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Laminin Isoforms Containing the γ3 Chain Are Unable to Bind to Integrins due to the Absence of the Glutamic Acid Residue Conserved in the C-terminal Regions of the γ1 and γ2 Chains*[S]

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Laminins are the major cell adhesive proteins in basement membranes, and consist of three subunits termed α, β, and γ. Recently, we found that the Glu residue at the third position from the C termini of the γ1 and γ2 chains is critically involved in integrin binding by laminins. However, the γ3 chain lacks this Glu residue, suggesting that laminin isoforms containing the γ3 chain may be unable to bind to integrins. To address this possibility, we expressed the E8 fragment of laminin-213 and found that it was incapable of binding to integrins. Similarly, the E8 fragment of laminin-113 was expressed and also found to be inactive in binding to integrins, confirming the distinction between the integrin binding activities of γ3 chain-containing isoforms and those containing the γ1 or γ2 chain. To further address the importance of the Glu residue, we swapped the C-terminal four amino acids of the γ3 chain with the C-terminal nine amino acids of the γ1 chain, which contain the Glu residue. The resulting chimeric E8 fragment of laminin-213 became fully active in integrin binding, whereas replacement with the nine amino acids of the γ1 chain after substitution of Gln for the conserved Glu residue failed to restore the integrin binding activity. These results provide both loss-of-function and gain-of-function evidence that laminin isoforms containing the γ3 chain are unable to bind to integrins due to the absence of the conserved Glu residue, which should play a critical role in integrin binding by laminins.

Laminins are heterotrimeric glycoproteins found in basement membranes and consist of three covalently linked chains termed α, β, and γ. There are five α chains (α1–α5), three β chains (β1–β3), and three γ chains (γ1–γ3) that can give rise to at least 15 different functional laminin isoforms (1–3). These isoforms have been implicated in a wide variety of biological processes involving cell-basement membrane interactions through binding to cell surface receptors including integrins, syndecans, and dystroglycan (1, 4–9).

Integrins are αβ transmembrane receptors that play critical roles in cell matrix adhesion in multicellular organisms. Several members of the integrin family proteins, including α3β1, α6β1, α6β4, and α7β1, serve as laminin receptors on a variety of cell types (10). The putative binding sites for these integrins have been mapped to the globular (G)[3] domain of the laminin α chains (11–16), although trimerization with β and γ chains is necessary for the G domain to exert its integrin binding activity (17–19). Recently, we found that the C-terminal regions of the γ chains are critically involved in integrin binding by laminins (20). Briefly, deletion of the C-terminal three but not two amino acids of the γ1 chain completely abrogated the integrin binding activity of laminin-511 (α5β1γ1), while 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; thereby underscoring a critical role of Glu-1607 in integrin binding by this laminin. Furthermore, a Glu residue is conserved between the γ1 and γ2 chains at the third position from the C terminus. Deletion of the C-terminal three amino acids from the γ2 chain or substitution of Gln for this Glu in the γ2 chain completely abrogated the integrin binding activity of laminin-332 (α3β3γ2), suggesting that the same mechanism operates in the modulation of the integrin binding activities of laminins containing either the γ1 or γ2 chain.

A novel γ chain isoform, γ3, is the eleventh laminin subunit to be identified (21–23). Studies on the tissue distribution of the γ3 chain have shown that it is broadly expressed in the skin, kidney, retina, and testis (21, 22, 24–26). It has been reported that the γ3 chain associates with the α2 and β1 chains to form laminin-213 (α2β1γ3) in the placenta (21) and with the α3 and β3 chains to form laminin-333 (α3β3γ3) in adult rat testes (27). The predicted primary and secondary structures of the γ3 chain suggest that it is more closely related to γ1 than to γ2 (21). However, the C-terminal region of the human laminin γ3 chain

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[3] The abbreviations used are: G domain, globular domain; mAb, monoclonal antibody; BSA, bovine serum albumin; LG, laminin G-like.

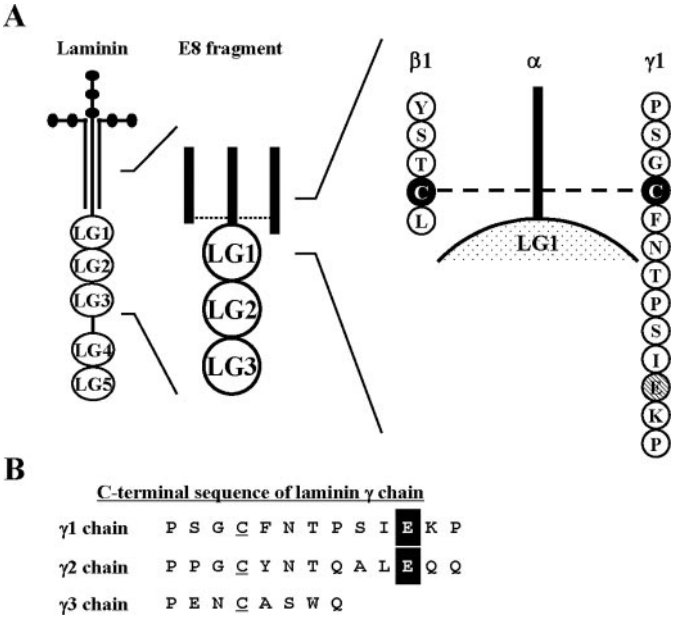

FIGURE 1. **Schematic representations of laminin and the C-terminal amino acid sequences of the human laminin γ1, γ2, and γ3 chains.** *A*, schematic diagrams of recombinant laminin, its E8 fragment and the C-terminal amino acid sequences of its β1 and γ1 chains. Cys residues are *circled* in *black*, and disulfide bonds are depicted by *broken lines*. The C-terminal Glu residue of the γ1 chain is *shaded*. *B*, C-terminal amino acid sequences of the human laminin γ1, γ2, and γ3 chains. Cys residues are *underlined*. The *black boxes* represent the Glu residues conserved between the γ1 and γ2 chains.

consists of only four amino acid residues after the Cys residue conserved among the three γ chains, and lacks the Glu residue conserved in the C-terminal regions of the γ1 and γ2 chains (*see* Fig. 1). The unique features of the γ3 chain, *i.e.* the short C-terminal tail and the absence of the Glu residue, are conserved among the γ3 homologues from humans, mice, rats, and zebrafish. Furthermore, there is no evidence for alternative splicing that confers the critical Glu residue on the C-terminal region of the γ3 chain. The absence of the conserved Glu residue in the γ3 chain raises the possibility that laminin isoforms containing the γ3 chain may be unable to bind to integrins, although the γ3 chain-containing laminins have never been directly examined for their cell adhesive and integrin binding activities.

