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Molecular Dissection of the α -Dystroglycan- and Integrin-binding Sites within the Globular Domain of Human Laminin-10*

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The adhesive interactions of cells with laminins are mediated by integrins and non-integrin-type receptors such as α -dystroglycan and syndecans. Laminins bind to these receptors at the C-terminal globular domain of their α chains, but the regions recognized by these receptors have not been mapped precisely. In this study, we sought to locate the binding sites of laminin-10 ($\alpha 5\beta 1\gamma 1$) for $\alpha 3\beta 1$ and $\alpha 6\beta 1$ integrins and α -dystroglycan through the production of a series of recombinant laminin-10 proteins with deletions of the LG (laminin G-like) modules within the globular domain. We found that deletion of the LG4–5 modules did not compromise the binding of laminin-10 to $\alpha 3\beta 1$ and $\alpha 6\beta 1$ integrins but completely abrogated its binding to α -dystroglycan. Further deletion up to the LG3 module resulted in loss of its binding to the integrins, underlining the importance of LG3 for integrin binding by laminin-10. When expressed individually as fusion proteins with glutathione *S*-transferase or the N-terminal 70-kDa region of fibronectin, only LG4 was capable of binding to α -dystroglycan, whereas neither LG3 nor any of the other LG modules retained the ability to bind to the integrins. Site-directed mutagenesis of the LG3 and LG4 modules indicated that Asp-3198 in the LG3 module is involved in the integrin binding by laminin-10, whereas multiple basic amino acid residues in the putative loop regions are involved synergistically in the α -dystroglycan binding by the LG4 module.

Laminins are the major basement membrane proteins expressed ubiquitously throughout the metazoa. Laminins are heterotrimers of three subunits, termed α , β , and γ chains, which assemble into cross-shaped molecules with three short arms and one long rod-like arm. To date, five α chains, three β chains, and three γ chains have been identified, combinations of which yield at least 12 isoforms with distinct subunit compositions (1). These isoforms have been shown to be involved in

many biological processes, including cell adhesion, proliferation, migration, and differentiation (1, 2).

The interaction of cells with laminins is mediated by a variety of cell surface receptors including integrins and non-integrin-type receptors such as α -dystroglycan and syndecans (1, 3). Integrins are of crucial importance among these receptors with respect to controlling the growth and differentiation of cells. There are more than 20 integrins with distinct subunit compositions, of which $\alpha 3\beta 1$ and $\alpha 6\beta 1$ integrins have been shown to be the major laminin receptors expressed in many cell types (4–6). α -Dystroglycan is a highly glycosylated protein containing novel *O*-mannosyl-type oligosaccharides (7) and forms a complex with a single pass transmembrane protein called β -dystroglycan (3). α -Dystroglycan binds to various types of laminin isoforms including laminin-1 ($\alpha 1\beta 1\gamma 1$) and laminin-2 ($\alpha 2\beta 1\gamma 1$) in a Ca^{2+} -dependent manner (8–11). Binding sites for integrins and α -dystroglycan have been mapped to the G domain,¹ the C-terminal globular domain of the laminin α chain (12–14). The G domain consists of five tandem repeats of LG modules of ~200 amino acid residues, designated LG1 through LG5. However, the binding sites within the G domain for integrins and α -dystroglycan remain to be defined.

Laminin-10 ($\alpha 5\beta 1\gamma 1$) is a major laminin isoform widely expressed in adult tissues (15). Mice lacking the laminin $\alpha 5$ gene exhibit embryonic lethality resulting from severe developmental abnormalities, such as syndactyly, exencephaly, and placental dysmorphogenesis (16). Laminin-10 also seems to be essential for hair morphogenesis because ablation of laminin-10 results in arrest of hair follicle development at the hair germ elongation phase (17). Previously, we purified laminin-10/11 from the conditioned medium of human lung carcinoma cells and demonstrated that adhesion of epithelial cells to laminin-10/11 was mediated mainly by $\alpha 3\beta 1$ integrin (4), although adhesion of fibroblastic cells was mediated through both $\alpha 3\beta 1$ and $\alpha 6\beta 1$ integrins (5). The roles of $\alpha 3\beta 1$ and $\alpha 6\beta 1$ integrins as major receptors for laminin-10/11 were confirmed further by direct binding of laminin-10/11 to $\alpha 3\beta 1$ and $\alpha 6\beta 1$ integrins (6). Recently, Yu and Talts (18) produced recombinant fragments modeled after the G domain of the mouse $\alpha 5$ chain and demonstrated that the fragment consisting of the LG1–3 modules had cell adhesive activity dependent on $\alpha 3\beta 1$ and $\alpha 6\beta 1$ inte-

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¹ The abbreviations used are: G domain, globular domain; BSA, bovine serum albumin; FN70K, N-terminal 70-kDa region of human fibronectin; GST, glutathione *S*-transferase; LG, laminin G-like; mAb, monoclonal antibody; rLN10, recombinant laminin-10; TBS, Tris-buffered saline.

grins, whereas the fragment consisting of the LG4–5 modules was capable of mediating cell adhesion via interaction with α -dystroglycan. However, precise mapping of the binding sites for integrins and α -dystroglycan within the G domain of the $\alpha 5$ chain remains undefined.

In the present study, we produced a panel of recombinant laminin-10 mutants with serial deletions of the LG1–5 modules and examined their binding activities to purified $\alpha 3\beta 1$ and $\alpha 6\beta 1$ integrins, α -dystroglycan, and heparin. Our results show that the LG3 module is indispensable for binding to $\alpha 3\beta 1$ and $\alpha 6\beta 1$ integrins, although the LG3 module alone is not sufficient to recapitulate the integrin binding activity. In contrast, the binding site(s) for α -dystroglycan and heparin have been mapped to the LG4 module, which alone can exhibit potent binding activities toward α -dystroglycan and heparin. We also attempted to identify the amino acid residues involved in integrin and α -dystroglycan binding by site-directed mutagenesis of the LG3 and LG4 modules, respectively.

EXPERIMENTAL PROCEDURES

cDNA Cloning and Construction of Expression Vectors—A full-length cDNA encoding the human laminin $\alpha 5$ subunit (GenBank accession AF443072) was amplified by reverse transcription-PCR as a series of ~1.2-kb fragments, and each fragment was subcloned into pGEM-T (Promega, Madison, WI) or pCRscript (Stratagene, La Jolla, CA) according to the manufacturer's instructions. RNA was extracted from A549 human lung adenocarcinoma cells and used as a template for reverse transcription-PCR. The list of primer sequences is available upon request. After sequence verification, error-free cDNA fragments were ligated in tandem to construct a cDNA encompassing the whole open reading frame. The $\alpha 5$ cDNA was inserted into the NheI/PmeI sites of pcDNA3.1 (Invitrogen), yielding the $\alpha 5$ chain expression vector pcDNA- $\alpha 5$. Expression vectors for laminin $\beta 1$ (pCEP- $\beta 1$) and $\gamma 1$ (pcDNA3.1- $\gamma 1$) were prepared as described previously (19). Expression vectors for laminin $\alpha 5$ chains lacking LG5 (nucleotides 1–10554; pcDNA- $\alpha 5\Delta$ LG5), LG4–5 (nucleotides 1–9891; pcDNA- $\alpha 5\Delta$ LG4–5), LG3–5 (nucleotides 1–9360; pcDNA- $\alpha 5\Delta$ LG3–5), LG2–5 (nucleotides 1–8802; pcDNA- $\alpha 5\Delta$ LG2–5), and LG1–5 (nucleotides 1–8241; pcDNA- $\alpha 5\Delta$ LG1–5) were constructed as follows. cDNA fragments encompassing nucleotides 5795–8241 (for the deletion of LG1–5), 5795–8802 (for the deletion of LG2–5), 5795–9360 (for the deletion of LG3–5), 9365–9891 (for the deletion of LG4–5), and 9365–10554 (for the deletion of LG5) were amplified by PCR using KOD DNA polymerase (TOYOBO, Osaka, Japan) with an NotI (for the deletions of LG1–5, LG2–5, and LG3–5) or AscI (for the deletions of LG4–5 and LG5) site at the 5'-end and a stop codon and a PmeI site at the 3'-end. The PCR products were digested with NotI/PmeI or AscI/PmeI, and the resultant cDNA fragments were recloned into the pcDNA- $\alpha 5$ vector cleaved with the same restriction enzymes.

