). The columns were washed with Tris-buffered saline (TBS) containing 1 mM EDTA, and bound proteins were eluted with 100 μ g/ml FLAG peptide (Sigma) and dialyzed against TBS containing 1 mM EDTA.

Recombinant integrins were produced using the FreeStyle[™] 293 expression system and purified from conditioned media as previously described (14). Purified recombinant integrins were dialyzed against TBS without divalent cations.

Recombinant E8 fragments of laminin-111, -121, -211, -221, -511, and -521, and those of laminin-511/521 swap mutants were produced using the FreeStyle[™] 293 expression system. The recombinant proteins were purified from conditioned media by sequential chromatography on Ni-NTA-agarose (Qiagen) and anti-FLAG[®] M2-agarose columns, and then dialyzed against TBS. The protein concentrations of all the recombinant products were determined using a BCA protein assay kit (Thermo Scientific, Rockford, IL) using bovine serum albumin (BSA) as a standard.

SDS-PAGE and Immunoblotting—SDS-PAGE was carried out according to Laemmli (39). The separated proteins were visualized by Coomassie Brilliant Blue staining or transferred onto polyvinylidene difluoride membranes. The membranes were probed with antibodies against individual laminin chains or tag peptides, and the resulting antigen-antibody complexes were visualized using ECL Western blotting detection reagents (GE Healthcare).

Solid-phase Binding Assays—Solid-phase assays for binding of integrins with laminins and their E8 fragments were carried out as described previously (14). Briefly, 96-well microtiter plates were coated with recombinant proteins overnight at 4 °C, blocked with 1% BSA for 1 h at room temperature, and incubated with integrins for 3 h at room temperature in the presence of 1 mM MnCl₂ or 10 mM EDTA. Following washes with TBS containing 0.1% BSA and 0.02% Tween 20 (buffer-W), the plates were incubated with the biotinylated pAb against ACID/BASE peptides in buffer-W containing 1 mM MnCl₂ for 30 min, washed with the same buffer, and incubated with horseradish peroxidase-conjugated streptavidin for 15 min. Bound antibodies were quantified by measuring the absorbance at 490 nm after incubation with *o*-phenylenediamine. The amounts of integrin ligands adsorbed on the plates were quantified by enzyme-linked immunosorbent assays using mAbs 1F11 (for laminin-111/121), 3F12 (for laminin-211/221), and 5D6 (for laminin-511/521) or anti-FLAG mAb (for E8 fragments) to confirm the equality of the adsorbed proteins. The apparent dissociation constants were determined as described previously (40). Statistical analyses were performed using Student's *t* test.

RESULTS

Laminin-521 Binds More Avidly to α 3 β 1 and α 7X2 β 1 Integrins Than Laminin-511—To investigate the potential functional distinctions between laminins containing the β 1 and β 2 chains in integrin binding, we produced recombinant laminin-511 and -521 by simultaneous transfection of cDNAs encoding the laminin α 5/ β 1/ γ 1 and α 5/ β 2/ γ 1 chains into 293-F cells, respectively, and we examined their binding activities toward a panel of laminin-binding integrins. 293-F cells expressed a small amount of the laminin β 1 chain (data not shown), and the resulting laminin-521 became contaminated with laminin-511 containing the endogenously expressed β 1 chain, which cannot be removed by immunoaffinity chromatography using antibodies against the α 5 chain. To avoid such contaminants in β 2

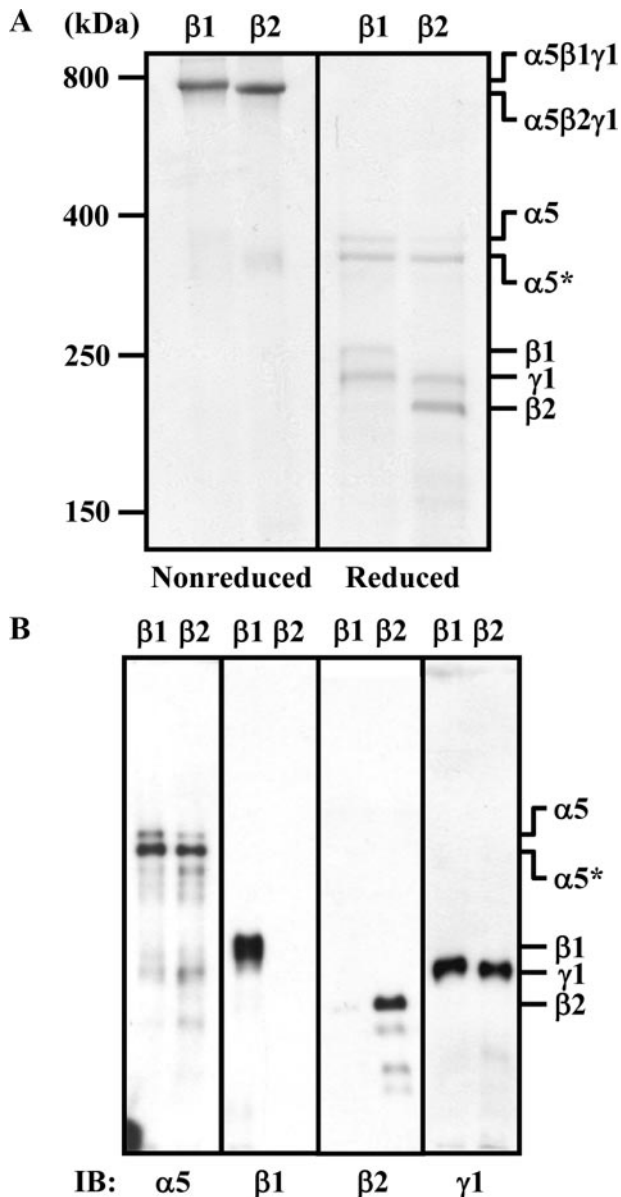


FIGURE 1. SDS-PAGE and immunoblot analyses of purified laminin-511 and -521. A and B, purified laminin-511 and -521 (labeled $\beta 1$ and $\beta 2$, respectively) were analyzed by SDS-PAGE in 4% gels under nonreducing or reducing conditions. The separated proteins were visualized by Coomassie Brilliant Blue staining (A) or transferred onto polyvinylidene difluoride membranes followed by immunoblotting (IB) with mAbs against the $\alpha 5$ (15H5), $\beta 1$ (DG10), $\beta 2$ (6A3) and, $\gamma 1$ (C125W) chains (B). The positions of molecular size markers, including nonreduced and reduced Engelbreth-Holm-Swarm-laminin (800 and 400 kDa, respectively), are shown in the left margin. The asterisk denotes the laminin $\alpha 5$ chain lacking LG4–5.

chain-containing laminins, a FLAG tag was fused to the N terminus of the $\beta 2$ chain to facilitate purification of the recombinant laminin-521 using an anti-FLAG mAb column. The authenticities of the purified proteins were verified by Coomassie Brilliant Blue staining (Fig. 1A) and immunoblotting with antibodies against individual laminin chains (Fig. 1B). Under nonreducing conditions, both laminin-511 and -521 gave high molecular weight bands migrating at similar positions to that of Engelbreth-Holm-Swarm-laminin (~800 kDa), confirming that both laminins were purified as disulfide-bonded heterotrimers (Fig. 1A). Under reducing conditions, laminin-511 gave

four bands. The two upper bands were full-length and LG4–5-deleted $\alpha 5$ chains (16) and the two lower bands were the $\beta 1$ and $\gamma 1$ chains, based on immunoblotting with antibodies against individual chains (Fig. 1, A and B). Similarly, laminin-521 also gave four bands under reducing conditions. The two upper bands were the same as those of laminin-511, and the two lower bands were the $\gamma 1$ and $\beta 2$ chains.