In the present study, we expressed and purified recombinant laminin isoforms containing the γ3 chain and several mutant γ3 chains, and examined their integrin binding and cell adhesive activities. Our results clearly showed that γ3 chain-containing laminins are devoid of integrin binding and cell adhesive activities, and that the Glu residue at the third position from the C terminus is a prerequisite for laminin recognition by integrins.

EXPERIMENTAL PROCEDURES

Antibodies—Monoclonal antibodies (mAbs) against the human laminin α1 (5A3), α2 (22W2-F9), α4 (2-11H), and α5 (15H5) chains were produced in our laboratory (28, 29). A hybridoma secreting a mAb against the laminin β2 chain (C4), developed by Dr. Joshua Sanes (Washington University School of Medicine, St. Louis, MO), was obtained from the Developmental Studies Hybridoma Bank (University of Iowa, Iowa City, IA). A mAb against the human laminin β1 chain (DG10) was kindly provided by Dr. Ismo Virtanen (University of Helsinki, Helsinki, Finland). A polyclonal antibody against the human laminin α3 chain was produced in our laboratory (30). A polyclonal antibody against the human laminin γ3 chain (C-19) was purchased from Santa Cruz Biotechnology (Santa Cruz, CA). An anti-FLAG mAb was purchased from Sigma. A polyclonal antibody against the ACID/BASE coiled-coil peptides (31, 32) was generously provided by Dr. Junichi Takagi (Institute for Protein Research, Osaka University, Osaka, Japan). Horseradish peroxidase-conjugated streptavidin was purchased from Zymed Laboratories Inc. (San Francisco, CA).

Construction of Expression Vectors—Soluble clasped α6, α7X2, and β1 integrin expression vectors were prepared as described previously (10, 33). Expression vectors for the human laminin α1, α3, α4, α5, β1, β3, and γ1 chains were constructed as described (16, 20, 33, 34). Expression vectors for the human laminin α2 (GenBank™ accession number NM_000426), β2 (GenBank™ accession number NM_002292), and γ3 (GenBank™ accession number NM_006059) chains were prepared as follows. Full-length cDNAs encoding the α2, β2, and γ3 subunits were amplified by reverse transcription-polymerase chain reaction (PCR) as a series of ~1-kb fragments. After sequence verification, error-free cDNA fragments were ligated in tandem, and the resulting cDNAs of the α2, β2, and γ3 chains were inserted into the expression vectors pcDNA3.1 (Invitrogen, Carlsbad, CA) for the α2 and β2 chains and pSecTag2A (Invitrogen) for the γ3 chain. A FLAG tag sequence was added to the N termini of the γ1 and γ3 chains by extension PCR, followed by insertion into the expression vectors.

Expression vectors for the E8 fragments of the β1 and γ1 chains (designated β1E8 and γ1E8, respectively) were prepared as described previously (20). Expression vectors for the E8 fragments of the α1, α2, and γ3 chains (designated α1E8, α2E8, and γ3E8, respectively) and their mutant forms were prepared as follows. cDNAs encoding α1E8 (Phe^{1878}-Gln^{2700}), α2E8 (Leu^{1900}-Ala^{2722}), and γ3E8 (Met^{1345}-Gln^{1587}) were amplified by PCR using α1, α2, and γ3 expression vectors as templates. The His_6 tag (for α1E8 and α2E8) and FLAG tag (for γ3E8) sequences were added by extension PCR with a HindIII site at the 5′-end and an EcoRI site at the 3′-end. The PCR products were digested with HindIII/EcoRI and inserted into the corresponding restriction sites of the expression vector pSecTag2A (Invitrogen). cDNAs encoding γ3E8 mutants were amplified by PCR, and the PCR products were inserted into the γ3E8 expression vector using the following primers: 5′-AAGGACAGTGCCAAGCTTGCCAAGGCC-3′ (forward primer for γ3 + 9AA and γ3 + 9AA(EQ)); 5′-TGCAGAATTCCTAGGGCTTTTCAATGGACGGGGTGTTGAAACAGTTCTCGGGCAG-3′ (reverse primer for γ3 + 9AA) and 5′-TGCAGAATTCCTAGGGCTTCTGAATGGACGGGGTGTTGAAACAGTTCTCGGGCAG-3′ (reverse primer for γ3 + 9AA(EQ)).

Immunoprecipitation and Western Blotting—293-F cells were simultaneously transfected with different combinations of expression vectors encoding various α, β, and γ chains using 293fectin (Invitrogen). At 72 h after transfection, the cells were collected and washed with phosphate-buffered saline. Cell pel-

lets were lysed with SDS sample buffer (50 mM Tris-HCl, pH 6.8, 2% SDS, 10% glycerol, 6% β-mercaptoethanol) and sonicated. The conditioned media were clarified by centrifugation and immunoprecipitated with anti-FLAG M2 agarose (Sigma) for 6 h at 4 °C. Equal amounts of cell lysates and immunoprecipitates were separated by SDS-polyacrylamide gel electrophoresis (PAGE) using 4 or 5% gels under reducing or nonreducing conditions, followed by immunoblotting with mAbs against the FLAG tag or individual laminin subunits. Immunoreactive proteins were detected using Can-Get-Signal Immunoreaction Enhance Solution (Toyobo, Tokyo, Japan) and enhanced chemiluminescence (ECL Plus; GE Healthcare, Piscataway, NJ).

Expression and Purification of Recombinant Proteins—Recombinant laminins were produced using the Free-Style™ 293 Expression System (Invitrogen) as described previously (16). Recombinant α6β1 and α7X2β1 integrins were produced using the same expression system and purified from conditioned media using anti-FLAG columns (Sigma) as described previously (10, 33). Recombinant E8 fragments of laminin-111, -113, -211, and -213, and their mutants were produced using the same expression system and purified from conditioned media as described previously (20). After dialysis against Tris-buffered saline (TBS; 50 mM Tris-HCl, pH 7.4, 150 mM NaCl), the purities of the recombinant proteins were verified by SDS-PAGE followed by Coomassie Brilliant Blue staining or immunoblotting.