Expression vectors for individual LG modules of the laminin $\alpha 5$ chain as GST fusion proteins were prepared as follows. cDNAs encoding the individual modules were amplified by PCR using pcDNA- $\alpha 5$ as a template. The PCR products were digested with EcoRI and XhoI and inserted into the corresponding restriction sites of the pGEX4T-1 expression vector (Amersham Biosciences). The PCR primers used were 5'-TTCAATGAATTCTCAGGGGTGCAGC-3' and 5'-CGGGTCCTCGAGCTACTTGGAGCGGGC-3' (for LG1), 5'-GCCCCGAATTCTCGACCGGGGACCCG-3' and 5'-GGCGCGCTCGAGCTACAGGTGGCGGGTGC-3' (for LG2), 5'-ACCCCGCAATTCTGGTGGGGCGCCG-3' and 5'-CGGGGTCTCAGACTCAGGGCGGGTGC-3' (for LG3), 5'-AATGAATTCAGGACACCCGAGAC-3' and 5'-TATCTCGAGCTAGGGGCCCAAGATGCAG-3' (for LG4), and 5'-GTCACAGAATTCATCTTGGGCCCTG-3' and 5'-CTGCAGGCTCGAGCTAGGCGGCTGG-3' (for LG5).

Expression vectors for fusion proteins of the LG3 and LG4 modules with the N-terminal 70-kDa domain of human fibronectin (FN70K), designated FN70K-LG3 and FN70K-LG4, respectively, were prepared as follows. The expression vector pMTX-1 for the truncated form of human fibronectin (20) was digested with HindIII and NotI and inserted into the corresponding restriction sites of pFLAG-CMV-5c (Sigma), yielding the expression vector pFLAG-FN70K for FN70K with a FLAG tag. cDNA fragments encoding the LG3 and LG4 modules were amplified by PCR using pcDNA- $\alpha 5$ as a template with an NotI site at the 5'-end and an EcoRV site at the 3'-end. The PCR products were digested with NotI/EcoRV and inserted into the corresponding restriction sites of the expression vector pFLAG-FN70K. The PCR primers

used were 5'-ATATGCGGCCGACCGCCGACCTGCTG-3' and 5'-TATGATATCTTGCAGGGCGGGTGC-3' (for LG3) and 5'-ATATGCGGCCGACGACACCCCGAGAC-3' and 5'-ATAGATATCGGGGCCCAAGATGCAG-3' (for LG4).

Site-directed Mutagenesis—Site-directed mutagenesis of the LG4 module was accomplished by overlap extension PCR with KOD polymerase using pGEX-LG4 encoding the GST-LG4 fusion protein as a template. The list of primer sequences used for the site-directed mutagenesis is available upon request. For site-directed mutagenesis of the LG3 module, the cDNA fragment encoding LG3 was excised from pcDNA- $\alpha 5\Delta$ LG4–5 with AscI and PmeI and recloned into pSecTag2A (Invitrogen) at the AscI/PmeI sites. The resulting plasmid was used as a template for site-directed mutagenesis of the LG3 module by overlap extension PCR as described above. The list of primer sequences used for the site-directed mutagenesis is available upon request. The purified PCR products containing the mutations were digested with AscI and PmeI and inserted into the corresponding restriction sites of the expression vector pcDNA- $\alpha 5\Delta$ LG4–5.

Expression and Purification of Recombinant Proteins—Recombinant laminin-10 (rLN10) and its mutants were produced using the FreeStyle™ 293 Expression system (Invitrogen). Briefly, 293-F cells were simultaneously transfected with expression vectors for $\alpha 5$, $\beta 1$, and $\gamma 1$ using 293fectin (Invitrogen), and grown in serum-free FreeStyle™ 293 Expression medium for 72 h. For the expressions of rLN10 and rLN10 lacking LG5, 200 μ g/ml heparin was included in the medium to inhibit the proteolytic cleavage between the LG3 and LG4 modules (21). The conditioned media were clarified by centrifugation and passed through immunoaffinity columns conjugated with an anti-human laminin $\alpha 5$ mAb 5D6 (22). The columns were washed with 50 mM Tris-HCl, pH 7.4, containing 500 mM NaCl to remove bound heparin, and then bound laminins were eluted with 0.1 M triethylamine, neutralized, and dialyzed against TBS (50 mM Tris-HCl, pH 7.4, 150 mM NaCl).

Individual LG modules of the $\alpha 5$ chain produced as GST fusions were induced in *Escherichia coli* with 0.1 mM isopropyl- β -D-thiogalactopyranoside and purified on glutathione-Sepharose 4B columns (Amersham Biosciences) after lysis of the cells by sonication. Bound proteins were eluted with 50 mM Tris-HCl, pH 8.0, containing 10 mM glutathione. Purified proteins were dialyzed against TBS. GST-LG3 was recovered as insoluble aggregates upon sonication and then solubilized in TBS containing 8 M urea and dialyzed against TBS before passing through a glutathione-Sepharose 4B column. FN70K, FN70K-LG3, and FN70K-LG4 were produced using the FreeStyle™ 293 Expression system as described above. The conditioned media were applied to anti-FLAG M2 columns (Sigma), and the columns were washed with TBS. Bound proteins were competitively eluted from the columns with 100 μ g/ml FLAG peptide (Sigma) and dialyzed against TBS.

Proteins and Antibodies— $\alpha 3\beta 1$ and $\alpha 6\beta 1$ integrins were purified from human placenta and reconstituted into 3 H-labeled phosphatidylcholine liposomes as described previously (6). α -Dystroglycan was purified from rabbit skeletal muscle according to Brancaccio *et al.* (23) with the following modifications. α -Dystroglycan partially purified using DEAE-Sepharcel and wheat germ agglutinin-agarose chromatography was purified further by laminin-1 affinity chromatography, followed by CsCl gradient centrifugation (10). Heparin-BSA was purchased from Sigma. mAbs against the human laminin $\alpha 5$ chain (15H5 and 5D6) were produced in our laboratory (22). A mAb against human laminin $\gamma 1$ (mAb 1920) was purchased from Chemicon. A mAb against human fibronectin (FN9-1) was obtained from Takara Biomedicals (Kyoto, Japan). A mAb against human $\beta 1$ integrin (AIB2) developed by Dr. Caroline Damsky (University of California, San Francisco) was obtained from the Developmental Studies Hybridoma Bank (Iowa City, IA). A polyclonal anti-GST antibody was produced by immunizing rabbits with purified GST and affinity-purified on a GST-conjugated Sepharose column.

Binding Assays for Integrins, α -Dystroglycan, and Heparin—Integrin binding assays were performed using $\alpha 3\beta 1$ and $\alpha 6\beta 1$ integrins reconstituted into 3 H-labeled phosphatidylcholine liposomes as described previously (6). Briefly, 96-well microtiter plates were coated with the proteins to be tested at 20 nM, blocked with 1% BSA, and incubated with integrin-liposomes in the presence of 1 mM Mn^{2+} at room temperature for 6 h. Plates were washed with TBS containing 1 mM Mn^{2+} , and the bound integrin-liposomes were recovered with 1% SDS and quantified with a Packard Tri-Carb 1500 liquid scintillation analyzer. For binding assays for α -dystroglycan and heparin, 96-well microtiter plates were coated with 5 μ g/ml α -dystroglycan or 5 μ g/ml heparin-BSA, blocked with 1% BSA, and incubated with rLN10, its mutant forms, or individual LG modules expressed as GST or FN70K fusion proteins in TBS containing either 1 mM $CaCl_2/MgCl_2$ or 1 mM EDTA at room temperature for 1 h. After washing with TBS, bound proteins were quantified

with the anti-laminin γ 1 mAb (rLN10 and its mutants), anti-GST antibody (GST fusion proteins), or anti-fibronectin mAb FN9-1 (FN70K fusion proteins) followed by incubation with horseradish peroxidase-conjugated rabbit anti-mouse or goat anti-rabbit IgG antibodies.

