| **Title** | Molecular Dissection of the α-Dystroglycan- and Integrin-binding Sites within the Globular Domain of Human Laminin-10 |
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| **Citation** | Journal of Biological Chemistry. 279(12) P.10946-P.10954 |
| **Issue Date** | 2004-03 |
| **Text Version** | publisher |
| **URL** | http://hdl.handle.net/11094/71431 |
| **DOI** | 10.1074/jbc.M313626200 |
| **rights** | |
| **Note** | |

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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 (α5β1γ1) for ααβ5 and α5β2 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 ααβ5 and α5β2 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 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 ααβ5 and α5β2 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 (α1β1γ1) and laminin-2 (α2β1γ1) in a Ca2+-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 (α5β1γ1) is a major laminin isoform widely expressed in adult tissues (15). Mice lacking the laminin α5 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 ααβ5 integrin (4), although adhesion of fibroblastic cells was mediated through both ααβ5 and α5β2 integrins (5). The roles of ααβ5 and α5β2 integrins as major receptors for laminin-10/11 were confirmed further by direct binding of laminin-10/11 to ααβ5 and α5β2 integrins (6). Recently, Yu and Talts (18) produced recombinant fragments modeled after the G domain of the mouse α5 chain and demonstrated that the fragment consisting of the LG1–3 modules had cell adhesive activity dependent on ααβ5 and α5β2 inte-
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 α5 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 used the deletions to determine binding activity towards αβ-integrins, α-dystroglycan, and heparin. Our results showed that the LG3 module is indispensable for binding to αβ-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

α-Dystroglycan and Integrin Binding Sites of Laminin-10

Expression vectors for laminin α5 subunit (GenBank accession AF444822) were amplified by reverse transcription-PCR using primers 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 α5 DNA was inserted into the NheII/Pmel sites of pcDNA3.1 (Invitrogen), yielding the α5 chain expression vector pcDNA-α5. Expression vectors for laminin β1 (pCEP-β1) and γ1 (pcDNA3.1-γ1) were prepared as described previously (19). Expression vectors for laminin α5 chains lacking LG5 (nucleotides 1–10554), pcDNA-α5L(5G), LG4–5 (nucleotides 1–9891; pcDNA-α5L(4G–5), LG3–5 (nucleotides 1–9360; pcDNA-α5L(3G–5), LG2–5 (nucleotides 1–8892; pcDNA-α5L(2G–5), and LG1–5 (nucleotides 1–8241; pcDNA-α5L(1G–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), 5795–9802 (for the deletion of LG3–5), 9985–9989 (for the deletion of LG4–5), and 9985–10554 (for the deletion of LG5) were amplified by PCR using KOD DNA polymerase (TOYOBO, Osaka, Japan) with an NolI (for the deletions of LG1–5, LG2–5, and LG3–5) or Asel (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 NolI and Asel, and the resulting cDNA fragments were recombined into the pcDNA-α5 vector cleaved with the same restriction enzymes.

Expression vectors for individual LG modules of the laminin α5 chain as GST fusion proteins were prepared as follows. cDNAs encoding the individual modules were amplified by PCR using pcDNA-α5 as a template. The PCR products were digested with EcoRI and Xhol and inserted into the corresponding restriction sites of the expression vector pFLAG-FN70K. The PCR primers used were 5′-ATATGGGCGCAAGGGGACTCGTG-3′ and 5′-ATAGTATGGGCGCAAGGGGACTCGTG-3′ (for LG5) and 5′-ATATGGGCGCAAGGGGACTCGTG-3′ and 5′-ATAGTATGGGCGCAAGGGGACTCGTG-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 these 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-α5L(4G–5 with Asel and Pmel and recloned into pSeetTag2A (Invitrogen) at the Asel/Pmel 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 primers used for the site-directed mutagenesis is available upon request. The purified PCR products containing the mutations were digested with Asel and Pmel and inserted into the corresponding restriction sites of the expression vector pcDNA-α5L(4G–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 α5, β1, and γ1 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 μg/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 α5 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 PBS (0.05 M NaCl, 0.05 M sodium phosphate, 0.1 M NaCl, pH 7.4) and 10 mM CaCl2.

Individual LG modules of the α5 chain produced as GST fusions were sequenced in Escherichia coli with 0.1 mM isopropyl-β-thiogalactopyranoside and purified on glutathione-Sepharose 4B columns (Amer sham Biosciences) after lysis of the cells by sonication. Bound proteins were eluted with 50 mM Tris-HCl, pH 8.0, containing 10 mM glutathione. Individual LG modules were further purified by anion exchange as insoluble aggregates upon sonication and then solubilized in PBS containing 8 M urea and dialyzed against PBS 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 PBS. Bound proteins were competitively eluted from the columns with 100 μg/ml FLAG peptide (Sigma) and dialyzed against PBS.