The purified laminin-511 and -521 were assessed for their abilities to bind to a panel of recombinant laminin-binding integrins by solid-phase binding assays (Fig. 2). The assays were performed in the presence of 1 mM Mn^{2+} to fully activate the integrins. Although $\alpha 6\beta 1$, $\alpha 6\beta 4$, and $\alpha 7X1\beta 1$ integrins bound to laminin-511 and -521 equally well without any bias (Fig. 2, B–D), $\alpha 3\beta 1$ and $\alpha 7X2\beta 1$ integrins bound more avidly to laminin-521 than to laminin-511 (Fig. 2, A and E). The apparent dissociation constants of $\alpha 3\beta 1$ integrin were 3.28 ± 0.26 nM for laminin-511 and 0.84 ± 0.14 nM for laminin-521 (Table 1), demonstrating that $\alpha 3\beta 1$ integrin binds to laminin-521 with an affinity about four times higher than that for laminin-511. A similar difference in the affinities toward laminin-511 and -521 was observed for $\alpha 7X2\beta 1$ integrin, although the dissociation constant for laminin-511 could not be determined because of incomplete saturation of the integrin binding activity. In contrast, $\alpha 6\beta 1$ and $\alpha 7X1\beta 1$ integrins did not show any differences in their dissociation constants toward laminin-511 and -521 ($p = 0.78$ for $\alpha 6\beta 1$ and $p = 0.19$ for $\alpha 7X1\beta 1$), as was also the case for $\alpha 6\beta 4$ integrin, whose titration curves for laminin-511 and -521 overlapped with each other over the range of integrin concentrations examined (Fig. 2C). Binding of these integrins to laminin-511 and -521 was abolished in the presence of EDTA (data not shown), endorsing the specificity of the solid-phase binding assays. These results raised the possibility that laminin β chains modulate the binding affinities of laminins to some but not all laminin-binding integrins depending on the type of integrin α subunit.

Enhanced Integrin-binding Affinities of $\beta 2$ Laminins Are Reproduced by E8 Fragments—The integrin binding activities of laminins have been shown to be fully reproduced by their E8 fragments consisting of the C-terminal coiled-coil domains of the α , β , and γ chains with an extension of three LG domains in the α chain (17, 18, 20). To examine whether the distinctive integrin-binding affinities of laminin-511 and -521 could be reproduced by their E8 fragments, we generated recombinant E8 fragments of laminin-511 and -521 (designated laminin-511E8 and -521E8, respectively), which were comprised of truncated forms of the $\alpha 5$, $\beta 1/\beta 2$, and $\gamma 1$ chains (designated $\alpha 5E8$, $\beta 1E8/\beta 2E8$, and $\gamma 1E8$, respectively; Fig. 3A). His₆, HA, and FLAG tags were engaged at the N termini of the $\alpha 5E8$, $\beta 1E8/\beta 2E8$, and $\gamma 1E8$ chains, respectively, to facilitate purification of the resulting E8 fragments. The authenticities of the recombinant laminin-511E8 and -521E8 were verified by SDS-PAGE (Fig. 3B) and subsequent immunoblotting with antibodies against individual tags (Fig. 3C). Under nonreducing conditions, two bands corresponding to $\alpha 5E8$ and the heterodimer of $\beta 1E8$ – $\gamma 1E8$ or $\beta 2E8$ – $\gamma 1E8$ were detected (Fig. 3, B and C), consistent with our previous report (20). Under reducing conditions, the recombinant E8 fragments gave three bands, each corresponding to $\alpha 5E8$, $\gamma 1E8$, and $\beta 1E8/\beta 2E8$. The $\beta 2E8$ frag-

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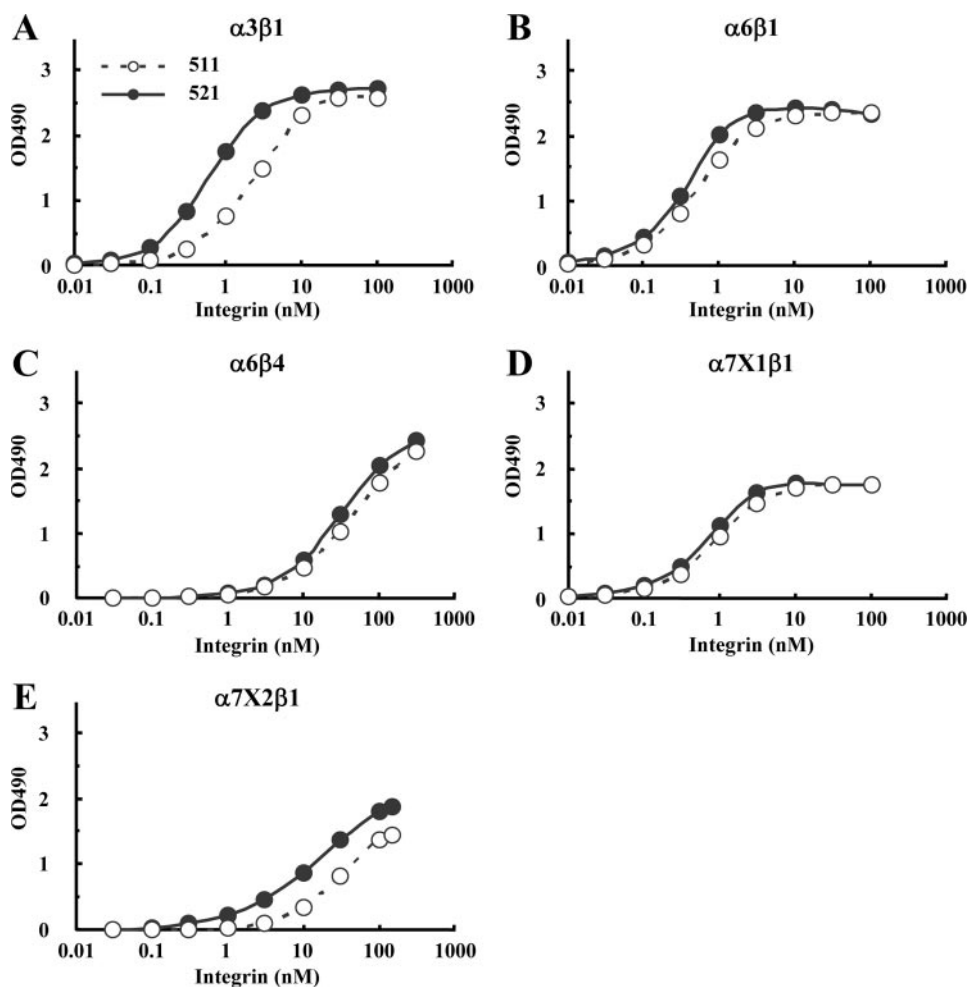


FIGURE 2. Titration curves of recombinant integrins bound to laminin-511 and -521. A–E, increasing concentrations of recombinant $\alpha 3\beta 1$ (A), $\alpha 6\beta 1$ (B), $\alpha 6\beta 4$ (C), $\alpha 7X1\beta 1$ (D), and $\alpha 7X2\beta 1$ (E) integrins were allowed to bind to microtiter plates coated with laminin-511 (open circles) or laminin-521 (closed circles) in the presence of 1 mM $MnCl_2$. Bound integrins were quantified using an antibody against the ACID/BASE peptides attached to the C termini of the α and β integrin subunits. The amounts of the integrins bound in the presence of 10 mM EDTA were taken as nonspecific binding and subtracted as background. The results shown are the means of duplicate determinations. The apparent dissociation constants of the recombinant integrins are summarized in Table 1.

TABLE 1
Dissociation constants of recombinant integrins toward laminin-511/-521 and their E8 fragments

	K_d^a				
	$\alpha 3\beta 1$	$\alpha 6\beta 1$	$\alpha 6\beta 4$	$\alpha 7X1\beta 1$	$\alpha 7X2\beta 1$
	HM	HM	HM	HM	HM
Laminin-511	3.28 ± 0.26	0.73 ± 0.06	ND ^b	1.05 ± 0.23	ND
Laminin-521	0.84 ± 0.14	0.74 ± 0.15	ND	0.81 ± 0.13	11.6 ± 1.97
Laminin-511E8	2.96 ± 0.54	0.83 ± 0.02	ND	1.01 ± 0.16	ND
Laminin-521E8	0.68 ± 0.08	0.79 ± 0.09	ND	0.75 ± 0.12	10.9 ± 2.17

^a The results are expressed as means \pm S.D. of three independent experiments.

^b Data were not determined because of only partial saturation at the highest integrin concentration examined.

ment produced a band that migrated faster than the band for $\beta 1E8$ because of the absence of an *N*-glycosylation site in $\beta 2E8$ (see Fig. 5A).

Solid-phase integrin-binding assays with laminin-511E8 and -521E8 gave essentially the same results as those obtained with intact laminin-511 and -521. Laminin-521E8 bound more avidly to $\alpha 3\beta 1$ and $\alpha 7X2\beta 1$ integrins than laminin-511E8 (Fig.