Cell Adhesion Assay—Cell adhesion assays were performed using HT1080 human fibrosarcoma cells (5). Briefly, 96-well microtiter plates were coated with 5 nM rLN10 or its mutants at 4 °C overnight and blocked with 1% BSA for 1 h at room temperature. HT1080 cells were harvested with phosphate-buffered saline containing 1 mM EDTA, suspended in serum-free Dulbecco's modified Eagle's medium at a density of 3×10^5 cells/ml, and then plated on the wells coated with rLN10 or its mutants at 3×10^4 cells/well. After incubation in a CO₂ incubator at 37 °C for 30 min, the attached cells were fixed and stained with Diff-Quik (International Reagents Corp., Japan), washed with distilled water, and extracted with 1% SDS for colorimetric quantification at 590 nm.

Cell adhesion inhibition assays were performed based on the cell adhesion assays. HT1080 cells were preincubated with a function-blocking anti- β_1 integrin mAb (AIB2) or control mouse IgG for 20 min at room temperature and then added to the precoated wells. After a 30-min incubation at 37 °C, the cells attached to the substrates were quantified as described above.

RESULTS

Production of Wild-type Laminin-10 and Its Deletion Mutants—To map the binding sites for integrin and α -dystroglycan within the G domain of the laminin α 5 chain, we expressed rLN10 and a series of its deletion mutants lacking LG modules (Fig. 1A) by triple transfection of cDNAs encoding the laminin α 5, β 1, and γ 1 subunits into 293-F cells. Secretion of endogenous laminins containing β 1 and/or γ 1 chains was undetectable in 293-F cells (data not shown). Both wild-type and mutant proteins were purified from conditioned media using immunoaffinity columns conjugated with an anti- α 5 chain mAb. To minimize the cleavage at the spacer segment between the LG3 and LG4 modules, wild-type rLN10 and its mutant lacking LG5 (rLN10 Δ LG5) were expressed in cells grown in medium containing heparin because heparin has been shown to inhibit partially the proteolytic processing of laminin-5 at the spacer region (21).

The authenticity of the recombinant proteins was verified by SDS-PAGE and immunoblotting with a mAb against the α 5 chain. Under reducing conditions, each recombinant protein gave three bands upon silver staining, one corresponding to the α 5 chain, with a molecular mass of 300,000–400,000 depending on the extent of the deletion, and two lower bands corresponding to the β 1 and γ 1 chains (Fig. 1B). When wild-type rLN10 and rLN10 Δ LG5 were expressed in 293-F cells grown in medium without heparin, the majority of the recombinant proteins were processed at the spacer region, yielding bands that comigrated with rLN10 lacking the LG4–5 modules (rLN10 Δ LG4–5; data not shown). Under nonreducing conditions, rLN10 and its deletion mutants barely entered the gel, confirming that they were purified as trimers of the α 5, β 1, and γ 1 chains.

Cell Adhesive and Integrin Binding Activities of Wild-type Laminin-10 and Its Deletion Mutants—The purified rLN10 and its deletion mutants were assayed for their cell adhesive activities using HT1080 cells. rLN10 Δ LG5 and rLN10 Δ LG4–5 exhibited potent cell adhesive activities equivalent to that of wild-type rLN10, but those lacking the LG3–5, LG2–5, and LG1–5 modules were barely able to mediate cell adhesion to substrates (Fig. 2). Cell adhesion to wild-type rLN10 and the mutants lacking LG5 and LG4–5 was strongly inhibited by a function-blocking mAb against β_1 integrin, confirming the role of β_1 integrins as major cell surface receptors for laminin-10 (5, 6). Cell adhesion to wild-type rLN10 and the mutants lacking LG4–5 and LG5 was also inhibited by a combination of anti- α_3 integrin and anti- α_6 integrin mAbs, but not by either mAb alone (data not shown), consistent with previous observations

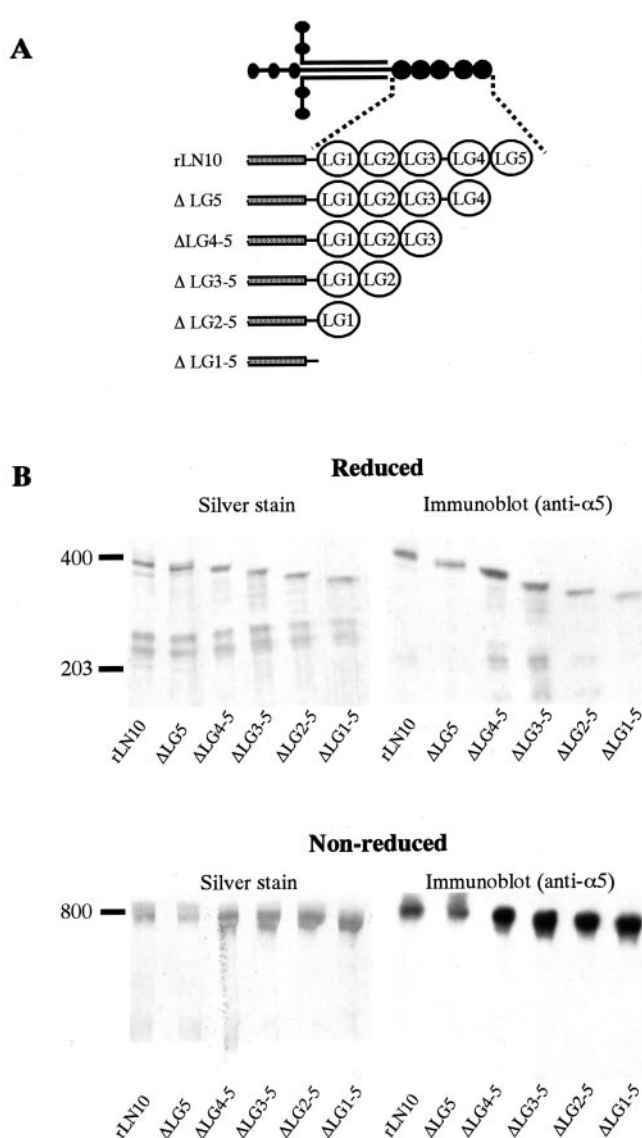


FIG. 1. SDS-PAGE and immunoblotting analyses of purified rLN10 and its deletion mutants. A, schematic diagrams of rLN10 and its deletion mutants. Δ LG5, rLN10 lacking the LG5 module; Δ LG4–5, rLN10 lacking the LG4–5 modules; Δ LG3–5, rLN10 lacking the LG3–5 modules; Δ LG2–5, rLN10 lacking the LG2–5 modules; Δ LG1–5, rLN10 lacking the LG1–5 modules. B, purified rLN10 and its deletion mutants were analyzed by SDS-PAGE on 4% gels under reducing (upper panels) or nonreducing (lower panels) conditions followed by silver staining of the gels (left panels) or immunoblotting with a mAb against the laminin α 5 chain (right panels). The positions of molecular size markers are shown in the left margin.

(5). A dramatic loss of the cell adhesive activity upon deletion of LG3 indicated that the LG3 module is indispensable for the potent cell adhesive activity of laminin-10 and that LG3-dependent cell adhesion is mainly mediated by $\alpha_3\beta_1$ and/or $\alpha_6\beta_1$ integrins.