Integrin Binding Assays—Solid-phase integrin binding assays of recombinant E8 fragments and their mutants were performed using purified recombinant α6β1 and α7X2β1 integrins (10, 33). Briefly, 96-well microtiter plates were coated with recombinant E8 fragments of laminin-111, -113, -211, and -213 and their mutants at the indicated concentrations. The amounts of the recombinant proteins adsorbed on the plates were quantified with an anti-His_6 mAb to confirm the presence of equal amounts of the adsorbed proteins. After blocking with 3% bovine serum albumin (BSA), the plates were incubated with 20 nM recombinant α6β1 or α7X2β1 integrin in the presence of 5 mM Mn^{2+} at 37 °C for 1 h. After washing with TBS containing 5 mM Mn^{2+} and 0.05% Tween 20, bound proteins were quantified by sequential incubations with the biotinylated anti-ACID/BASE antibody and horseradish peroxidase-conjugated streptavidin.

Cell Adhesion Assay—Cell adhesion assays were performed as described previously (16, 20) using K562 human leukemia cells transfected with a cDNA encoding an α7X2 integrin subunit (35), which were a generous gift from Dr. Arnoud Sonnenberg (The Netherlands Cancer Institute, Amsterdam, The Netherlands). The cells were maintained in RPMI1640 supplemented with 10% fetal bovine serum and used without Mn^{2+} pretreatment. After fixation with formaldehyde and 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

Production of Recombinant Laminins Containing the γ3 Chain—The γ3 chain was originally identified in heterotrimers with the α2 and β1 chains, *i.e.* laminin-213 (21). To elucidate the role of the γ3 chain in integrin binding by laminins, we first attempted to express recombinant laminin-213 by cotransfecting 293-F cells with a cDNA encoding the γ3 chain with an N-terminal FLAG-tag and cDNAs encoding the α2 and β1 chains. Secretion of endogenous γ3 chain-containing laminins by 293-F cells was undetectable (data not shown). We also expressed recombinant laminin-211, in which the γ1 chain was N-terminally FLAG-tagged, in 293-F cells as a control. Immunoblotting with an anti-FLAG mAb detected both the γ1 and γ3 chains in cell lysates, whereas only the γ1 chain was detected in the culture medium after immunoprecipitation of the recombinant γ chains with an anti-FLAG antibody (supplemental Fig. S1). Failure of recombinant laminin-213 secretion by cells was also observed after transfection of other cell lines, including JAR human choriocarcinoma cells originating from the placenta, A204 human rhabdomyosarcoma cells originating from skeletal muscle cells and NIH-3T3 cells (data not shown).

Given the scarcity of laminin-213 secreted by transfected 293-F cells, we sought to more comprehensively identify the α and β subunits that could assemble with the γ3 chain to yield αβγ heterotrimers for purification of γ3 chain-containing laminins. We transfected 293-F cells with FLAG-tagged γ1 chain (positive control) or γ3 chain in combination with different α (α1-α5) and β (β1-β3) chains, and analyzed the secreted laminin heterotrimers by immunoprecipitation with an anti-FLAG antibody followed by immunoblotting with antibodies against individual laminin subunit chains.

Immunoblotting with antibodies against α chains demonstrated that laminins containing the γ1 chain were immunoprecipitated from conditioned media with the anti-FLAG antibody irrespective of the types of α and β chains in the combinations, whereas laminins containing the γ3 chain were either only faintly detected after immunoprecipitation (laminins containing the α1, α2, and α4 chains) or undetectable even when 8-fold more culture medium was used for immunoprecipitation (laminins containing the α3 and α5 chains) (Fig. 2*A*). It was noted that the amounts of secreted laminin isoforms containing the β2 and γ3 chains (*i.e.* laminin-123, -223, and -423) were lower than those containing the β1 and γ3 chains (laminin-113, -213, and -413). A similar bias toward β1 chain-containing isoforms was also observed between the isoforms containing the β1/γ1 and β2/γ1 chains, suggesting that the β2-containing laminins were less efficient than the β1-containing laminins in assembly into heterotrimers and/or secretion of the resulting heterotrimers. Although the antibodies against the α3 and α5 chains failed to detect γ3 chain-containing heterotrimers in the immunoprecipitates, this could be due to the limited sensitivities of these antibodies in immunoblotting.

To further explore the secretion of the γ3 chain as laminin-113/123, -213/223, and -413/423 from 293-F cells, we examined the reactivities of the immunoprecipitates toward antibodies against the β1, β2, and γ3 chains (Fig. 2*B*). Laminin-111 and -121 were also immunoprecipitated and subjected to immunoblotting as controls for the expression of laminins containing the β1 and β2 chains. Under reducing conditions, the β1 and β2 chains were both detected in the immunoprecipitates of laminin-113/123, -213/223, and -413/423, although the amounts of both β chains in the precipitates were much lower than those in