4, A and E), whereas laminin-511E8 and -521E8 bound to $\alpha 6\beta 1$, $\alpha 6\beta 4$, and $\alpha 7X1\beta 1$ integrins with almost the same affinities (Fig. 4, B–D). The dissociation constants of the laminin-binding integrins toward laminin-511E8 and -521E8 were essentially the same as those obtained with intact laminin-511 and -521 (Table 1), confirming that $\alpha 3\beta 1$ and $\alpha 7X2\beta 1$ integrins bind with higher affinities to laminin-521 than to laminin-511, whereas the other laminin-binding integrins do not discriminate laminin-521 from laminin-511. These results also show that the region in the laminin β chains that modulates the binding affinities for $\alpha 3\beta 1$ and $\alpha 7X2\beta 1$ integrins is preserved in the E8 fragments, *i.e.* within the C-terminal 226 amino acid residues.

C-terminal 20 Amino Acid Residues of Laminin β Chains Modulate Integrin-binding Affinities—To define the region within the laminin β chains that modulates the integrin-binding affinities, we produced a panel of mutant E8 fragments of laminin-511/521, in which the $\beta 1$ and $\beta 2$ chains were differentially swapped from their N termini (Fig. 5, A and B). SDS-PAGE of the mutant E8 fragments of laminin-511/521 under nonreducing conditions gave two bands, one corresponding to the $\alpha 5E8$ chain and the other corresponding to the swapped $\beta E8$ chains linked to the $\gamma 1E8$ chain

by a disulfide bond (Fig. 5C, left panel). The relative intensities between the upper and lower bands remained unchanged among the control and mutant fragments, indicating that the heterotrimeric integrities of the mutant E8 fragments were not compromised after partial swapping between the $\beta 1$ and $\beta 2$ chains. The lower band of the mutant E8 fragments migrated to the identical position to the control $\beta 1E8$ - $\gamma 1E8$ or $\beta 2E8$ - $\gamma 1E8$ heterodimer, depending on the presence or absence of the putative *N*-glycosylation site (see Fig. 5, A and B). Consistent with the differences in the mobilities of the $\beta E8$ - $\gamma 1E8$ heterodimers under nonreducing conditions, the swapped β chains also migrated to the position of either the $\beta 1E8$ or $\beta 2E8$ chain under reducing conditions, depending on the presence or absence of the putative *N*-glycosylation site.

The binding affinities of the mutant E8 fragments to $\alpha 3\beta 1$ integrin were assessed by solid-phase binding assays (Table 2). Although the E8 fragments containing the Swap1–4 chains, in which different lengths of the N-terminal region of the $\beta 2E8$ chain were swapped with the corresponding regions of $\beta 1E8$,

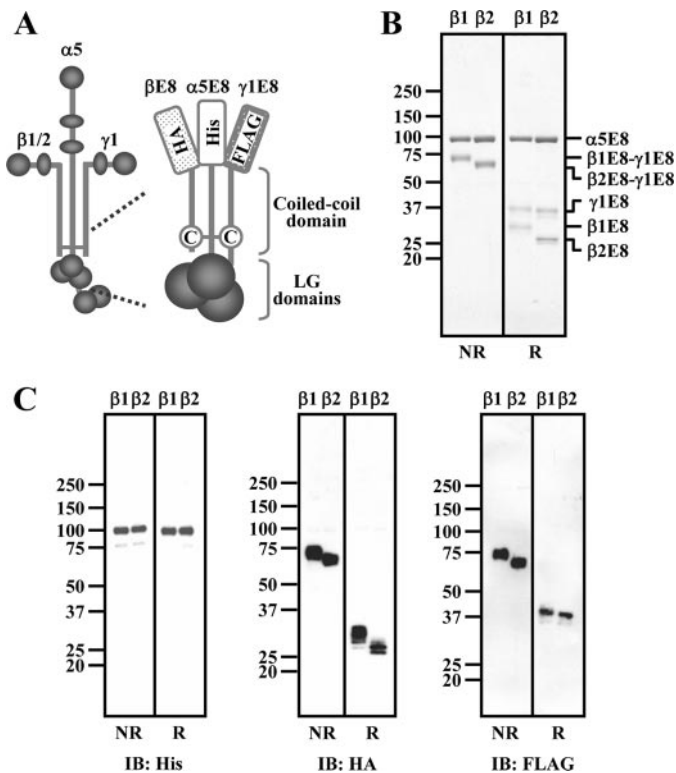


FIGURE 3. SDS-PAGE and immunoblot analyses of purified laminin-511E8 and -521E8. *A*, schematic diagrams of a full-length laminin and a recombinant E8 fragment. The cysteine (C) residues conserved in the C termini of the laminin β and γ chains are indicated. *B* and *C*, laminin-511E8 and -521E8 (labeled $\beta 1$ and $\beta 2$, respectively) were subjected to SDS-PAGE in 5–20% gradient polyacrylamide gels, followed by Coomassie Brilliant Blue staining (*B*) or immunoblotting (*IB*) with specific antibodies against the $\alpha 5 E 8$ (anti-His₆), $\beta 1 E 8$ / $\beta 2 E 8$ (anti-HA), and $\gamma 1 E 8$ (anti-FLAG) fragments (*C*) under nonreducing (NR) and reducing (R) conditions. The positions of molecular size markers are shown in the left margin.

exhibited no significant decreases in their integrin-binding affinities, the E8 fragment containing the Swap5 chain, in which the entire $\beta 2$ chain except for the C-terminal-most Gln residue was replaced with the $\beta 1$ chain, showed a significant reduction in the integrin-binding affinity, suggesting that the C-terminal 20 amino acid residues in the coiled-coil domain of the β chains were responsible for the modulation of laminin-integrin interactions by β chains. To further explore the role of the C-terminal 20 residues of the β chains, we generated Swap6, in which the C-terminal 20 residues of the $\beta 1$ chain in the coiled-coil domain were swapped with the corresponding region of $\beta 2$. The resulting E8 fragment containing Swap6 bound to $\alpha 3\beta 1$ integrin with a similar affinity to that of the control laminin-521E8 (K_d , 0.81 ± 0.05 nM), confirming the critical role of the C-terminal 20 residues in the modulation of integrin-binding affinity by β chains. Unlike $\alpha 3\beta 1$ integrin, $\alpha 6\beta 1$ did not show any significant differences in its affinities toward these swap mutants of the laminin-511/521 E8 fragments irrespective of the mutations introduced (Table 2). These results support the conclusion that the C-terminal region of the β chains modulates the binding affinities of laminins toward $\alpha 3\beta 1$ but not $\alpha 6\beta 1$ integrin.

β Chains Modulate Integrin-binding Affinities of Laminin Isoforms Containing Other α Chains—Given the modulation of the integrin-binding affinities of $\alpha 5$ chain-containing laminins

by β chains, we next examined whether the integrin binding activities of laminin isoforms containing other α chains were also modulated by β chains. We produced recombinant E8 fragments of laminin-211 and -221 (designated laminin-211E8 and -221E8, respectively) and examined their binding activities toward $\alpha 6\beta 1$, $\alpha 7X1\beta 1$, and $\alpha 7X2\beta 1$ integrins. Although these integrins have been shown to bind to laminin-211 and -221 (14, 15), our solid-phase binding assays failed to detect any significant binding of laminin-211E8 and -221E8 to $\alpha 6\beta 1$ and $\alpha 7X1\beta 1$ integrins (Fig. 6, *A* and *B*). Consistent with these results, recombinant full-length laminin-211 and -221 also failed to bind to $\alpha 6\beta 1$ and $\alpha 7X1\beta 1$ integrins (data not shown). These apparent discrepancies may result from potential contaminations by other laminin isoforms in the laminin-211/221 used in the previous reports (see “Discussion”). On the other hand, $\alpha 7X2\beta 1$ integrin was capable of binding to both laminin-211E8 and -221E8, although it bound more avidly to laminin-221E8 than to laminin-211E8, with an ~ 6 -fold difference in the dissociation constants (0.89 ± 0.09 nM for laminin-221E8 *versus* 5.51 ± 0.61 nM for laminin-211E8) (Fig. 6*C*), consistent with the higher affinity of $\alpha 7X2\beta 1$ integrin for laminin-521 than for laminin-511. To further extend these observations to other laminin isoforms, we produced recombinant E8 fragments of laminin-111 and -121 (designated laminin-111E8 and -121E8, respectively), and we found that laminin-121E8 bound more avidly to $\alpha 7X2\beta 1$ integrin than laminin-111E8 with dissociation constants of 2.04 ± 0.07 nM for laminin-111E8 and 0.50 ± 0.06 nM for laminin-121E8 (Fig. 6*D*). These results further support the conclusion that $\alpha 7X2\beta 1$ integrin binds to $\beta 2$ -laminins with higher affinities than to $\beta 1$ -laminins, independently of the type of laminin α chain.