Proteins and Antibodies—αβ-integrins and β3-integrins were purified from human placenta and reconstituted into 3H-labeled phosphatidylcholine liposomes as described previously (6). α-Dystroglycan was purified from rabbit muscle membranes according to the published method with the following modifications. α-Dystroglycan partially purified using DEAE- Sephadex and wheat germ agglutinin-agarose chromatography was purified further by laminin-1 affinity chromatography, followed by CaCl2 gradient centrifugation (10). Heparin-BSA was purchased from Sigma. mAbs against the human laminin α5 chain (1H5 and 5D6) were produced in our laboratory (22). A mAb against human laminin γ1 (mAb 1920) was purchased from Chemicon. A mAb against human fibronectin (FN9-1) was obtained from Takara Biomedicals (Kyoto, Japan). A mAb against human β3 integrin (AIIB2) developed by Dr. Caroline Dansky (University of California, San Francisco) was obtained from the Developmental Studies Hybridoma Bank (Iowa City, IA). A polyclonal anti-human α-dystroglycan was raised in rabbits immunizing rabbit anti-human α-dystroglycan affinity purified and γ1 affinity-purified on a GST-conjugated Sepharose column.

Binding Assays for Integrins, α-Dystroglycan, and Heparin—Integrin binding assays were performed using αβ-integrins and β3-integrins reconstituted into 3H-labeled phosphatidylcholine liposomes as described previously (6). Briefly, 96-well microtiter plates were coated with the purified fibronectin (FN70K), designated FN70K-LG3 and FN70K-LG4, respectively, as described above. 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-5 (Sigma), yielding the expression vector pFLAG-FN70K for FN70K with a C-terminus modified by the expression vectors for fusion proteins of the LG3 and LG4 modules with the N-terminus modified by the expression vectors for fusion proteins of the LG3 and LG4 modules. The plate was coated with 50 μM α-dystroglycan (prepared 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 CaCl2/MgCl2 or 1 mM EDTA at room temperature for 1 h. After washing with TBS, bound proteins were quantified using 1% SDS and quantified by a Packard Tri-Carb 1500 liquid scintillation analyzer. For binding assays for α-dystroglycan and heparin, 96-well microtiter plates were coated with the purified fibronectin (FN70K) at 0.5 μg/ml. After incubating the coated plate with 0.05 mg/ml heparin, the wells were washed with TBS containing 1 mM CaCl2 and 1 mM NaCl, and the bound heparin-ligands were recovered with 1% SDS and quantified by a Packard Tri-Carb 1500 liquid scintillation analyzer. For binding assays for α-dystroglycan and heparin, 96-well microtiter plates were coated with the purified fibronectin (FN70K) at 0.5 μg/ml. After incubating the coated plate with 0.05 mg/ml heparin, the wells were washed with TBS containing 1 mM CaCl2 and 1 mM NaCl, and the bound heparin-ligands were recovered with 1% SDS and quantified by a Packard Tri-Carb 1500 liquid scintillation analyzer.
with the anti-lamin γ1 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 CO_2 incubator at 37 °C for 30 min, the attached cells were fixed and stained with Diff-Quick (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-β1 integrin mAb (AIIB2) 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 α5 chain, we expressed rLN10 and a series of its deletion mutants lacking LG modules (Fig. 1A) by triple transfection of cDNAs encoding the laminin α5, β1, and γ1 subunits into 293-F cells. Secretion of endogenous laminins containing β1 and/or γ1 chains was undetectable in 293-F cells (data not shown). Both wild-type and mutant proteins were purified from conditioned media using immunopurification columns conjugated with an anti-α5 chain mAb. To minimize the cleavage at the spacer segment between the LG3 and LG4 modules, wild-type rLN10 and its mutant lacking LG5 (rLN10ΔLG5) 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 α5 chain. Under reducing conditions, each recombinant protein gave three bands upon silver staining, one corresponding to the α5 chain, with a molecular mass of 300,000–400,000 depending on the extent of the deletion, and two lower bands corresponding to the β1 and γ1 chains (Fig. 1B). When wild-type rLN10 and rLN10ΔLG5 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 (rLN10ΔLG4–5; data not shown). Under nonreducing conditions, rLN10 and its deletion mutants barely entered the gel, confirming that they were purified as trimers of the α5, β1, and γ1 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 adhesion activities on HT1080 cells. rLN10ΔLG5 and rLN10ΔLG4–5 exhibited potent cell adhesion 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 β1 integrin, confirming the role of β1 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-α5 integrin and anti-α6 integrin mAbs, but not by either mAb alone (data not shown), consistent with previous observations (5). A dramatic loss of the cell adhesion 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 α5β1 and/or α6β1 integrins.