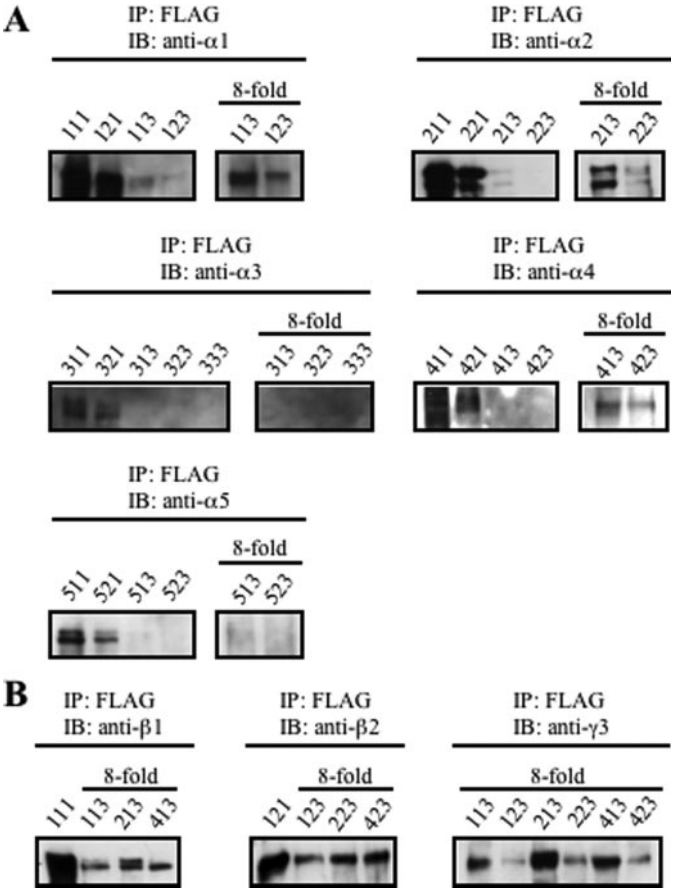

FIGURE 2. **Combinatorial expressions of γ3 chain-containing laminins in 293-F cells.** 293-F cells were transfected with expression vectors for the γ1 or γ3 chain together with various combinations of expression vectors for α and β chains. At 72 h after transfection, conditioned media (1 or 8 ml) were immunoprecipitated with an anti-FLAG M2 antibody. *8-fold* indicates that 8-ml aliquots of conditioned media were immunoprecipitated with the anti-FLAG M2 antibody. Equal amounts of the immunoprecipitates were separated by SDS-PAGE in 4 or 5% gels under reducing conditions, followed by immunoblotting with mAbs against the α1-α5 chains (*A*) and β1, β2, and γ3 chains (*B*).

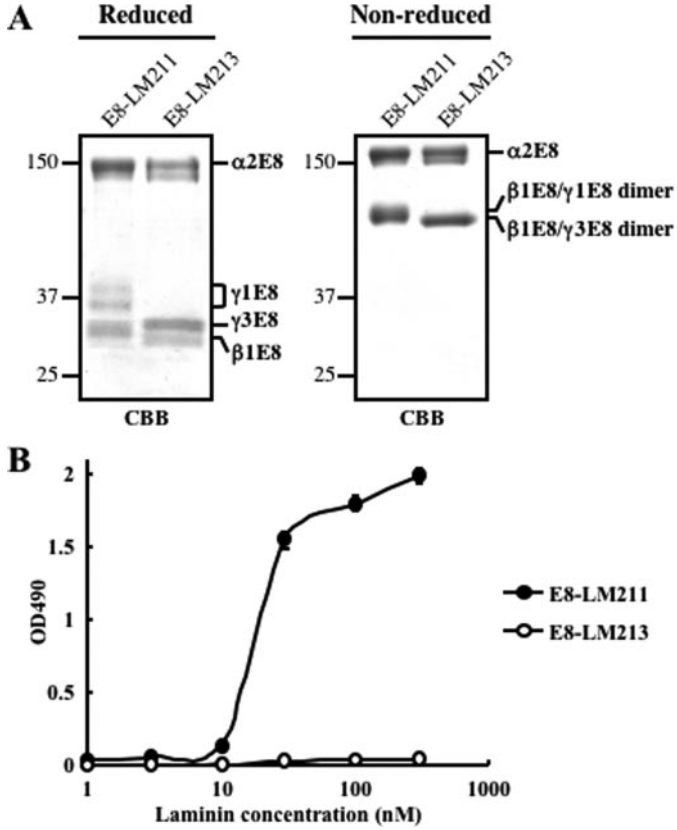

FIGURE 3. **Binding of α7X2β1 integrin to the E8 fragment of laminin-213.** *A*, purified E8 fragments of laminin-211 (E8-LM211) and laminin-213 (E8-LM213) were separated by SDS-PAGE in 12% gels under reducing (*left panel*) and non-reducing (*right panel*) conditions followed by Coomassie Brilliant Blue (*CBB*) staining. The positions of molecular size markers are shown on the *left*. *B*, binding of α7X2β1 integrin to the E8 fragments of laminin-211 and laminin-213. 96-well microtiter plates were coated with increasing concentrations of the E8 fragments of laminin-211 (E8-LM211, positive control) and laminin-213 (E8-LM213), blocked with BSA and incubated with 20 nM α7X2β1 integrin. The amounts of bound α7X2β1 integrin were quantified as described under "Experimental Procedures." Each point represents the mean ± S.D. of triplicate assays.

the precipitates of laminin-111 and -121. Immunoprecipitation of the recombinant γ3 chain was verified by immunoblotting with an anti-γ3 antibody, and showed a bias toward the β1 chain-containing isoforms over the β2 chain-containing laminins, consistent with the observation that laminin isoforms containing the β2 chain were less efficiently secreted by 293-F cells than those containing the β1 chain. Under nonreducing conditions, the γ3 chain was detected at the position of laminin heterotrimers (data not shown), confirming that the γ3 chain was secreted and immunoprecipitated as heterotrimers in combination with the α1/β1, α1/β2, α2/β1, α2/β2, α4/β1, and α4/β2 chains, yielding laminin-113/123, -213/223, and -413/423.

Integrin Binding Activities of the E8 Fragments of Laminin-213 and -113—Because the recombinant γ3 chain-containing laminins were not amenable for purification in sufficient quantities for functional assays, we set out to produce a recombinant E8 fragment of laminin-213 consisting of the three truncated subunits, modeled after the E8 fragment of laminin-111 (17–19). The E8 fragment of laminin-211 was also produced as a control. To obtain the heterotrimeric E8 fragments, the E8 fragments of both laminin-211 and -213 were enriched by sequential affinity purification using a nickel column that captured the truncated α2 chain with an N-terminal His_6 tag and an anti-FLAG column that captured the truncated γ1 and γ3 chains with an N-terminal FLAG tag. The authenticities of the resulting recombinant E8 fragments were verified by SDS-PAGE and Coomassie Brilliant Blue staining. Under reducing conditions, each recombinant protein gave three bands, one corresponding to the truncated α2 chain and two lower bands corresponding to the truncated β1 and γ1 or γ3 chains (Fig. 3*A*). The truncated β1 chain of the E8 fragment of laminin-213 gave a band that migrated slightly faster than the truncated β1 chain of laminin-211, suggesting that the β1 chains were differentially glycosylated or modified by other types of post-translational modifications when assembled with different γ chains. Under nonreducing conditions, the E8 fragments of laminin-211 and -213 gave two bands, one corresponding to the truncated α2 chain and the other corresponding to a heterodimer of the truncated β1/γ1 or β1/γ3 chains. The relative intensities of these two bands were essentially the same for the E8 fragments of laminin-211 and -213. These results indicated that both E8