X2-type Integrins Preferentially Bind to $\beta 2$ -Laminins—Because the integrin $\alpha 3$ gene contains only the X2-like exon and has been classified into X2-type integrins along with $\alpha 7X2\beta 1$ (12), we hypothesized that X2-type but not X1-type integrins might be able to discriminate between $\beta 1$ -laminins and $\beta 2$ -laminins and bind more avidly to $\beta 2$ -laminins than to $\beta 1$ -laminins. Consistent with this hypothesis, $\alpha 6\beta 1$ and $\alpha 6\beta 4$ integrins, which did not show any preference for $\beta 2$ -laminins, are classified as X1-type integrins because the major form of the integrin $\alpha 6$ subunit contains the X1 exon-derived sequence (12). A minor form of the integrin $\alpha 6$ transcript that contains both the X1 and X2 sequences has also been reported (38) (Fig. 7*A*), but transcripts containing only the X2 exon-derived sequence have not been found. To corroborate our hypothesis, we set out to clone the putative $\alpha 6X2$ cDNA by replacing the sequence encoded by the X1 exon with that of the X2 exon in the $\alpha 6X1$ cDNA and expressed a recombinant truncated form of $\alpha 6X2\beta 1$ integrin in 293-F cells.

Upon SDS-PAGE, the resulting $\alpha 6X2\beta 1$ integrin gave bands migrating at ~ 250 kDa under nonreducing conditions and ~ 150 kDa under reducing conditions, as was the case with the control $\alpha 6X1\beta 1$ integrin (Fig. 7*B*, left panel). The authenticity of the $\alpha 6X2\beta 1$ integrin was further confirmed by immunodetection of the FLAG and His₆ tags added to the C termini of the $\alpha 6$ and $\beta 1$ chains, respectively (Fig. 7*B*, middle and right panels). Because the integrin $\alpha 6$ subunit splits into heavy and light chains under reducing conditions, only the light chain derived

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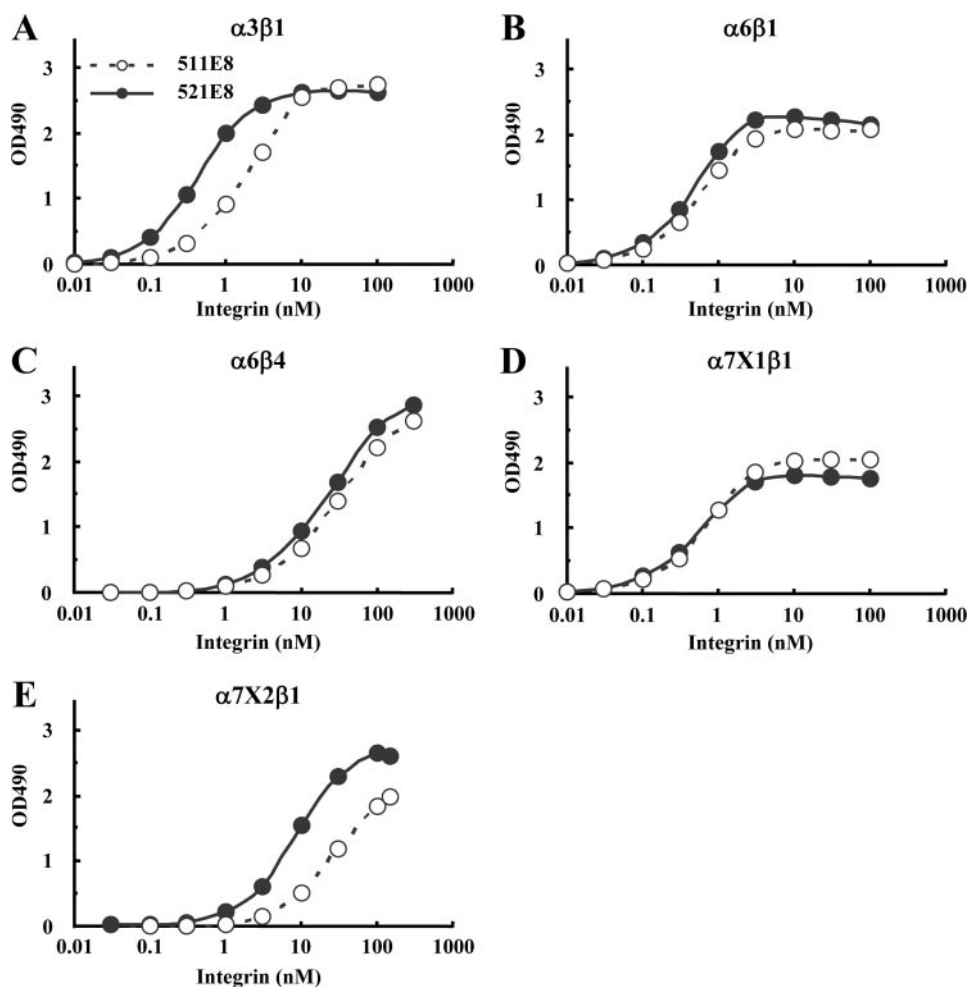


FIGURE 4. Titration curves of recombinant integrins bound to laminin-511E8 and -521E8. A–E, increasing concentrations of recombinant $\alpha 3\beta 1$ (A), $\alpha 6\beta 1$ (B), $\alpha 6\beta 4$ (C), $\alpha 7X1\beta 1$ (D), and $\alpha 7X2\beta 1$ (E) integrins were allowed to bind to microtiter plates coated with laminin-511E8 (open circle) and -521E8 (closed circle) in the presence of 1 mM $MnCl_2$. The amounts of the integrins bound in the presence of 10 mM EDTA were taken as nonspecific binding and subtracted as background. The results shown are the means of duplicate determinations. Apparent dissociation constants of recombinant integrins are summarized in Table 1.

from the C-terminal region was detected with the anti-FLAG antibody.

The laminin binding activities of the $\alpha 6X2\beta 1$ integrin were assessed by solid-phase binding assays using three pairs of E8 fragments, namely laminin-111E8/121E8, -211E8/221E8, and -511E8/521E8 (Fig. 7C). Although the control $\alpha 6\beta 1$ containing the X1 sequence was highly active in binding to laminin-511E8/521E8 but inactive in binding to laminin-211E8/221E8 (Figs. 4 and 6), the $\alpha 6X2\beta 1$ integrin was capable of binding to laminin-211E8/221E8 but not to laminin-511E8/521E8 (Fig. 7C). Therefore, the ligand specificities of $\alpha 6X2\beta 1$ differed significantly from those of $\alpha 6X1\beta 1$. Nevertheless, $\alpha 6X2\beta 1$ exhibited higher affinities toward $\beta 2$ -laminins (*i.e.* laminin-121E8/221E8) than toward $\beta 1$ -laminins (*i.e.* laminin-111E8/211E8), corroborating our hypothesis that X2-type integrins discriminate between $\beta 1$ -laminins and $\beta 2$ -laminins and bind to the latter isoforms with higher affinities.

DISCUSSION

Despite a number of attempts to identify the integrin recognition sites in laminins by molecular dissection using limited

proteolysis, recombinant protein expression, and synthesis of an array of peptides, the mechanism by which laminins bind to integrins remained only poorly understood until recently. This situation has been in striking contrast to the interactions of collagens and Arg-Gly-Asp (RGD)-containing adhesive proteins with their integrin receptors, in which the amino acid residues that directly coordinate the divalent metal ions in the metal ion-dependent adhesion site of integrins, designated MIDAS, have been defined by x-ray crystallography (41, 42). Several lines of evidence indicate that three LG domains, LG1–3, in laminin α chains are prerequisites for the integrin binding activities of laminins (16, 36, 43, 44). However, the α chain monomers alone are functionally inactive (16–19), thereby underscoring the importance of heterotrimerization of the α chains with the β and γ chains for laminin recognition by integrins. Recently, the conserved Glu residue in the C-terminal regions of the $\gamma 1$ and $\gamma 2$ chains was shown to play a crucial role in laminin recognition by integrins (20), and laminin isoforms containing the $\gamma 3$ chain that lacks the conserved Glu residue are unable to bind to integrins (21). Despite the accumulating evidence for roles of

the α and γ chains, it has remained unknown whether the β chains also contribute to laminin recognition by integrins. In this study, we compared the binding activities of $\beta 2$ -laminins (*i.e.* laminin-121, -221, and -521) toward a panel of laminin-binding integrins with those of $\beta 1$ -laminins (*i.e.* laminin-111, -211, and -511). We found that $\beta 2$ -laminins were capable of binding to $\alpha 3\beta 1$ and $\alpha 7X2\beta 1$ integrins, both of which contain X2 exon-derived sequences, with higher affinities than $\beta 1$ -laminins. The other laminin-binding integrins, $\alpha 6\beta 1$, $\alpha 6\beta 4$, and $\alpha 7X1\beta 1$, all of which contain X1 exon-derived sequences, did not exhibit any preference for $\beta 2$ -laminins over $\beta 1$ -laminins, indicating that only X2-type integrins discriminate between the $\beta 1$ and $\beta 2$ chains. In support of our conclusion, a constructed integrin containing an X2-type $\alpha 6$ subunit, $\alpha 6X2\beta 1$, was capable of recognizing the difference in the β chains of laminins and bound with higher affinities to $\beta 2$ -laminins than to $\beta 1$ -laminins. We mapped the region within the laminin β chains responsible for the affinity modulation toward X2-type integrins to the C-terminal 20 amino acid residues, demonstrating that not only the C-terminal regions of the α and γ chains (*i.e.* the LG1–3 domains in α chains and the Glu