To confirm the importance of the LG3 module in the binding of laminin-10 to α5β1 and α6β1 integrins, we examined the binding of rLN10 and its mutants to these integrins. We purified α5β1 and α6β1 integrins from placenta and examined their binding to laminin-10 and its mutants after reconstitution into 3H-labeled phosphatidyicholine liposomes (Fig. 3). As expected, the mutants lacking the LG5 and LG4–5 modules were capable of binding to both α5β1 and α6β1 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...
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.

a-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 a-dystroglycan and heparin by solid phase binding assays using microtiter plates coated with a-dystroglycan or heparin-BSA. Wild-type rLN10 was capable of binding to a-dystroglycan in the presence of Ca2+ ions but not in the presence of EDTA (Fig. 4), consistent with previous reports that the binding of laminin-1/2 to a-dystroglycan is Ca2+-dependent (8–11). In contrast, wild-type rLN10 bound to heparin irrespective of the presence of Ca2+ or EDTA, confirming that heparin binding to laminin-10 does not require divalent cations, e.g. Ca2+ (12, 13, 24). Among the deletion mutants tested, rLN10ΔLG5 retained full binding activities toward a-dystroglycan and heparin, but other mutants including rLN10ΔLG4–5 were only marginally active at binding to either a-dystroglycan or heparin (Fig. 4). These results provide loss-of-function evidence that both a-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 a/β1 integrins and a-dystroglycan/heparin suggested that the LG3 module was the likely binding site for a/β1 and a/β1 integrins, whereas the LG4 module was the likely binding site for a-dystroglycan and heparin. To explore these possibilities further, we expressed the individual LG modules of the a5 chain in bacteria as GST fusion proteins (Fig. 5A) and assayed their abilities to bind a/β1 integrins as well as a-dystroglycan/heparin. Only GST-LG4 was capable of binding to a-dystroglycan and heparin among the five GST-LG modules (Fig. 5B), confirming that LG4 is the major binding site for both a-dystroglycan and heparin. In contrast, neither LG3 nor any of the other LG modules showed any significant binding to a/β1 or a/β1 integrins (Fig. 5C), except that GST-LG1 exhibited a very weak integrin binding activity. Because the failure of LG3 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 a-dystroglycan (Fig. 6A) and heparin (data not shown), but FN70K-LG3 did not show any significant binding to either a/β1 or a/β1 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 a-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 a1 chain and the LG5 module of the a2 chain participate in binding to a-dystroglycan and heparin (12, 13). These basic amino acid residues are predicted to be within the loops connecting adjacent β strands, based on the
amined whether heparin could compete with mostly overlap each other. To explore this possibility, we ex-

/H9251 binding to

and its deletion mutants. 96-well microtiter plates were coated with

MgCl2 (H9251 to

C

96-well microtiter plates were coated with

MgCl2 (H9251 to

C

represents the mean of triplicate assays

module (mutants designated L1

acid residues predicted within the loop regions of the LG4

and were indistinguishable from wild-type GST-LG4 (data not

GST-LG4 mutants showed identical electrophoretic mobilities

crystal structure of the LG4

residues involved in binding to

heparin were very similar, it is likely that the amino acid

binding profiles of the LG4 mutants toward

module to

–

L9 mutants (Fig. 7

), suggesting that the basic amino acid

B

glycan and heparin were observed with the L2, L3, L5, L6, and

L5 modules are

DISCUSSION

In the present study, we attempted to locate the binding sites

for α2β1/α2β1 integrins and α-dystroglycan within the G do-

main of laminin-10 by producing a series of deletion and sub-

stitution 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 en-

coding 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

α2β1/α2β1 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 α1 (12), α2 (13, 29), α4 (14), and α5

chains (18) have been shown to bind to α-dystroglycan, al-

though the LG1–3 modules of the α2 and α4 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 α1

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 α5 chain spans at least two LG modules in a manner

analogous to the interaction of the LG1–3 and LG4–5 modules of

the α2 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-dys-

troglycan was supported further by the observation that rLN10

lacking LG5 retained the full binding activity to α-dystrogly-

can, but rLN10 lacking both LG4 and LG5 was barely active.

The reason for this discrepancy remains to be clarified, al-

though it may be possible that the α-dystroglycan binding activity of LG4 is conformation-dependent and that the puta-

tive active conformation is stabilized by LG5 when connected in

both α-dystroglycan and heparin through closely overlapping,

if not identical, basic amino acid residues within LG4.
The expression of LG4 as fusion proteins with GST or FN70K could mimic the neighboring effect of LG5, allowing us to detect the \( \alpha / H9251 \)-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 \( \alpha / H9251 \)-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 \( \alpha / H9251 \)-dystroglycan because of masking of the epitope(s) by the bound \( \alpha / H9251 \)-dystroglycan.