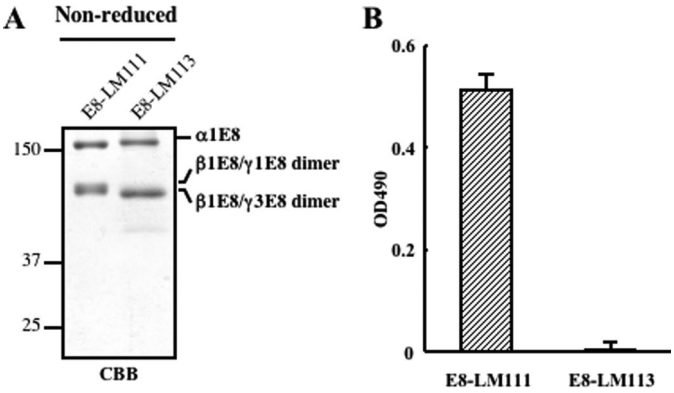

FIGURE 4. **Binding of α6β1 integrin to the E8 fragment of laminin-113.** *A*, purified E8 fragments of laminin-111 (E8-LM111) and laminin-113 (E8-LM113) were separated by SDS-PAGE in 12% gels under non-reducing conditions followed by Coomassie Brilliant Blue (*CBB*) staining. The positions of molecular size markers are shown on the *left*. *B*, binding of α6β1 integrin to the E8 fragments of laminin-111 and laminin-113. 96-well microtiter plates were coated with the E8 fragments of laminin-111 (E8-LM111; positive control) and laminin-113 (E8-LM113) at 100 nM, blocked with BSA, and incubated with 20 nM α6β1 integrin. The amounts of bound α6β1 integrin were quantified as described under "Experimental Procedures." Each *column* and *bar* represents the mean ± S.D. of triplicate assays, respectively.

fragments were purified as heterotrimers, although the truncated α2 chain was not covalently linked to the truncated β1/γ1 and β1/γ3 heterodimers.

The α2 chain-containing laminins including laminin-211 have been shown to be potent ligands for α7X2β1 integrin (10, 36). Therefore, we examined whether the E8 fragment of laminin-213 had α7X2β1 integrin binding activity. Solid-phase binding assays revealed that the E8 fragment of laminin-211 was fully active in binding to α7X2β1 integrin, whereas the E8 fragment of laminin-213 exhibited only marginal activity (Fig. 3*B*). The failure of the E8 fragment of laminin-213 to bind integrins was not unique to α7X2β1 integrin, but reproduced with other laminin-binding integrins, *i.e.* α3β1, α6β1, and α6β4 (data not shown), raising the possibility that laminin-213 and possibly other laminin isoforms containing the γ3 chain are unable to bind to integrins.

To further investigate this possibility, we expressed and purified the E8 fragment of laminin-113 (Fig. 4*A*). The E8 fragment of laminin-111, which is a ligand for α6β1 integrin (10), was also produced as a control. As expected, the E8 fragment of laminin-111 was active in binding to α6β1 integrin, whereas the E8 fragment of laminin-113 was almost devoid of binding activity (Fig. 4*B*). These results are consistent with the conclusion that laminin isoforms containing the γ3 chain are incapable of binding to integrins.

Replacement of the C-terminal Region of the γ3 Chain with the C-terminal Region of the γ1 Chain Restores Integrin Binding Activity to Laminin-213—Because the C-terminal tail of the γ3 chain is truncated and lacks the Glu residue that is conserved between the γ1 and γ2 chains and critical for integrin binding activity, we hypothesized that the γ3 chain-containing laminins would regain integrin binding activities when furnished with a Glu residue at the appropriate position in the γ3 chain. To address this possibility, we produced two mutant forms of the E8 fragment of laminin-213: one mutant containing the γ3 chain in which the C-terminal four amino acids were replaced