To confirm the importance of the LG3 module in the binding of laminin-10 to $\alpha_3\beta_1$ and $\alpha_6\beta_1$ integrins, we examined the binding of rLN10 and its mutants to these integrins. We purified $\alpha_3\beta_1$ and $\alpha_6\beta_1$ integrins from placenta and examined their binding to laminin-10 and its mutants after reconstitution into ³H-labeled phosphatidylcholine liposomes (Fig. 3). As expected, the mutants lacking the LG5 and LG4–5 modules were capable of binding to both $\alpha_3\beta_1$ and $\alpha_6\beta_1$ integrins with potencies that were comparable with that of wild-type rLN10, although mutants lacking LG3–5, LG2–5, or LG1–5 were almost devoid of

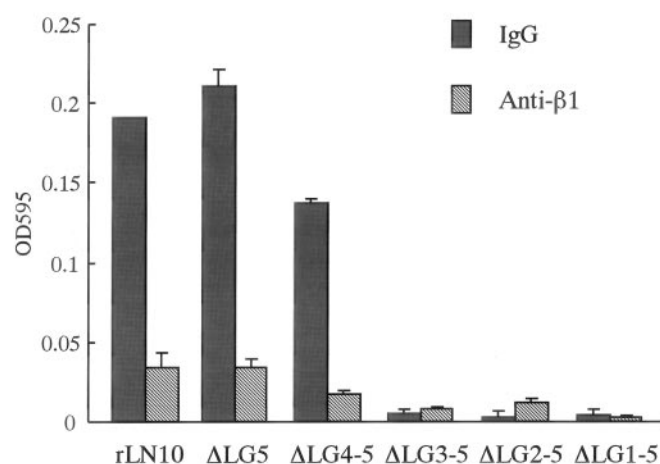


FIG. 2. Cell adhesive activities of rLN10 and its deletion mutants. HT1080 cells were preincubated with either a function-blocking mAb against β_1 integrin (20 μ g/ml, hatched bars) or control IgG (20 μ g/ml, solid bars) for 20 min at room temperature and then added to 96-well microtiter plates coated with rLN10 or its deletion mutants at 5 nM. After a 30-min incubation at 37 °C, the cells attached to the substrates were fixed and quantified as described under "Experimental Procedures." The deletion mutants are abbreviated as described in the legend for Fig. 1. Each bar represents the mean of triplicate assays \pm S.D. Pretreatment with control IgG did not affect the cell adhesive activity of rLN10.

any activity. The binding activities of mutants lacking LG5 and LG4–5 to these integrins were completely abrogated in the presence of EDTA (data not shown), confirming that the mutants lacking LG5 and LG4–5 retained the same integrin binding properties as intact laminin-10.

α -Dystroglycan and Heparin Binding Activities of Wild-type Laminin-10 and Its Deletion Mutants—We also examined the binding of wild-type laminin-10 and its deletion mutants to α -dystroglycan and heparin by solid phase binding assays using microtiter plates coated with α -dystroglycan or heparin-BSA. Wild-type rLN10 was capable of binding to α -dystroglycan in the presence of Ca^{2+} ions but not in the presence of EDTA (Fig. 4), consistent with previous reports that the binding of laminin-1/2 to α -dystroglycan is Ca^{2+} -dependent (8–11). In contrast, wild-type rLN10 bound to heparin irrespective of the presence of Ca^{2+} or EDTA, confirming that heparin binding to laminin-10 does not require divalent cations, e.g. Ca^{2+} (12, 13, 24). Among the deletion mutants tested, rLN10ΔLG5 retained full binding activities toward α -dystroglycan and heparin, but other mutants including rLN10ΔLG4–5 were only marginally active at binding to either α -dystroglycan or heparin (Fig. 4). These results provide loss-of-function evidence that both α -dystroglycan and heparin bind to the LG4 module of laminin-10.

Receptor Binding Activities of Individual LG Modules—The binding profiles of the deletion mutants of laminin-10 toward $\alpha_3\beta_1/\alpha_6\beta_1$ integrins and α -dystroglycan/heparin suggested that the LG3 module was the likely binding site for $\alpha_3\beta_1$ and $\alpha_6\beta_1$ integrins, whereas the LG4 module was the likely binding site for α -dystroglycan and heparin. To explore these possibilities further, we expressed the individual LG modules of the α_5 chain in bacteria as GST fusion proteins (Fig. 5A) and assayed their abilities to bind $\alpha_3\beta_1/\alpha_6\beta_1$ integrins as well as α -dystroglycan/heparin. Only GST-LG4 was capable of binding to α -dystroglycan and heparin among the five GST-LG modules (Fig. 5B), confirming that LG4 is the major binding site for both α -dystroglycan and heparin. In contrast, neither LG3 nor any of the other LG modules showed any significant binding to $\alpha_3\beta_1$ or $\alpha_6\beta_1$ integrins (Fig. 5C), except that GST-LG1 exhibited a very weak integrin binding activity. Because the failure of LG3

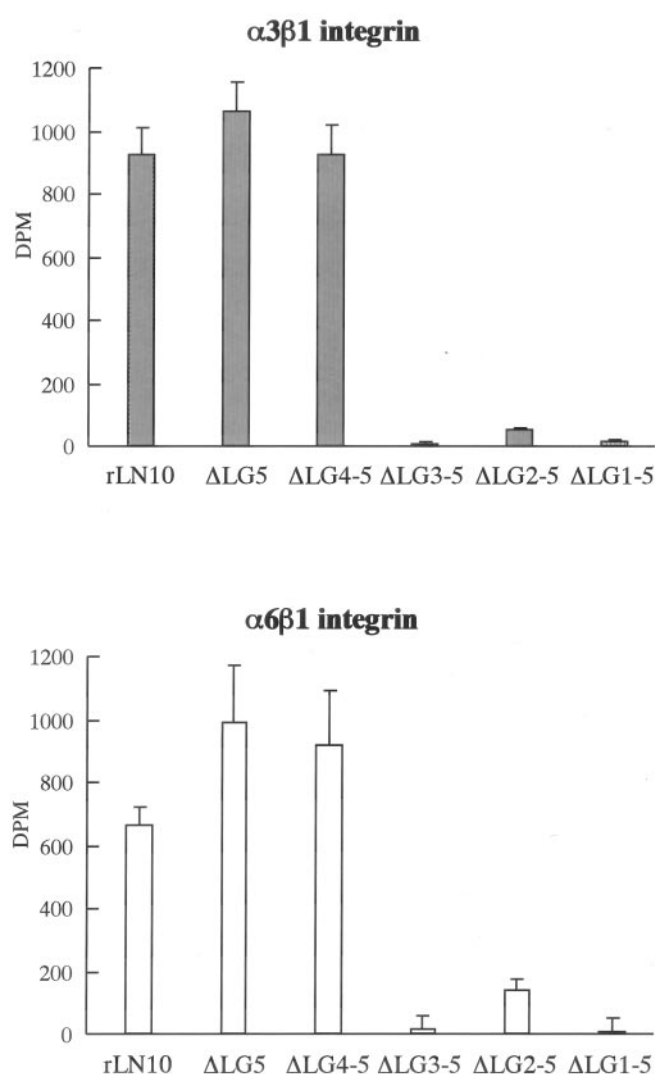


FIG. 3. Integrin binding activities of rLN10 and its deletion mutants. 96-well microtiter plates were coated with rLN10 or its deletion mutants at 20 nM. After blocking with BSA, the wells were incubated with ^3H -labeled phosphatidylcholine liposomes containing $\alpha_3\beta_1$ integrin (upper panel) or $\alpha_6\beta_1$ integrin (lower panel) in the presence of 1 mM Mn^{2+} at room temperature for 6 h. Bound integrin-containing liposomes were quantified with a Packard Tri-Carb 1500 liquid scintillation analyzer. Each bar represents the mean of triplicate assays \pm S.D.

and the other LG modules to bind to these integrins could result from misfolding and/or the absence of glycosylation resulting from their expression in bacteria, we expressed the LG3 and LG4 modules in 293-F cells as fusion proteins with FN70K, which serves as a vehicle for the secretion of recombinant proteins in mammalian expression systems (20). FN70K-LG4 retained the ability to bind α -dystroglycan (Fig. 6A) and heparin (data not shown), but FN70K-LG3 did not show any significant binding to either $\alpha_3\beta_1$ or $\alpha_6\beta_1$ integrin (Fig. 6B). These results raise the possibility that LG3 is necessary, but not sufficient, for the integrin binding activity of laminin-10 (see "Discussion").