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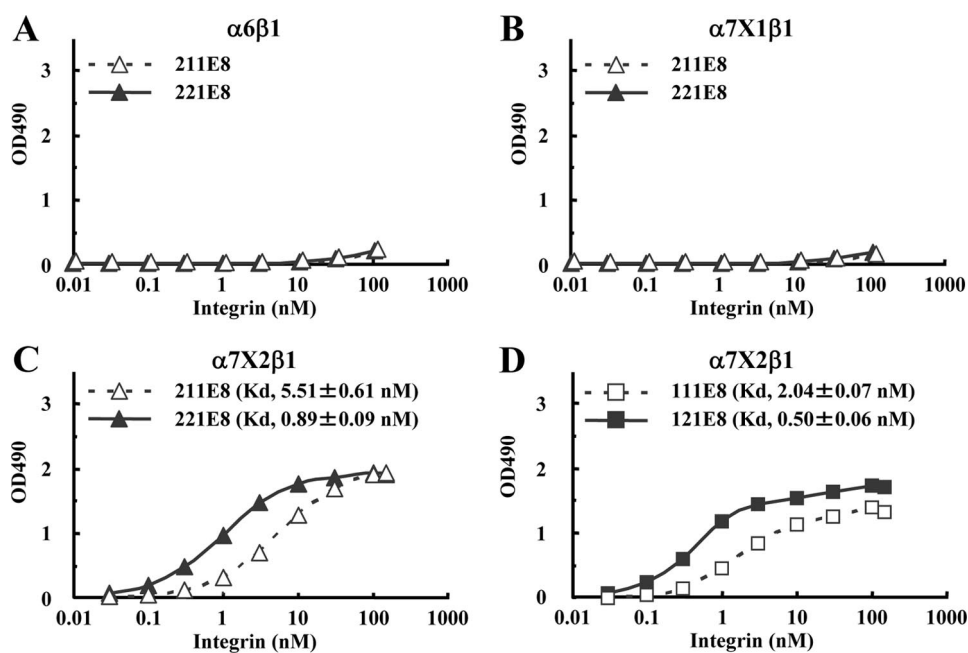


FIGURE 6. Titration curves of recombinant integrins bound to laminin-111E8/121E8 and laminin-211E8/221E8. A–D, increasing concentrations of recombinant $\alpha 6\beta 1$ (A), $\alpha 7X1\beta 1$ (B), and $\alpha 7X2\beta 1$ (C and D) integrins were allowed to bind to microtiter plates coated with laminin-211E8 (open triangles) and -221E8 (closed triangles) (A–C) and laminin-111E8 (open squares) and -121E8 (closed squares) (D) in the presence of 1 mM MnCl₂. The amounts of the integrins bound in the presence of 10 mM EDTA were taken as nonspecific binding and subtracted as background. The results shown are the means of duplicate determinations. The apparent dissociation constants are expressed as the means \pm S.D. of three independent experiments.

models for the β -propeller domains of the integrin $\alpha 7X1$ and $\alpha 7X2$ subunits by molecular dynamics simulations based on the crystal structure of $\alpha v\beta 3$ integrin. They showed that clusters of acidic residues as well as solvent-exposed hydrophobic residues within the X1 and X2 regions involved in defining the ligand-binding specificities of $\alpha 7\beta 1$ integrin are located at the periphery of the β -propeller domain of the $\alpha 7$ subunit, distant from the I-like domain of the integrin β subunit, which is closely associated with the β -propeller domain. Given that the solvent-exposed hydrophobic residues (e.g. Tyr²⁰⁸ in the $\alpha 7X2$ subunit) are at equivalent positions to Tyr²⁰⁸ in the β -propeller domain of the integrin $\alpha 5$ subunit, which has been shown to interact with the so-called “synergy site” of fibronectin (15, 49), it is tempting to speculate that the X2 region interacts directly with laminins at the C-terminal