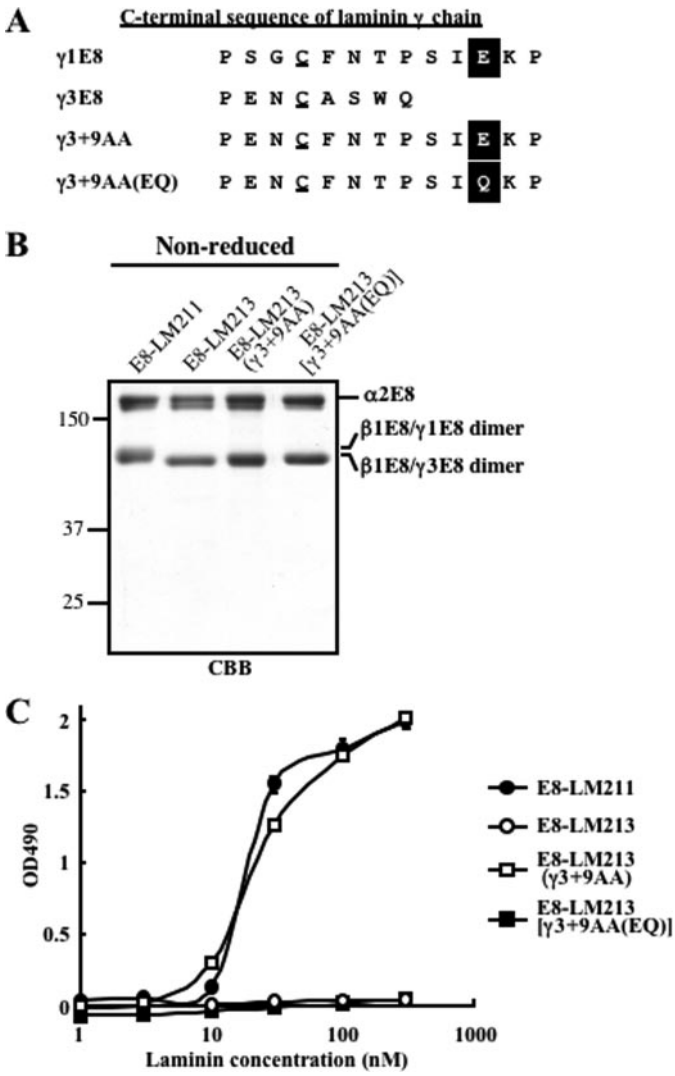

FIGURE 5. **Binding of α7X2β1 integrin to the E8 fragments of laminin-213 mutants.** *A*, C-terminal amino acid sequences of the γ1 and γ3 chains and γ3 mutants with substitutions within the C-terminal tail. The Cys residues are *underlined*. *γ1E8*, control E8 fragment of the γ1 chain; *γ3E8*, E8 fragment of the γ3 chain; *γ3 + 9AA*, E8 fragment of the γ3 chain with the C-terminal nine amino acids of the γ1 chain; *γ3 + 9AA (EQ)*, γ3 + 9AA in which the Glu residue (*E*) is replaced by Gln (*Q*). The *black boxes* represent the conserved Glu residues and the substituted Gln residue. *B*, the E8 fragments of laminin-211 (E8-LM211), laminin-213 (E8-LM213), and the laminin-213 mutant proteins E8-LM213 (γ3 + 9AA) and E8-LM213 (γ3 + 9AA (EQ)) were separated by SDS-PAGE in 12% gels under non-reducing conditions followed by Coomassie Brilliant Blue (*CBB*) staining. The positions of molecular size markers are shown on the *left*. *C*, binding of α7X2β1 integrin to the E8 fragments of laminin-211, laminin-213, and the laminin-213 mutant proteins. 96-well microtiter plates were coated with increasing concentrations of the E8 fragments of laminin-211, laminin-213, and laminin-213 mutant proteins, blocked with BSA, and incubated with 20 nM α7X2β1 integrin. The amounts of bound α7X2β1 integrin were quantified as described under "Experimental Procedures." Each point represents the mean ± S.D. of triplicate assays.

with the C-terminal nine amino acids of the γ1 chain (designated γ3 + 9AA), and another mutant containing a similar γ3 chain to γ3 + 9AA, except that the Glu residue at the third position from the C terminus was replaced with Gln (designated γ3 + 9AA(EQ), Fig. 5*A*). The purities and authenticities of these mutant E8 fragments were verified by SDS-PAGE and Coomassie Brilliant Blue staining of the gels (Fig. 5*B*). The control and mutant E8 fragments of laminin-213 gave two bands

under nonreducing conditions, one corresponding to the truncated α2 chain and another corresponding to a heterodimer of the truncated β1 and γ3 chains. Given that both the β1 and γ3 chains of the E8 fragment contain only one Cys residue conserved near their C termini, these results confirmed that heterodimerization of the β1 and γ3 chains through a disulfide bridge was not compromised by the mutation introduced into the C-terminal region of the γ3 chain.

As expected, solid-phase binding assays revealed that the mutant E8 fragment containing the γ3 + 9AA chain was fully active in binding to α7X2β1 integrin (Fig. 5*C*). The other mutant E8 fragment containing the γ3 + 9AA(EQ) chain did not show any residual integrin binding activity, similar to the case for the control E8 fragment of laminin-213. These results clearly demonstrated that the failure of γ3 chain-containing laminins to bind integrins was due to the absence of the C-terminal Glu residue conserved between the γ1 and γ2 chains, thereby endorsing the importance of this Glu residue for integrin binding by laminins.

Cell-adhesive Activities of Laminin-213 and Its Mutants—The importance of the Glu residue for integrin binding was further verified by cell adhesion assays using K562 cells stably transfected with α7X2 integrin (35). The control E8 fragment of laminin-211 and the E8 fragment of the laminin-213 mutant containing the γ3 + 9AA chain were fully active in mediating cell adhesion of K562 cells expressing α7X2β1 integrin, whereas the E8 fragments of laminin-213 and its mutant containing the γ3 + 9AA(EQ) chain were almost devoid of cell adhesive activity (Fig. 6). Control K562 cells that were not transfected with α7X2 integrin did not adhere to any of the recombinant E8 fragments, confirming the specificity of the α7X2β1 integrin-dependent cell adhesion. These results further supported our conclusion that γ3 chain-containing laminins are unable to bind to integrins due to the absence of the conserved Glu residue at the third position from the C terminus.

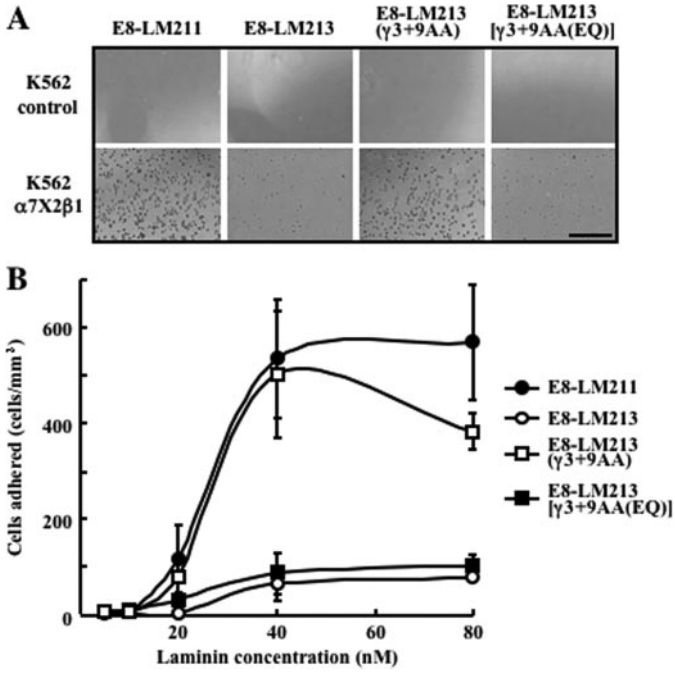

FIGURE 6. **Cell-adhesive activities of the E8 fragments of laminin-211, laminin-213, and mutant laminin-213 proteins.** K562 cells either left untransfected (*control*) or transfected with α7X2 integrin (*α7X2β1*) were incubated at 37 °C for 30 min on 96-well microtiter plates coated with the E8 fragments of laminin-211 (E8-LM211), laminin-213 (E8-LM213), or the mutant laminin-213 proteins E8-LM213 (γ3 + 9AA) and E8-LM213 (γ3 + 9AA(EQ)). Adherent cells were fixed and stained. *A*, representative images of control and α7X2-transfected K562 cells adhering to the substrates. *Bar* = 300 μm. *B*, cells adhering to the substrates were counted. Each point represents the mean ± S.D. of triplicate assays.