Mapping of α -Dystroglycan and Heparin Binding Sites within the LG4 Module by Site-directed Mutagenesis—Previous studies have demonstrated that the basic amino acid residues within the LG4 module of the α_1 chain and the LG5 module of the α_2 chain participate in binding to α -dystroglycan and heparin (12, 13). These basic amino acid residues are predicted to be within the loops connecting adjacent β strands, based on the

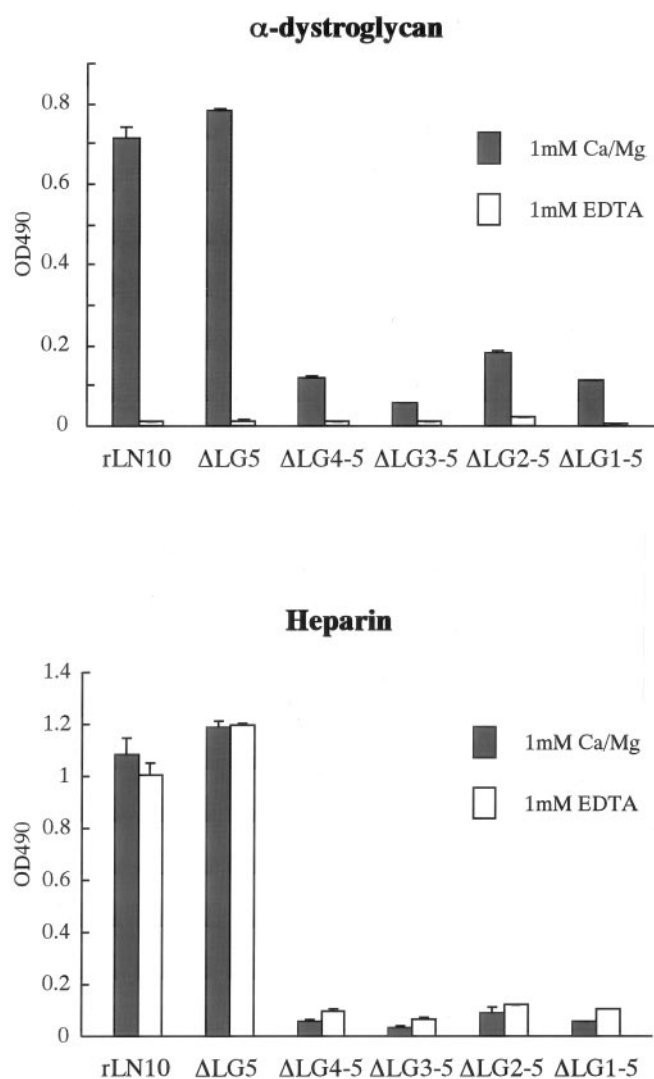


FIG. 4. α -Dystroglycan and heparin binding activities of rLN10 and its deletion mutants. 96-well microtiter plates were coated with α -dystroglycan (upper panel) or heparin-BSA (lower panel) and incubated with rLN10 or its mutant forms in the presence of 1 mM $\text{CaCl}_2/\text{MgCl}_2$ (closed bars) or 1 mM EDTA (open bars) at room temperature for 1 h. Bound proteins were detected with an anti- γ 1 mAb. Each bar represents the mean of triplicate assays \pm S.D.

crystal structure of the LG4–5 modules of the α 2 chain (25, 26). To specify the amino acid residues within LG4 involved in binding to α -dystroglycan and heparin, we produced a series of alanine substitution mutants of GST-LG4 for the basic amino acid residues predicted within the loop regions of the LG4 module (mutants designated L1–L11; Fig. 7A). All of these GST-LG4 mutants showed identical electrophoretic mobilities and were indistinguishable from wild-type GST-LG4 (data not shown). Partial reductions in the binding activities to α -dystroglycan and heparin were observed with the L2, L3, L5, L6, and L9 mutants (Fig. 7B), suggesting that the basic amino acid residues in these loops are involved in the binding of the LG4 module to α -dystroglycan and heparin. Because the overall binding profiles of the LG4 mutants toward α -dystroglycan and heparin were very similar, it is likely that the amino acid residues involved in binding to α -dystroglycan and heparin mostly overlap each other. To explore this possibility, we examined whether heparin could compete with α -dystroglycan for binding to GST-LG4 (Fig. 7C). As expected, GST-LG4 binding to α -dystroglycan was inhibited by increasing concentrations of heparin, supporting the possibility that laminin-10 binds to

both α -dystroglycan and heparin through closely overlapping, if not identical, basic amino acid residues within LG4.

Production of Laminin-10 Mutants with Amino Acid Substitutions within the LG3 Module and Their Integrin Binding Activities—We also produced a series of rLN10 mutants with single amino acid substitutions within the LG3 module to explore the amino acid residue(s) involved in its binding to $\alpha_3\beta_1$ and $\alpha_6\beta_1$ integrins. Because Asp and/or Glu residues have been shown to be the key residues within the known integrin recognition motifs (27, 28), we focused on the Asp and Glu residues within the predicted loop regions of the LG3 module of the α 5 chain and replaced them with Ala (Fig. 8A). Because of the failure to detect integrin binding activity of GST-LG3, we expressed rLN10 Δ LG4–5, which retained the full integrin binding activity of rLN10, and its mutants with alanine substitutions in 293-F cells and purified them on immunoaffinity columns. The integrin binding assays with these rLN10 Δ LG4–5 mutants demonstrated that alanine replacement of Asp-3198 resulted in significant reductions in the binding activities toward both $\alpha_3\beta_1$ and $\alpha_6\beta_1$ integrins (Fig. 8B). Other mutants exhibited only marginal decreases in the integrin binding activities, suggesting that Asp-3198 is involved in integrin recognition by the G domain of laminin-10, although other residues within the LG3 and/or LG1–2 modules may also serve as part of integrin recognition sites.

DISCUSSION

In the present study, we attempted to locate the binding sites for $\alpha_3\beta_1/\alpha_6\beta_1$ integrins and α -dystroglycan within the G domain of laminin-10 by producing a series of deletion and substitution mutants of rLN10 as well as individual LG modules expressed as GST or FN70K fusion proteins. We employed the FreeStyle™ 293 Expression system for the production of rLN10 to maximize the yields of the transiently expressed rLN10 and its mutants upon triple transfection of cDNAs encoding α , β , and γ chains. We also improved the yields of intact rLN10 by blocking the proteolytic processing at the linker segment between the LG3 and LG4 modules with heparin included in the medium (21). Our data clearly show that LG4 harbors the binding site(s) for α -dystroglycan and heparin, whereas LG3 is necessary, but not sufficient, for the binding to $\alpha_3\beta_1/\alpha_6\beta_1$ integrins.

There is accumulating evidence that the LG4–5 modules are involved in laminin binding to α -dystroglycan. Thus, the LG4–5 modules of the α 1 (12), α 2 (13, 29), α 4 (14), and α 5 chains (18) have been shown to bind to α -dystroglycan, although the LG1–3 modules of the α 2 and α 4 chains were also active in binding to α -dystroglycan to variable extents (13, 14). Further dissection of LG4–5 into individual modules identified LG4 as the major α -dystroglycan binding site within the α 1 chain (12) but often failed to detect any significant activities with either the LG4 or LG5 modules of other α chains (13, 18). Yu and Talts (18) argued that the α -dystroglycan binding site within the α 5 chain spans at least two LG modules in a manner analogous to the interaction of the LG1–3 and LG4–5 modules of the α 2 chain, based on their results that neither LG4 nor LG5 exhibited any significant binding to α -dystroglycan. Our results that the LG4 module alone, expressed as a GST or FN70K fusion protein, was active in binding to α -dystroglycan are apparently controversial to these previous observations. The importance of LG4 in the binding of laminin-10 to α -dystroglycan was supported further by the observation that rLN10 lacking LG5 retained the full binding activity to α -dystroglycan, but rLN10 lacking both LG4 and LG5 was barely active. The reason for this discrepancy remains to be clarified, although it may be possible that the α -dystroglycan binding activity of LG4 is conformation-dependent and that the putative active conformation is stabilized by LG5 when connected in