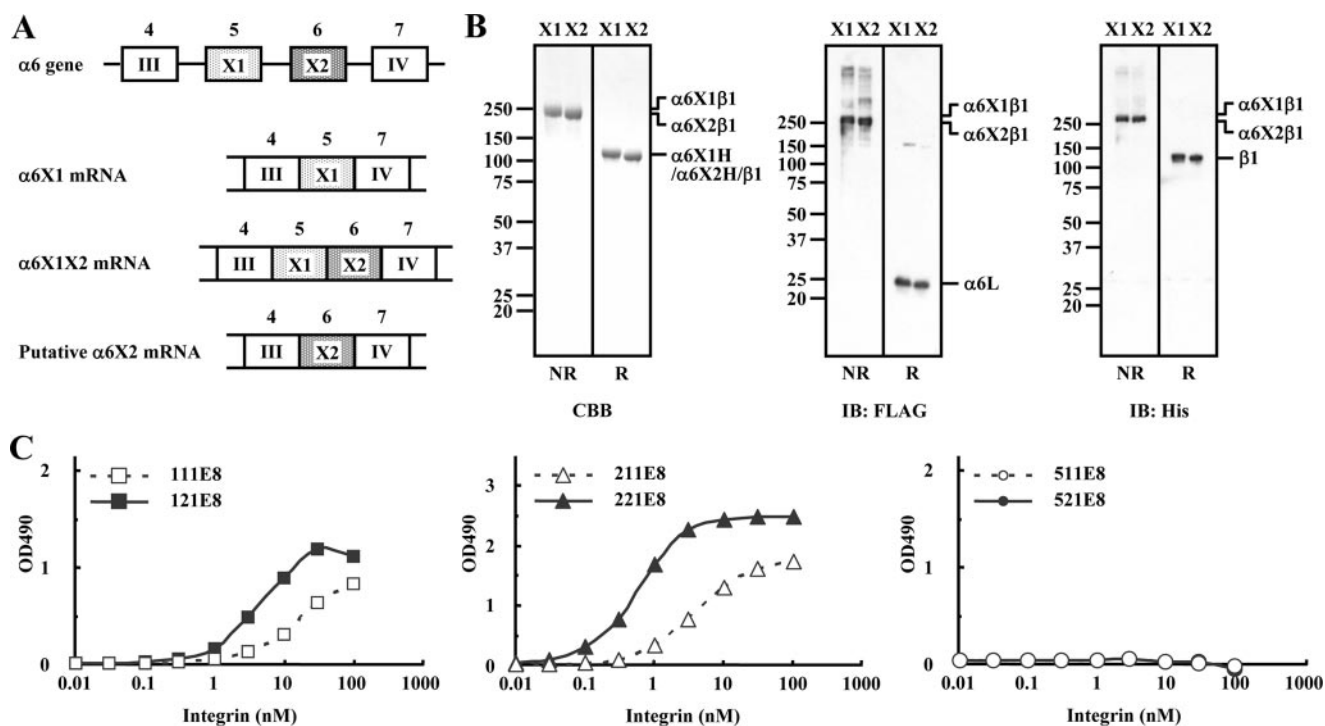


FIGURE 7. Ligand-binding specificities of recombinant $\alpha 6X2\beta 1$ integrin. A, schematic diagrams of the exon/intron structures of the part of the human integrin $\alpha 6$ gene encompassing domains 4–7 and the RNA transcripts arising from alternative splicing at the X1/X2 exons. Exons 4–7 (open boxes or dotted boxes) encode the blade III, X1, X2, and blade IV regions, respectively. B, recombinant $\alpha 6X1\beta 1$ and $\alpha 6X2\beta 1$ integrins (labeled X1 and X2, respectively) were subjected to SDS-PAGE in 5–20% gradient gels under nonreducing (NR) and reducing (R) conditions, followed by Coomassie Brilliant Blue (CBB) staining (left panel) or immunoblotting with antibodies against the FLAG tag (for detection of the $\alpha 6$ light chain; middle panel) and His₅ tag (for detection of the $\beta 1$ subunit; right panel). The positions of molecular size markers are shown in the left margin. C, titration curves of recombinant $\alpha 6X2\beta 1$ integrin bound to laminin-111E8/121E8, -211E8/221E8, and -511E8/521E8. Increasing concentrations of recombinant $\alpha 6X2\beta 1$ integrins were allowed to bind to microtiter plates coated with laminin-111E8 (open squares), laminin-121E8 (closed squares), laminin-211E8 (open triangles), laminin-221E8 (closed triangles), laminin-511E8 (open circles), or laminin-521E8 (closed circles) in the presence of 1 mM MnCl₂. The amounts of the integrins bound in the presence of 10 mM EDTA were taken as nonspecific binding and subtracted as background. The results shown are the means of duplicate determinations.

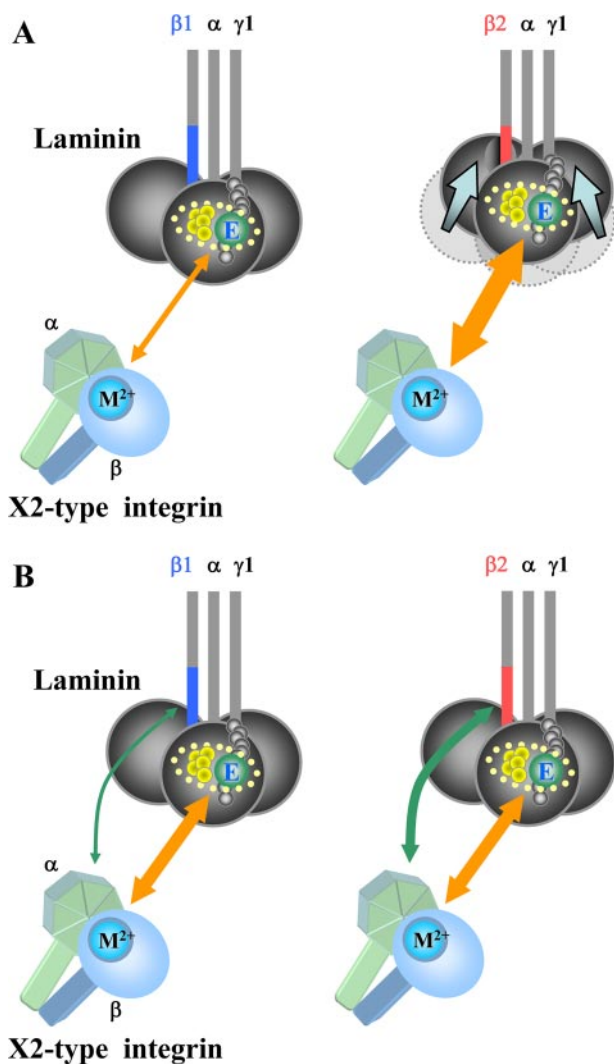


FIGURE 8. Models for the mechanism by which the laminin β_2 chain potentiates the binding affinity toward X2-type laminin-binding integrins. A, interaction between the C-terminal region of the β_2 chain and the LG1–3 modules of the α chain fine-tunes the conformation of LG1–3, which are assembled into a cloverleaf shape, and thereby up-regulates the binding affinities of β_2 -laminins for X2-type laminin-binding integrins. The items encircled with dotted lines are the putative integrin-binding site comprised of the glutamic acid (E) residue in the C-terminal region of the γ_1 chain (green) and undefined residues within the LG1–3 modules of the α chain (yellow), the former coordinating the divalent metal ion (M^{2+}) in the MIDAS motif of the integrin β subunit, whereas the latter possibly interacts with the β -propeller domain of the integrin α subunit. The differences in the affinities between the putative integrin-binding site and X2-type integrins are reflected by the width of the orange two-way arrows connecting them. B, C-terminal region of the laminin β_2 chain interacts directly with X2-type integrins and thereby potentiates the binding affinities of β_2 -laminins for X2-type laminin-binding integrins. The C-terminal region of the β_1 chain may also interact with X2-type integrins with significantly lower affinities than those of the β_2 chain. The width of the green two-way arrows reflects the strength of the interaction between the C-terminal region of the β chains and X2-type integrins. The orange two-way arrows refer to the interaction between the integrin and the putative integrin-binding site of laminin comprised of the LG1–3 modules of the α chain and the C-terminal tail of the γ_1 chain.

region of the β_2 chain, in a similar manner to the interaction of $\alpha_5\beta_1$ integrin with the synergy site of fibronectin, thereby potentiating the binding affinities of X2-type integrins toward β_2 -laminins.

It is interesting to note that the proposed mechanisms by which the β chains modulate the binding affinities toward

X2-type integrins are analogous to those proposed for the synergy site of fibronectin to secure the specific high affinity binding of fibronectin to $\alpha_5\beta_1$ integrin. Although the RGD motif in the III-10 module is a prerequisite for fibronectin to bind to $\alpha_5\beta_1$ integrin, the synergy site in the III-9 module, which is defined by the Pro-His-Ser-Arg-Asn sequence, is required for the high affinity binding of fibronectin to $\alpha_5\beta_1$ integrin (50). There is evidence that the synergy site in the III-9 module modulates the conformation of the cell-binding domain, including III-10 toward $\alpha_5\beta_1$ integrin (51, 52), representing an analogous scenario to our first model depicted in Fig. 8A. On the other hand, Mould *et al.* (49) provided evidence that the III-9 module interacts with the β -propeller domain of the integrin α_5 subunit, consistent with our second model (Fig. 8B). Currently, there is no conclusive evidence available for either of the models shown in Fig. 8. However, determination of the three-dimensional structures of β_2 -laminins or their E8 fragments either alone or as complexes with X2-type integrins will provide definite evidence for the mechanism by which the C-terminal region of the β_2 chain potentiates the interactions of β_2 -laminins with X2-type integrins.

There is accumulating evidence that the X1/X2 regions define the ligand specificities of $\alpha_7\beta_1$ integrins. It has been shown that $\alpha_7X_1\beta_1$ integrin binds more avidly to laminin-511 and -411 than to laminin-111 and -211, whereas $\alpha_7X_2\beta_1$ integrin binds to laminin-111 and -211/221 with higher affinities than to laminin-511 and -411 (Refs. 14, 15 and this study). Notably, the X1- and X2-type $\alpha_6\beta_1$ integrins exhibited similar ligand-binding specificities to those of $\alpha_7X_1\beta_1$ and $\alpha_7X_2\beta_1$ integrins. Specifically, the $\alpha_6\beta_1$ integrin containing the X1-derived sequence bound more avidly to laminin-511/521 than to laminin-111, whereas $\alpha_6X_2\beta_1$, a constructed $\alpha_6\beta_1$ integrin containing the X2-derived sequence, was capable of binding to laminin-211/221 and -111/121 but not to laminin-511/521. Consistent with the similarities in their ligand specificities, the α_6 and α_7 subunits exhibit the highest sequence homology among the integrin α subunits (12). In contrast, $\alpha_3\beta_1$ integrin, the third X2-type laminin-binding integrin, exhibits ligand specificities that are clearly distinct from those of $\alpha_7X_2\beta_1$ and $\alpha_6X_2\beta_1$, and it is capable of binding to laminin-511/521 with the highest affinity but incapable of binding to laminin-111/121. Multiple alignment of the X2 regions of the integrin α_3 , α_6 , and α_7 subunits reveals that the X2 region of the α_3 subunit is shorter than those of the α_6 and α_7 subunits and differs significantly in its amino acid sequence (Fig. 9). The clear distinction in the ligand specificities between $\alpha_3\beta_1$ and the X2-type $\alpha_6\beta_1$ and $\alpha_7\beta_1$ integrins may therefore reflect, at least partially, the differences in the lengths and amino acid sequences of their X2 regions. Despite such differences, the X2 regions of the three subunits share the $DX_{(4-5)}Y(K/R)$ motif (15) (Fig. 9), which is not found in the X1 region of the α_6 and α_7 subunits. Because the tyrosine residue conserved in the consensus motif is equivalent in position to Tyr²⁰⁸ of the integrin α_5 subunit, which has been shown to interact with the synergy site of fibronectin (15, 49), this motif may be involved in the recognition of the laminin β_2 chain by X2-type integrins.