DISCUSSION

No previous studies have addressed the functions of γ3 chain-containing laminins at the protein level, other than their immunohistochemical localizations in tissues. Here, we have provided evidence that the γ3 chain-containing laminins, typically laminin-213, are functionally distinct from other laminin isoforms containing the γ1 or γ2 chain, and lack the ability to bind to integrins. Our data have revealed that the failure of the γ3 chain-containing laminins to bind integrins is due to the absence of the Glu residue conserved in the other γ chains at the third position from their C termini, since integrin binding activity was restored to laminin-213 upon substitution of the C-terminal four amino acids of the γ3 chain with the C-terminal nine amino acids of the γ1 chain. Only the intact nine-amino acid sequence, and not that with substitution of Gln for the Glu residue, could fully restore the integrin binding activity to laminin-213, underscoring the importance of the conserved Glu residue in laminin recognition by integrins. To the best of our knowledge, this is the first demonstration of a functional distinction between the γ3 chain-containing laminins and other laminin isoforms.

Despite the importance of the Glu residue at the C-terminal regions of the γ chains for integrin binding by laminins (Ref. 20 and this study), it remains unclear whether this Glu residue is directly involved in laminin recognition by integrins through coordination with the metal ion within the MIDAS (metal ion-dependent adhesion site) motif of laminin-binding integrins, as has been demonstrated for the Glu residue in the GFOG**E**R integrin-binding motif in collagens (37, 38) and the Asp residue in many cell-adhesive proteins containing the RG**D** motif (39–42). Given that not only the Glu residue within the γ1 or γ2 chain but also the laminin G-like (LG) 1–3 modules of the α chain are indispensable for integrin binding by laminins (11–19, 43), the LG1–3 modules, but not the γ chains, may well provide the critical acidic residues that coordinate the metal ion in the MIDAS motif of integrins, while the Glu residue in the γ chains could be required for stabilization of the functionally active conformation of the LG1–3 modules, possibly through direct interaction with the LG1–3 modules. However, our preliminary results indicated that substitution of any of the acidic amino acid residues conserved within the LG1–3 modules of the different laminin α chains with Ala did not compromise the integrin binding activity of the E8 fragment of laminin-511 as severely as Gln substitution for the conserved Glu residue of the γ1 or γ2 chain, unless the Ala substitutions severely impaired the conformational integrity of the LG1–3 modules, as evidenced by significantly reduced reactivities toward mAb 4C7 recognizing the LG1–3 domains of the α5

chain (Refs. 16, 33)[4]; thereby arguing against the possibility that the LG1–3 modules harbor a critical acidic residue that coordinates the metal ion in the MIDAS motif of integrins. Recently, Navdaev *et al.* (44) reported that C-terminal truncation of the γ2 chain induced opening of the compact supradomain assembly of the LG1–3 modules of the E8 fragment of laminin-332, as revealed by electron microscopy, and proposed a role for the C-terminal tail of the γ2 chain in stabilizing the integrin binding-competent conformation of the LG1–3 modules. However, it remains to be explored whether a single mutation of the Glu residue within the C-terminal region of the γ2 chain can destabilize the supradomain assembly of the LG1–3 modules of laminin-332, because some other amino acid residues within the C-terminal nine amino acid residues are also conserved between the γ1 and γ2 chains.

Unlike other laminin isoforms, the γ3 chain-containing laminins lack integrin binding activity, a hallmark of laminins in basement membranes. This unique feature of the γ3 chain-containing laminins raises a question as to their physiological functions *in vivo*. It is widely accepted that laminins serve as potent ligands for integrins in basement membranes and thereby provide anchorage-dependent signals that prevent apoptosis, regulate cell proliferation and differentiation, and maintain tissue integrity. One of the likely consequences of the absence of integrin binding activity in γ3 chain-containing laminins could be loosened anchorage of cells onto the basement membrane and resultant weakening of integrin-mediated signaling events. Thus, the γ3 chain-containing laminins may function as dominant-negative laminins that negatively regulate cell-basement membrane interactions during embryonic development and in tissue organization. Given that the LG4–5 modules of the laminin α chains are capable of binding to cell surface receptors other than integrins, *i.e.* α-dystroglycan, heparan sulfate proteoglycans, and sulfated glycolipids (9, 11–13, 15, 16, 45, 46), it is conceivable that the γ3 chain-containing laminins compromise integrin-mediated signals but maintain their functions exerted through non-integrin laminin receptors. It is interesting to note that a variant form of the γ2 transcript (variant-2), which arises by alternative splicing of the 3′-most exon, encodes a γ2 chain that is shorter than the normal γ2 chain and lacks the critical Glu residue at the third position from the C terminus (47, 48). The laminin isoform containing the variant-2 γ2 transcript is therefore assumed to be incapable of binding to integrins due to the absence of the Glu residue required for integrin binding by laminin-332 (20, 44). Airenne *et al.* (49) showed that the variant-2 γ2 transcript was expressed in the mesenchyme of the developing kidney, whereas the normal γ2 transcripts were exclusively detected in the epithelium of the Wolffian duct and ureteric buds, suggesting distinctive functions for the two γ2 chain variants in early kidney development. Although no transcript variants have been reported for the γ1 and γ3 chains, the expression of two kinds of laminin isoforms with and without integrin binding activity may be a novel mechanism for modulating the interactions of cells with basement membranes possessing both γ1 and γ3 chain-containing laminins.

It has been reported that *Lamc1* and *Lamc2* knock-out mice both exhibit severe phenotypes due to failure of endoderm differentiation (50) and skin blistering (51), respectively, whereas *Lamc3* knock-out mice appear to be fertile and do not show any apparent developmental defects (52). Given the similarity in the protein structures between the γ1 and γ3 chains, deficiency of the γ3 chain may be largely compensated for by the γ1 chain. Because *Lamc3* knock-out mice did not show any apparent phenotypes, the γ3 chain-containing laminins may be dispensable for the maintenance and function of basement membranes throughout embryonic development. Recently, Brunken and co-workers (52) reported that the combined absence of β2 and γ3 chain-containing laminins was associated with substantial alterations in the development of inner retinal neurons, particularly those positive for tyrosine hydroxylase activity, disruption of the basement membrane that lines the vitreal surface of the retina, and marked disarray of the vitreal end feet of Müller cells. These alterations are similar to those observed in the β2-null retina (52). Because the γ3 chain is most prominently expressed in the nervous system, including the mouse retina (24, 52, 53), it may participate in the development of inner retinal neurons as an auxiliary basement membrane component that modulates the functions of β2 chain-containing laminins.