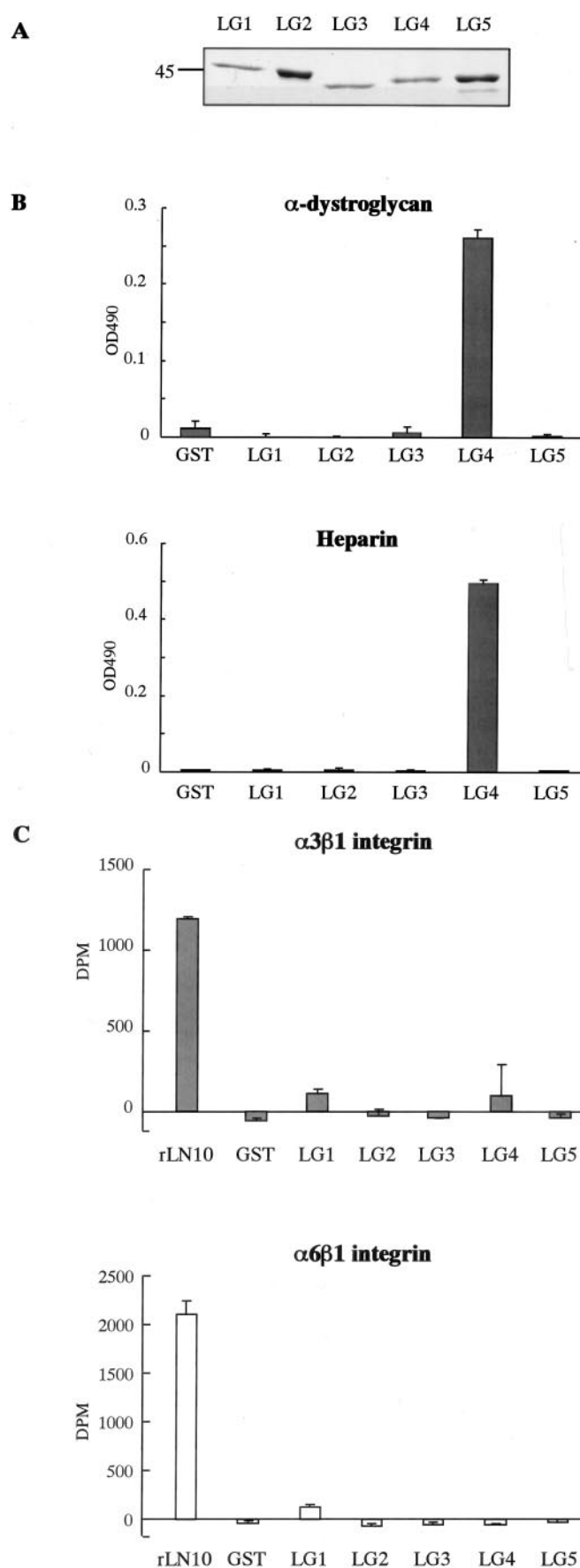


FIG. 5. α -Dystroglycan, heparin, and integrin binding activities of individual LG modules. A, SDS-PAGE profiles of individual LG modules of the $\alpha 5$ chain expressed and purified as GST fusion proteins. Proteins were stained with Coomassie Brilliant Blue. The

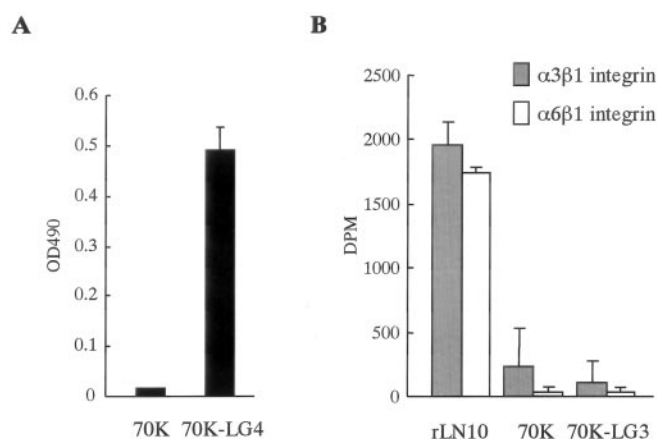


FIG. 6. Receptor binding activities of the LG3 and LG4 modules expressed in mammalian cells. A, α -dystroglycan binding activities of FN70K (70K) and FN70K-LG4 (70K-LG4). Bound proteins were detected with a mAb against human fibronectin. B, binding activities of rLN10, FN70K, and FN70K-LG3 (70K-LG3) to $\alpha_3\beta_1$ (shaded bars) and $\alpha_6\beta_1$ (open bars) integrins. Each bar represents the mean of triplicate assays \pm S.D.

tandem. The expression of LG4 as fusion proteins with GST or FN70K could mimic the neighboring effect of LG5, allowing us to detect the α -dystroglycan binding activity of LG4 even in the absence of LG5. Alternatively, the apparent discrepancy might reflect the specificity of the antibodies used in the α -dystroglycan binding assays of LG4 and/or LG5. Yu and Talts (18) used a polyclonal antibody raised against LG4–5 tandem modules, which may not be able to recognize LG4 when it is bound to α -dystroglycan because of masking of the epitope(s) by the bound α -dystroglycan.

Binding of laminin-1 and -2 to α -dystroglycan has been shown to be strictly Ca^{2+} -dependent. The crystal structure of the LG4–5 tandem modules of the $\alpha 2$ chain revealed that two aspartic acid residues conserved in the LG modules of the $\alpha 1$ and $\alpha 2$ chains are involved in the Ca^{2+} binding and are therefore considered to be important in the Ca^{2+} -dependent α -dystroglycan binding (25, 26). However, these two aspartic acid residues do not seem to be conserved in the LG4 modules of other laminin α chains including $\alpha 5$ (25). Nevertheless, our results clearly show that binding of rLN10 to α -dystroglycan is strictly Ca^{2+} -dependent, making it likely that the Ca^{2+} binding site(s) involved in the Ca^{2+} -dependent α -dystroglycan binding of laminin-10 are different from those in laminin-1 and -2. Consistent with this possibility, binding of laminin-8 to α -dystroglycan is also Ca^{2+} -dependent,² although laminin-8 lacks the two aspartic acid residues equivalent to those conserved in the LG4–5 modules of the $\alpha 2$ chain.

The LG4 module has been shown to be the major heparin binding region within the G domain of most laminin α chains,

² H. Ido, unpublished observation.

position of the 45-kDa molecular size marker is shown in the left margin. B, α -dystroglycan and heparin binding activities of GST-LG modules. 96-well microtiter plates were coated with 5 $\mu\text{g}/\text{ml}$ α -dystroglycan or 5 $\mu\text{g}/\text{ml}$ heparin-BSA (upper panel) and incubated with 10 nM individual GST-LG modules at room temperature for 1 h. Bound proteins were detected with an anti-GST polyclonal antibody. Binding assays for α -dystroglycan were performed in the presence of 1 mM $\text{CaCl}_2/\text{MgCl}_2$. C, integrin binding activities of individual GST-LG modules. Microtiter plates were coated with 20 nM individual GST-LG modules and then incubated with ^3H -labeled phosphatidylcholine liposomes containing $\alpha_3\beta_1$ integrin (upper panel) or $\alpha_6\beta_1$ integrin (lower panel) in the presence of 1 mM Mn^{2+} at room temperature for 6 h. Each bar represents the mean of triplicate assays \pm S.D.