Binding of laminin-1 and -2 to \( \alpha / H9251 \)-dystroglycan has been shown to be strictly Ca\(^{2+}\)-dependent. The crystal structure of the LG4–5 tandem modules of the \( \alpha / H9251 \)-2 chain revealed that two aspartic acid residues conserved in the LG modules of the \( \alpha / H9251 \) and \( \alpha / H9252 \) chains are involved in the Ca\(^{2+}\) binding and are therefore considered to be important in the Ca\(^{2+}\)-dependent \( \alpha / H9251 \)-dystroglycan binding (25, 26). However, these two aspartic acid residues do not seem to be conserved in the LG4 modules of other laminin \( \alpha \) chains including \( \alpha / H9255 \) (25). Nevertheless, our results clearly show that binding of rLN10 to \( \alpha / H9251 \)-dystroglycan is strictly Ca\(^{2+}\)-dependent, making it likely that the Ca\(^{2+}\) binding site(s) involved in the Ca\(^{2+}\)-dependent \( \alpha / H9251 \)-dystroglycan binding of laminin-10 are different from those in laminin-1 and -2. Consistent with this possibility, binding of laminin-8 to \( \alpha / H9251 \)-dystroglycan is also Ca\(^{2+}\)-dependent, although laminin-8 lacks the two aspartic acid residues equivalent to those conserved in the LG4–5 modules of the \( \alpha / H9252 \) chain.

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

\begin{footnote}{H. Ido, unpublished observation.}
\end{footnote}

position of the 45-kDa molecular size marker is shown in the left margin. B, \( \alpha / H9251 \)-dystroglycan and heparin binding activities of GST-LG modules. 96-well microtiter plates were coated with 5 \( \mu \)g/ml \( \alpha / H9251 \)-dystroglycan (upper panel) or 5 \( \mu \)g/ml heparin-BSA (lower 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 \( \alpha / H9251 \)-dystroglycan were performed in the presence of 1 mM CaCl\(_2/MgCl_2\). C, integrin binding activities of individual GST-LG modules. Microtiter plates were coated with 20 nM individual GST-LG modules and then incubated with \( 3^H \)-labeled phosphatidylcholine liposomes containing \( \alpha / \beta \) integrin (upper panel) or \( \alpha / \beta \) 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 ± S.D.
except for α2 (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 H9251 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 H9251-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 H9251-dystroglycan bind to overlapping sites in LG4. In support of this view, heparin competed with H9251-dystroglycan for the binding sites within LG4.

**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 α5 chain. Putative β-sheets were deduced from the crystal structure of the LG4 module of the laminin α2 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 μg/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 ± 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 α5 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 rLN10LG4–5 and its alanine substitution mutants. Binding assays with \(^{3}H\)-labeled phosphatidylcholine liposomes containing α5β1 (upper panel) or α6β1 (lower panel) integrins were performed as described under “Experimental Procedures.” The averaged integrin binding activity of rLN10LG4–5 was taken as 100%. Each bar represents the mean of triplicate assays ± S.D.
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 α2 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 specific binding to integrins (18, 33, 34). Despite the similarity between laminin-5 and laminin-10 in their integrin binding specificities, i.e. both are high affinity ligands for αβ1 and αβ2 integrins (6), none of the single amino acid residues of the Lys-Arg-Asp sequence serves as part of the common recognition sites for αβ3/αβ1 integrins. In contrast, the Asp residue corresponding to Asp-3198 in the α5 chain is conserved in the LG3 module of the α5 chain, making it unlikely that the Lys-Arg-Asp sequence serves as part of the common recognition sites for αβ3/αβ1 integrins. The putative synergy sites in the LG1 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 βγ 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/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 alanes 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 αβ1 and αβ2 integrins. Despite the similarity between laminin-5 and laminin-10 in their integrin binding specificities, several negative charge of LG3. Recently, Kariya et al. (41) reported that the substitution of three consecutive alanes for the Lys-Arg-Asp sequence within 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 βγ dimer.

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 $\alpha_5\beta_1$ 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 $\alpha_3$ 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.

Acknowledgments—We thank Noriko Sanzen for establishing the hybridoma clones and purifying the mAbs. We also thank Chisei Shimono and Dr. Ki-Hwan Kim for providing the laminin $\beta_1$ and $\gamma_1$ expression vectors. We are grateful to Dr. Tomohiko Fukuda and Dr. Hiroebu Fujiwara for valuable comments.

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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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J. Biol. Chem. 2004, 279:10946-10954.
doi: 10.1074/jbc.M313626200 originally published online December 29, 2003

Access the most updated version of this article at doi: 10.1074/jbc.M313626200

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