The laminin γ3 chain was originally identified as a novel γ chain that combines with the α2 and β1 chains in the placenta (21). Because the γ3 chain was absent from the testes in α2 chain-deficient mice (26), the expression of γ3 chain-containing laminins may be at least partially dependent on the expression of the α2 chain. Consistent with this possibility, the γ3 chain was secreted by 293-F cells as a heterotrimer when cotransfected with the α2 and β1 chains. Our data also showed that the γ3 chain could be expressed and secreted by 293-F cells as laminin-113/123, -223, and -413/423, while combinations with the other α and β chains did not yield detectable amounts of γ3 chain-containing laminin heterotrimers. These results do not necessarily exclude the occurrence of γ3 chain-containing isoforms that failed to be secreted by 293-F cells in tissues, since such isoforms have been detected in hippocampal synapses (laminin-323), testes (laminin-333), and photoreceptor synapses (laminin-523) (24, 27, 53). The failure to detect secretion of γ3 chain-containing isoforms by 293-F cells could simply arise because the secretion levels of these isoforms were below the threshold for detecting laminins by immunoblotting.

Despite the low efficiency of secretion from 293-F cells, γ3 chain-containing laminins have been detected at the basement membranes of various mouse tissues (21, 22, 24–26), indicating that mouse isoforms containing the γ3 chain are competent in secretion from cells and deposition at basement membranes. Although the reason for this apparent discrepancy remains to be elucidated, the human and mouse γ3 chain isoforms may differ in their efficiencies of secretion and/or assembly with other subunit chains. In this respect, it should be noted that there are only two potential *N*-glycosy-

[4] S. Li, unpublished observations.

lation sites in the coiled-coil domain of the human γ3 chain, compared with four sites in the same domain of the mouse γ3 chain. Because the coiled-coil domain is responsible for the heterotrimeric assembly of laminin subunit chains, the reduced *N*-glycosylation at the coiled-coil domain of the human γ3 chain may lead to decreased stabilities of γ3 chain-containing heterotrimers and hence significant reductions in their secretion efficiencies.

In conclusion, we have provided evidence that laminin isoforms containing the γ3 chain are unable to bind to integrins due to the absence of the Glu residue at the third position from the C terminus, which underscores the critical role of this Glu residue conserved in the C-terminal regions of the γ1 and γ2 chains in integrin binding by laminins. Although the physiological functions of the γ3 chain-containing laminins remain to be elucidated, the spatiotemporally regulated expression of such apparently dormant laminins in basement membranes may be an auxiliary mechanism that regulates the adhesive interactions of cells with underlying basement membranes during embryonic development. Further studies on the signals transduced from these laminins as well as their effects on cell proliferation, migration and apoptosis should bring about new insights into the regulatory mechanisms operating in cell-basement membrane interactions during physiological as well as pathological processes.

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Laminin Isoforms Containing the γ3 Chain Are Unable to Bind to Integrins due to the Absence of the Glutamic Acid Residue Conserved in the C-terminal Regions of the γ1 and γ2 Chains

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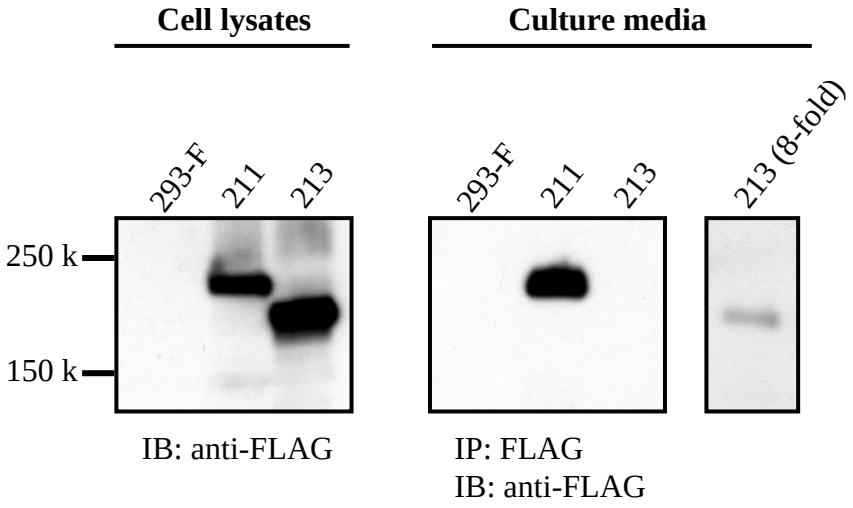
SUPPLEMENTAL FIGURE S1.



Immunoblot analysis of laminin-213 expressed in 293-F cells. 293-F cells were transfected with expression vectors for FLAG-tagged γ1 or γ3 chains together with expression vectors for the α2 and β1 chains. At 72 h after transfection, the cells were lysed with SDS sample buffer and sonicated. Conditioned media (1 ml) were immunoprecipitated with an anti-FLAG M2 antibody, followed by SDS-PAGE in 4% gels under reducing conditions and immunoblotting with an anti-FLAG mAb. An equal amount of each cell lysate (i.e., lysate of cells recovered from 1 ml of culture) was subjected to the same immunoblotting analysis for comparison. The cell lysate and anti-FLAG immunoprecipitate of untransfected 293-F cells were also analyzed as negative controls (*293-F*). For detection of the low levels of recombinant laminin-213 expression, 8-fold more conditioned medium was immunoprecipitated and analyzed by immunoblotting (*8-fold*). Although the predicted molecular masses of γ1 and γ3 chains are ~170 kDa, the γ3 chain gave a band migrating faster than the γ1 chain under reducing conditions. The difference in their apparent molecular masses may reflect the difference in their *N*-linked glycans attached, since the γ3 chain contains 9 potential *N*-glycosylation sites, compared with 14 sites in the γ1 chain.