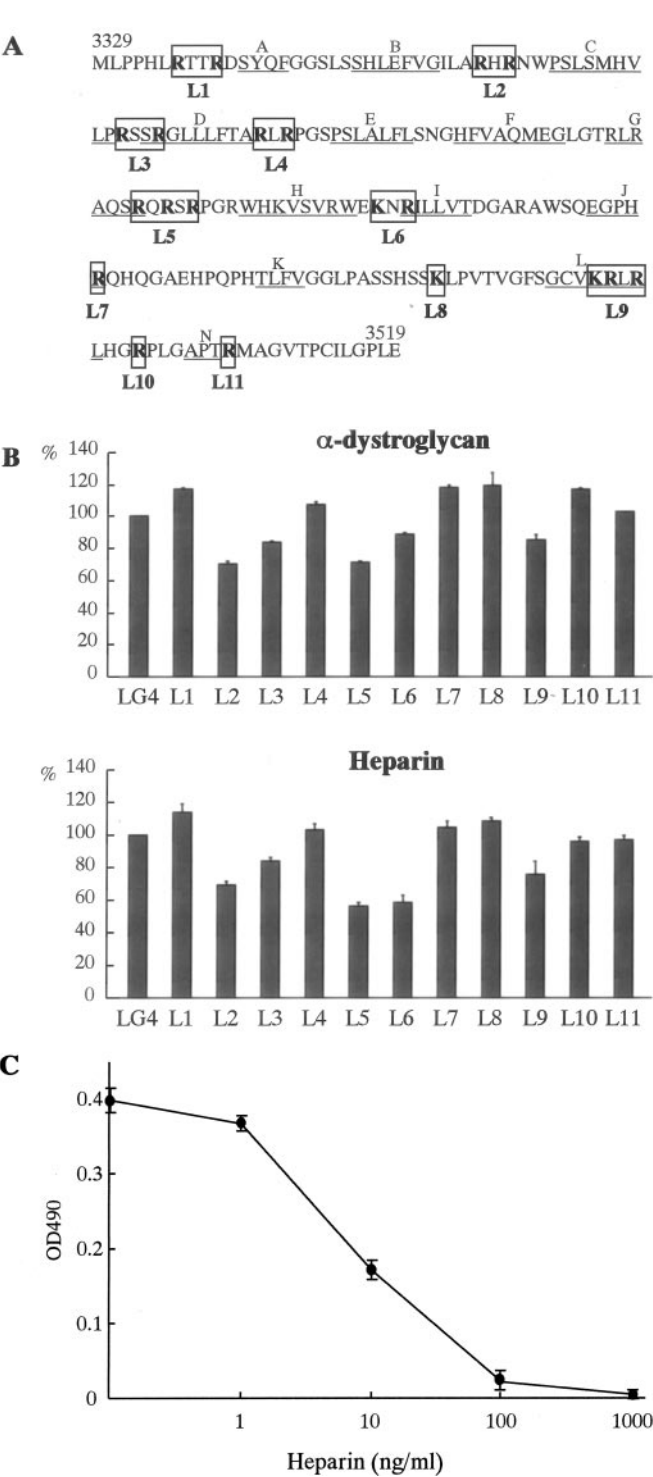


FIG. 7. α -Dystroglycan and heparin binding activities of LG4 mutants with alanine substitutions of basic amino acid residues. A, amino acid sequence of the LG4 module of the $\alpha 5$ chain. Putative β -sheet structures were deduced from the crystal structure of the LG4 module of the laminin $\alpha 2$ chain (25, 26). β -Sheets are indicated by underlined regions and letters A–N. The Arg and Lys residues indicated by bold letters in the boxed regions were substituted to alanine (designated L1–L11). B, binding activities of individual LG4 mutants for α -dystroglycan (upper panel) and heparin-BSA (lower panel). The averaged α -dystroglycan binding activity of GST-LG4 was taken as 100%. C, inhibition of α -dystroglycan binding of GST-LG4 by heparin. 10 nM GST-LG4 was incubated with microtiter plates coated with 5 μ g/ml α -dystroglycan in the presence of increasing concentrations of heparin for 1 h. Bound GST-LG4 was quantified with an anti-GST polyclonal antibody. Each point represents the mean of triplicate assays \pm S.D.

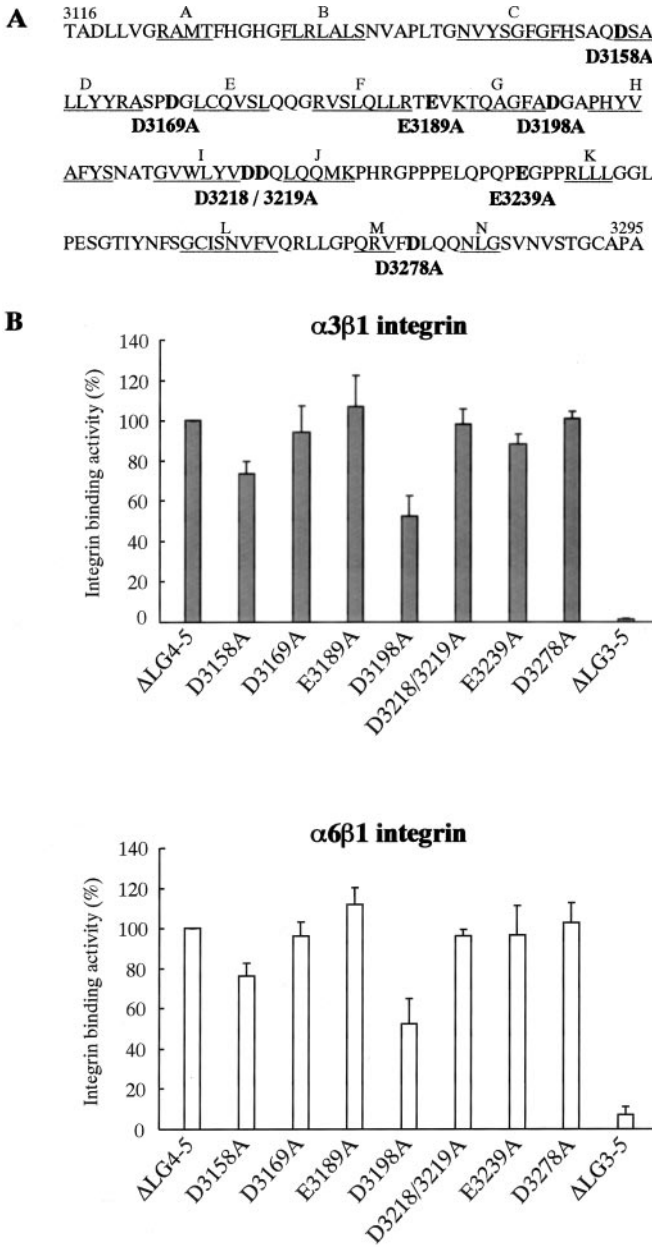


FIG. 8. Mapping the amino acid residues within the LG3 module involved in integrin binding. A, amino acid sequence of the LG3 module of the $\alpha 5$ chain. Putative β -sheets are indicated by underlined regions and letters A–N (25, 26). The Asp and Glu residues indicated by bold letters were substituted to alanine. B, integrin binding activities of rLN10 Δ LG4–5 and its alanine substitution mutants. Binding assays with 3 H-labeled phosphatidylcholine liposomes containing $\alpha 3 \beta 1$ (upper panel) or $\alpha 6 \beta 1$ (lower panel) integrins were performed as described under “Experimental Procedures.” The averaged integrin binding activity of rLN10 Δ LG4–5 was taken as 100%. Each bar represents the mean of triplicate assays \pm S.D.

except for $\alpha 2$ (12, 13, 18, 24, 30, 31). Consistent with previous observations, only the LG4 module was capable of binding to heparin among the five LG modules of the $\alpha 5$ chain when they were expressed as GST fusion proteins. The critical role of LG4 in heparin binding of laminin-10 was confirmed further by the absence of heparin binding activity in rLN10 lacking the LG4–5 modules. Although heparin and α -dystroglycan differ in their dependence on Ca^{2+} for binding to laminin-10, site-directed mutagenesis of the LG4 module indicated that both heparin and α -dystroglycan bind to overlapping sites in LG4. In support of this view, heparin competed with α -dystroglycan for the binding sites within LG4. Overlapping of the binding sites

for heparin and α -dystroglycan has also been documented for laminin-1 and -2 (12, 13, 25). Because alanine substitution of multiple basic amino acid residues within any single stretch of the oligopeptide sequences predicted to form loops resulted in only moderate reduction in the α -dystroglycan and heparin binding activities, it seems likely that the binding activities of LG4 toward α -dystroglycan and heparin are elicited by a cooperative interplay of multiple basic amino acid residues situated discretely over a broad range of the LG4 module, consistent with previous studies using site-directed mutagenesis of the LG4–5 modules of the $\alpha 2$ chain (13).