Our solid-phase binding assays showed that α_2 -containing laminins hardly bound to $\alpha_6\beta_1$ and $\alpha_7X_1\beta_1$ integrins. These

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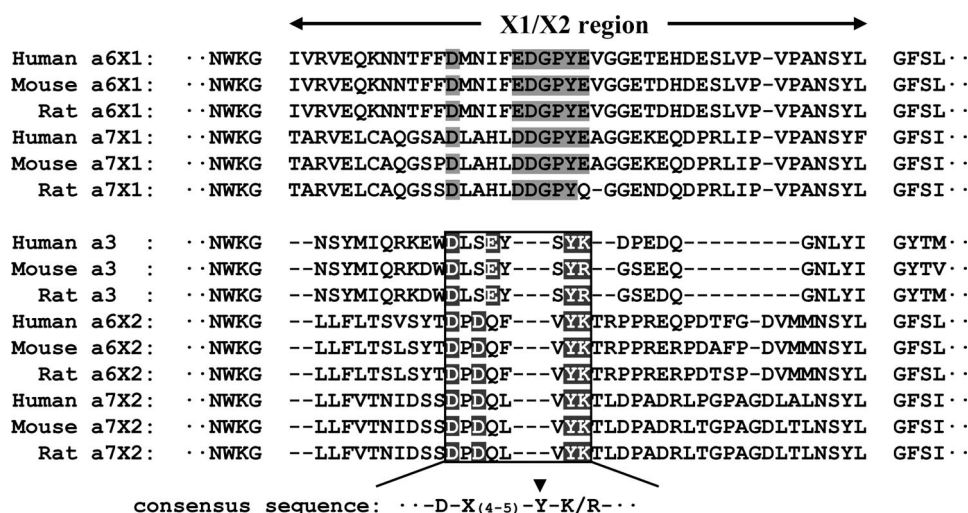


FIGURE 9. Multiple sequence alignment of the variable regions of the integrin $\alpha 3$, $\alpha 6$, and $\alpha 7$ subunits. The amino acid sequences of the X1 and X2 regions of the integrin $\alpha 3$, $\alpha 6$, and $\alpha 7$ subunits of different vertebrate species were aligned using ClustalX (35). The highly conserved sequence in the variable region of the X2-type integrins is boxed. The consensus residues in the conserved sequence are highlighted in black. The conserved sequence in the X1 region that corresponds to the consensus sequence in the X2 region is shaded. The arrowhead indicates the tyrosine residue equivalent to Tyr²⁰⁸ in the integrin $\alpha 5$ subunit.

results are apparently in conflict with our previous results that these integrins are capable of binding to laminin-211/221 with moderate affinities (14). In our previous study, we extracted laminin-211/221 from human placenta with EDTA followed by purification by immunoaffinity chromatography using antibodies against the laminin $\alpha 2$ chain. The resulting $\alpha 2$ chain laminins were found to contain small amounts of other laminin isoforms, including laminin-511/521,⁴ which may partially override the intrinsic integrin binding activities of laminin-211/221 and thereby result in moderate binding of the placental laminin-211/221 to $\alpha 6\beta 1$ and $\alpha 7X1\beta 1$ integrins.

The binding of X2-type integrins to $\beta 2$ -laminins with higher affinities than to $\beta 1$ -laminins raises a question as to the physiological relevance of these findings. The laminin $\beta 2$ chain has been shown to be dominantly expressed in the basement membranes of kidney glomeruli and neuromuscular junctions (26, 28, 29), where ablation of the $\beta 2$ chain leads to aberrant neuromuscular junctions and dysfunction of glomerular basement membranes (27, 30). Although expression of the $\beta 1$ chain was up-regulated in the basement membranes of $\beta 2$ knock-out mice to compensate for the absence of the $\beta 2$ chain, aberrant phenotypes persisted in the mice (27, 28), suggesting that the functions of $\beta 2$ -laminins cannot simply be compensated by $\beta 1$ -laminins (53). Consistent with the expression patterns of $\beta 2$ -laminins in the kidney glomeruli, podocytes and mesangial cells express $\alpha 3\beta 1$ integrin, one of the X2-type integrins, as their major laminin-binding integrin for adherence to glomerular basement membranes (10, 54). In neuromuscular junctions, $\alpha 7\beta 1$ is the major integrin type (55), although it remains unclear which type of $\alpha 7$ subunit, $\alpha 7X1$ or $\alpha 7X2$, is expressed in the cells at these junctions. Because the $\alpha 7X2$ subunit is the major integrin α subunit expressed in adult skeletal muscle (12), it seems likely that $\alpha 7X2\beta 1$ will be mainly expressed in cells at neuromuscular junctions. Given the close association of

⁴ Y. Taniguchi, unpublished data.

the expression of X2-type integrins with cells adhering to basement membranes dominated by $\beta 2$ -laminins, the high affinity interactions between X2-type integrins and $\beta 2$ -laminins may contribute to the maintenance of the structural and/or functional integrities of the junctions between apposed cells, where the deficiency of $\beta 2$ -laminins cannot be compensated for by up-regulation of $\beta 1$ -laminins.

In conclusion, we have provided evidence that $\beta 2$ -laminins bind with higher affinities to X2-type integrins than $\beta 1$ -laminins, whereas $\beta 1$ -laminins and $\beta 2$ -laminins bind to X1-type integrins with similar affinities. The region responsible for the enhanced binding of $\beta 2$ -laminins to X2-type integrins has been mapped to the C-terminal 20 amino

acid residues of the $\beta 2$ chain, highlighting the auxiliary role of the C-terminal region of the β chains in the modulation of the integrin-binding affinities of laminins. The fact that the regions critically involved in integrin binding are all localized at the interface between the heterotrimeric coiled-coil domains and the LG1–3 modules led us to propose two alternative models for the β chain-dependent modulation of laminin-integrin interactions, wherein integrins recognize multiple sites at the interface, including the Glu residue in the C-terminal tail of the γ chains, the ill-defined residues within the LG1–3 modules and the C-terminal 20 amino acid residues of the β chains, although it remains to be explored whether the C-terminal residues of the β chains interact directly with integrins. Further studies, including the determination of the three-dimensional structures of laminins and their E8 fragments, are awaited to better understand the mechanistic basis of the recognition of laminins in basement membranes by integrins.

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REFERENCES

1. Tunggal, P., Smyth, N., Paulsson, M., and Ott, M. C. (2000) *Microsc. Res. Tech.* **51**, 214–227
2. Colognato, H., and Yurchenco, P. D. (2000) *Dev. Dyn.* **218**, 213–234
3. Miner, J. H., and Yurchenco, P. D. (2004) *Annu. Rev. Cell Dev. Biol.* **20**, 255–284
4. Smyth, N., Vatansever, H. S., Murray, P., Meyer, M., Frie, C., Paulsson, M., and Edgar, D. (1999) *J. Cell Biol.* **144**, 151–160
5. Miner, J. H., Cunningham, J., and Sanes, J. R. (1998) *J. Cell Biol.* **143**, 1713–1723