Integrin-mediated cell adhesion is the hallmark of the biological functions of laminin G domains. Many studies on the functional dissection of G domains have addressed the regions of various laminin isoforms that are responsible for the integrin-mediated cell adhesion. One approach to map the integrin binding sites within the G domain is to express individual LG modules separately or in tandem arrays and examine their cell adhesive activities. This approach has been successful in defining the binding sites for non-integrin-type receptors such as α -dystroglycan (12, 13) and syndecans (30, 32), as described above, but has suffered from difficulties in reproducing the full cell adhesive activities of intact laminins characterized by their specific binding to integrins (18, 33, 34). Similar difficulties have also been encountered in reproducing the activities of G domains with vast arrays of oligopeptides which together cover the entire G domains (35, 36). An alternative approach to circumvent these drawbacks is to produce mutant laminins with deletions or amino acid substitutions within the G domain in their trimeric configuration. This approach has been successful in narrowing down the major cell adhesive activities to the LG1–3 modules in laminin-2 (29) and laminin-5 (37), consistent with the previous observation that the E8 fragment of laminin-1 consisting of the $\alpha 1$ chain fragment containing LG1–3, but not LG4–5, exhibited a potent integrin-mediated cell adhesive activity upon heterotrimer formation with a truncated version of the β - γ dimer (38). Hirosaki *et al.* (37) demonstrated that further deletion of LG3 from recombinant laminin-5 resulted in an almost complete loss of cell adhesive activity, underscoring the importance of LG3 in the integrin-mediated cell adhesive activity of laminin-5, although they did not examine the direct integrin binding of their recombinant laminin-5. Our results are consistent with their report in that the LG3 module is indispensable for reproducing the potent cell adhesive activity of laminin-10 but provide further direct evidence that LG3 is required for the binding of laminin-10 to $\alpha_3\beta_1$ and $\alpha_6\beta_1$ integrins, the major adhesion receptors for laminin-10 (6). Given that cell adhesion to laminin-10 is mediated not only by these integrins but also by other non-integrin-type receptors such as α -dystroglycan and syndecans, cell adhesive activities *per se* may not be a reliable measure for defining the region(s) involved in integrin recognition by laminins.

Recently, Shang *et al.* (34) demonstrated that a bacterially expressed LG3 module of rat laminin-5 was active in promoting cell adhesion and migration in an $\alpha_3\beta_1$ integrin-dependent manner and capable of binding directly to $\alpha_3\beta_1$ integrin from cell lysates when immobilized on a column. The cell adhesive activity of the LG3 module was, however, significantly lower than that of intact laminin-5, requiring 250-fold more LG3 than intact laminin-5 to attain half-maximal cell adhesion. We carefully examined the cell adhesive as well as integrin binding activities of the LG3 module expressed in bacteria as a GST fusion protein or in mammalian cells as a fusion protein with FN70K but failed to detect any significant activities except that a very weak integrin binding activity was reproducibly detected with LG1, but not with LG3. Given that the activity

detected with the rat LG3 module was significantly lower than that of intact laminin-5 and that heterotrimerization with a truncated β - γ dimer was required for integrin binding activity of the α chain-derived fragment containing the LG1–3 modules (38, 39), the integrin binding activity of the LG3 module of laminin-10 *per se* may be very low and beyond the technical limits for detection. Shang *et al.* (34) proposed the possibility that the LG1 and/or LG2 modules function as synergy sites for LG3 to produce fully active integrin binding sites, as has been demonstrated for the integrin binding domain of fibronectin, in which the III-10 module containing the RGD cell adhesive motif needs to be connected with its preceding III-9 module to exert its full integrin binding activity (40). The role of LG1–2 modules may possibly be more conformational, stabilizing the active conformation of LG3 when connected adjacent to LG3 and assembled with the β - γ dimer.

The importance of the LG3 module in integrin binding of rLN10 was underscored further by the significant reduction in the integrin binding activity upon alanine substitution for Asp-3198 in LG3. Among the six Asp residues within the LG3 module which were substituted with alanine, only Asp-3198 resulted in a reduction in the integrin binding activity of rLN10 Δ LG4–5 upon alanine substitution, making it unlikely that the effect of Asp-3198 substitution was nonspecific, *e.g.* because of the reduced negative charge of LG3. Recently, Kariya *et al.* (41) reported that the substitution of three consecutive alanines for the Lys-Arg-Asp sequence within the LG3 module of laminin-5 strongly compromised the cell adhesive activity of recombinant laminin-5, which was mainly dependent on $\alpha_3\beta_1$ and $\alpha_6\beta_1$ integrins. Despite the similarity between laminin-5 and laminin-10 in their integrin binding specificities, *i.e.* both are high affinity ligands for $\alpha_3\beta_1$ and $\alpha_6\beta_1$ integrins (6), none of the single amino acid residues of the Lys-Arg-Asp sequence is conserved in the corresponding region of the LG3 module of the $\alpha 5$ chain, making it unlikely that the Lys-Arg-Asp sequence serves as part of the common recognition sites for $\alpha_3\beta_1/\alpha_6\beta_1$ integrins. In contrast, the Asp residue corresponding to Asp-3198 in the $\alpha 5$ chain is conserved in the LG3 module of the $\alpha 3$ chain. Because the reduction in the integrin binding activity of rLN10 Δ LG4–5 by alanine substitution for Asp-3198 was still partial, it is unlikely that Asp-3198 is the sole recognition site for $\alpha_3\beta_1/\alpha_6\beta_1$ integrins, but rather comprises part of the recognition site for $\alpha_3\beta_1/\alpha_6\beta_1$ integrins, possibly with putative synergy sites within the LG1–2 modules. In this respect, it is interesting to note that the LG1 module expressed as a GST fusion protein exhibited a weak, but reproducible, binding activity to $\alpha_3\beta_1/\alpha_6\beta_1$ integrins. The putative synergy sites in the LG1 module remain to be explored.

Our data show that laminin-10 binds to integrins and α -dystroglycan through distinct LG modules within the G domain. Thus, laminin-10 may well be able to utilize both cell surface receptors simultaneously, although the linker segment between the LG3 and LG4 modules of laminin-10 and other laminin isoforms has been shown to be frequently cleaved *in vivo* and *in vitro* (14, 42, 43), resulting in the loss of the α -dystroglycan binding site. The physiological significance of this cleavage at the linker segment and the resulting inactivation of α -dystroglycan binding activity remain to be elucidated, although it may be relevant to the assembly of laminin-10 into the basement membrane. There is some evidence that α -dystroglycan serves as a major cell surface receptor involved in the basement membrane assembly of laminins (44). It is tempting, therefore, to speculate that when laminin-10 is secreted by the cell, it first binds to α -dystroglycan to facilitate its assembly to the basement membrane, but then subsequent cleavage between the LG3 and LG4 modules removes the α -dystroglycan

binding site, resulting in the transfer of laminin-10 to $\alpha_3\beta_1$ and/or $\alpha_6\beta_1$ integrins, the major laminin-10-binding integrins capable of eliciting a series of transmembrane signaling events (45, 46). Although it is not clear how the cleavage between LG3 and LG4 affects the integrin binding activity of LG3, Hirosaki *et al.* (47) reported that the cleavage between LG3 and LG4 was associated with enhanced cell adhesive activities of α_3 chain-containing laminins. It remains to be defined whether the processing at the linker segment between LG3 and LG4 regulates the integrin binding activity of laminin-10.

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Molecular Dissection of the α -Dystroglycan- and Integrin-binding Sites within the Globular Domain of Human Laminin-10

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