6. Nguyen, N. M., Kelley, D. G., Schlueter, J. A., Meyer, M. J., Senior, R. M., and Miner, J. H. (2005) *Dev. Biol.* **282**, 111–125
7. Miner, J. H., and Li, C. (2000) *Dev. Biol.* **217**, 278–289
8. Miyagoe, Y., Hanaoka, K., Nonaka, I., Hayasaka, M., Nabeshima, Y., Arahata, K., and Takeda, S. (1997) *FEBS Lett.* **415**, 33–39
9. Kuang, W., Xu, H., Vachon, P. H., and Engvall, E. (1998) *Exp. Cell Res.* **241**, 117–125
10. Kikkawa, Y., Virtanen, I., and Miner, J. H. (2003) *J. Cell Biol.* **161**, 187–196
11. Humphries, M. J. (2002) *Arthritis Res.* **4**, S69–S78
12. Ziober, B. L., Vu, M. P., Waleh, N., Crawford, J., Lin, C. S., and Kramer, R. H. (1993) *J. Biol. Chem.* **268**, 26773–26783
13. Belkin, A. M., and Stepp, M. A. (2000) *Microsc. Res. Tech.* **51**, 280–301
14. Nishiuchi, R., Takagi, J., Hayashi, M., Ido, H., Yagi, Y., Sanzen, N., Tsuji, T., Yamada, M., and Sekiguchi, K. (2006) *Matrix Biol.* **25**, 189–197
15. von der Mark, H., Poschl, E., Lanig, H., Sasaki, T., Deutzman, R., and von der Mark, K. (2007) *J. Mol. Biol.* **371**, 1188–1203
16. Ido, H., Harada, K., Futaki, S., Hayashi, Y., Nishiuchi, R., Natsuka, Y., Li, S., Wada, Y., Combs, A. C., Ervasti, J. M., and Sekiguchi, K. (2004) *J. Biol. Chem.* **279**, 10946–10954
17. Kunneken, K., Pohlentz, G., Schmidt-Hederich, A., Odenthal, U., Smyth, N., Peter-Katalinic, J., Bruckner, P., and Eble, J. A. (2004) *J. Biol. Chem.* **279**, 5184–5193
18. Deutzmann, R., Aumailley, M., Wiedemann, H., Pysny, W., Timpl, R., and Edgar, D. (1990) *Eur. J. Biochem.* **191**, 513–522
19. Sung, U., O'Rear, J. J., and Yurchenco, P. D. (1993) *J. Cell Biol.* **123**, 1255–1268
20. Ido, H., Nakamura, A., Kobayashi, R., Ito, S., Li, S., Futaki, S., and Sekiguchi, K. (2007) *J. Biol. Chem.* **282**, 11144–11154
21. Ido, H., Ito, S., Taniguchi, Y., Hayashi, M., Sato-Nishiuchi, R., Sanzen, N., Hayashi, Y., Futaki, S., and Sekiguchi, K. (2008) *J. Biol. Chem.* **283**, 28149–28157
22. Hunter, D. D., Shah, V., Merlie, J. P., and Sanes, J. R. (1989) *Nature* **338**, 229–234
23. Champlaud, M. F., Virtanen, I., Tiger, C. F., Korhonen, M., Burgeson, R., and Gullberg, D. (2000) *Exp. Cell Res.* **259**, 326–335
24. Brown, J. C., Wiedemann, H., and Timpl, R. (1994) *J. Cell Sci.* **107**, 329–338
25. Champlaud, M. F., Lunstrum, G. P., Rousselle, P., Nishiyama, T., Keene, D. R., and Burgeson, R. E. (1996) *J. Cell Biol.* **132**, 1189–1198
26. Miner, J. H., Patton, B. L., Lentz, S. I., Gilbert, D. J., Snider, W. D., Jenkins, N. A., Copeland, N. G., and Sanes, J. R. (1997) *J. Cell Biol.* **137**, 685–701
27. Noakes, P. G., Miner, J. H., Gautam, M., Cunningham, J. M., Sanes, J. R., and Merlie, J. P. (1995) *Nat. Genet.* **10**, 400–406
28. Patton, B. L., Miner, J. H., Chiu, A. Y., and Sanes, J. R. (1997) *J. Cell Biol.* **139**, 1507–1521
29. Sasaki, T., Mann, K., Miner, J. H., Miosge, N., and Timpl, R. (2002) *Eur. J. Biochem.* **269**, 431–442
30. Noakes, P. G., Gautam, M., Mudd, J., Sanes, J. R., and Merlie, J. P. (1995) *Nature* **374**, 258–262
31. Zenker, M., Aigner, T., Wendler, O., Tralau, T., Muntefering, H., Fenski, R., Pitz, S., Schumacher, V., Royer-Pokora, B., Wuhl, E., Cochat, P., Bouvier, R., Kraus, C., Mark, K., Madlon, H., Dotsch, J., Rascher, W., Maruniak-Chudek, I., Lennert, T., Neumann, L. M., and Reis, A. (2004) *Hum. Mol. Genet.* **13**, 2625–2632
32. Nishimune, H., Sanes, J. R., and Carlson, S. S. (2004) *Nature* **432**, 580–587
33. Fujiwara, H., Kikkawa, Y., Sanzen, N., and Sekiguchi, K. (2001) *J. Biol. Chem.* **276**, 17550–17558
34. Hayashi, Y., Kim, K. H., Fujiwara, H., Shimono, C., Yamashita, M., Sanzen, N., Futaki, S., and Sekiguchi, K. (2002) *Biochem. Biophys. Res. Commun.* **299**, 498–504
35. Thompson, J. D., Gibson, T. J., Plewniak, F., Jeanmougin, F., and Higgins, D. G. (1997) *Nucleic Acids Res.* **25**, 4876–4882
36. Ido, H., Harada, K., Yagi, Y., and Sekiguchi, K. (2006) *Matrix Biol.* **25**, 112–117
37. Takagi, J., Erickson, H. P., and Springer, T. A. (2001) *Nat. Struct. Biol.* **8**, 412–416
38. Delwel, G. O., Kuikman, I., and Sonnenberg, A. (1995) *Cell Adhes. Commun.* **3**, 143–161
39. Laemmli, U. K. (1970) *Nature* **227**, 680–685
40. Nishiuchi, R., Murayama, O., Fujiwara, H., Gu, J., Kawakami, T., Aimoto, S., Wada, Y., and Sekiguchi, K. (2003) *J. Biochem. (Tokyo)* **134**, 497–504
41. Emsley, J., Knight, C. G., Farnedale, R. W., Barnes, M. J., and Liddington, R. C. (2000) *Cell* **101**, 47–56
42. Xiong, J. P., Stehle, T., Zhang, R., Joachimiak, A., Frech, M., Goodman, S. L., and Arnaout, M. A. (2002) *Science* **296**, 151–155
43. Hirotsaki, T., Mizushima, H., Tsubota, Y., Moriyama, K., and Miyazaki, K. (2000) *J. Biol. Chem.* **275**, 22495–22502
44. Kikkawa, Y., Sasaki, T., Nguyen, M. T., Nomizu, M., Mitaka, T., and Miner, J. H. (2007) *J. Biol. Chem.* **282**, 14853–14860
45. Timpl, R., Tisi, D., Talts, J. F., Andac, Z., Sasaki, T., and Hohenester, E. (2000) *Matrix Biol.* **19**, 309–317
46. Lupas, A., Van Dyke, M., and Stock, J. (1991) *Science* **252**, 1162–1164
47. Wolf, E., Kim, P. S., and Berger, B. (1997) *Protein Sci.* **6**, 1179–1189
48. Delorenzi, M., and Speed, T. (2002) *Bioinformatics (Oxf.)* **18**, 617–625
49. Mould, A. P., Symonds, E. J., Buckley, P. A., Grossmann, J. G., McEwan, P. A., Barton, S. J., Askari, J. A., Craig, S. E., Bella, J., and Humphries, M. J. (2003) *J. Biol. Chem.* **278**, 39993–39999
50. Aota, S., Nomizu, M., and Yamada, K. M. (1994) *J. Biol. Chem.* **269**, 24756–24761
51. Altroff, H., Choulier, L., and Mardon, H. J. (2003) *J. Biol. Chem.* **278**, 491–497
52. Takagi, J., Strokovich, K., Springer, T. A., and Walz, T. (2003) *EMBO J.* **22**, 4607–4615
53. Miner, J. H., Go, G., Cunningham, J., Patton, B. L., and Jarad, G. (2006) *Development (Camb.)* **133**, 967–975
54. Sterk, L. M., de Melker, A. A., Kramer, D., Kuikman, I., Chand, A., Claessen, N., Weening, J. J., and Sonnenberg, A. (1998) *Cell Adhes. Commun.* **5**, 177–192
55. Martin, P. T., Kaufman, S. J., Kramer, R. H., and Sanes, J. R. (1996) *Dev. Biol.* **174**, 125–139
56. Aumailley, M., Bruckner-Tuderman, L., Carter, W. G., Deutzmann, R., Edgar, D., Ekblom, P., Engel, J., Engvall, E., Hohenester, E., Jones, J. C., Kleinman, H. K., Marinkovich, M. P., Martin, G. R., Mayer, U., Meneguzzi, G., Miner, J. H., Miyazaki, K., Patarroyo, M., Paulsson, M., Quaranta, V., Sanes, J. R., Sasaki, T., Sekiguchi, K., Sorokin, L. M., Talts, J. F., Tryggvason, K., Uitto, J., Virtanen, I., von der Mark, K., Wewer, U. M., Yamada, Y., and Yurchenco, P. D. (2005) *Matrix Biol.* **24**, 326–332