Laminin-6 Is Activated by Proteolytic Processing and Regulates Cellular Adhesion and Migration Differently from Laminin-5*

Received for publication, November 20, 2001, and in revised form, September 27, 2002
Published, JBC Papers in Press, October 11, 2002, DOI 10.1074/jbc.M111096200

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Laminin-6 (LN6) and laminin-5 (LN5), which share the common integrin-binding domain in the laminin α3 chain, are thought to cooperatively regulate cellular functions, but the former has poorly been characterized. Human fibrosarcoma HT1080 cells expressing an exogenous α3 chain were found to secrete LN6 with the full-length α3 chain and a smaller amount of its processed form lacking the carboxyl-terminal G4-5 domain, besides mature LN5 without G4-5 (mat-LN5). We prepared the unprocessed LN6 and mat-LN5, as well as LN6 mutants without G4-5 (LN6ΔG4-5) or G5 (LN6ΔG5). These laminins supported attachment of HT1080 cells and human keratinocytes (HaCaT) through integrins αβ1 and/or αβ1, LN6ΔG4-5, LN6ΔG5, and mat-LN5 promoted rapid cell spreading, whereas LN6 did hardly. A purified G4-5 fragment of the laminin α3 chain supported cell attachment through interaction with heparan sulfate proteoglycans and promoted cell spreading in combination with mature LN5 (mat-LN5). These results imply that the G4-5 domain within the LN6 molecule suppresses cell adhesion, while the released G4-5 promotes it. The presence of G5 rather than the heparin-binding domain G4 was responsible for the impaired cell spreading activity of LN6. However, the unprocessed LN6 promoted cell spreading in the presence of mat-LN5. Unlike mat-LN5, both LN6ΔG4-5 and LN6 did weakly or did not stimulate cell motility. These findings demonstrate that LN6 and LN5 have distinct biological activities, but they may cooperatively support cell adhesion. The proteolytic processing of the α3 chain seems to regulate the physiological functions of LN6.

The components of basement membranes not only support tissue architectures but also regulate various cellular functions, such as adhesion, migration, differentiation, growth, and apoptosis. The regulatory functions of basement membranes can be largely attributed to the interaction of laminins with their receptors. Genetically distinct five α, three β, and three γ subunits of laminin form specialized heterotrimers (laminins-1 to -15) that are expressed in a tissue-specific manner during embryonic development as well as in the adult (1). All laminin α subunits share a large globular domain at their carboxyl-terminal region (G domain), which consists of five homologous globular subdomains (or modules) of about 200 amino acids each (G1–G5 or LG1–LG5). This region contains binding sites for cellular matrix proteins (e.g. perlecain and fibulin-1), as well as cellular receptors including integrins, syndecans, and α-dystroglycan (1).

Laminin-5 (LN5),1 which consists of α3, β3, and γ2 chains, was originally found as an anchoring filament component of keratinocytes (kalinin/epiligrin/nicein) (2–4) and as a cell-scattering factor secreted by gastric carcinoma cells (ladsin) (5). LN5 is unique in its biological activity and structure. It potently stimulates both cell adhesion and cell motility in culture (5–7). LN5 is currently the only laminin molecule with truncations in the amino-terminal regions (or the short arms) of all three subunits. The laminin β3 and γ2 subunits are found only in LN5, whereas the α3 chain is found in laminin-6 (LN6, α3β1γ1), laminin-7 (LN7, α3β2γ1), and laminin-13 (LN13, α3β2γ3). LN5 is secreted by various types of epithelial cells in tissues and in culture, and assembled into the basement membrane structures in vivo. The interaction of LN5 with integrin αβ1 in the hemidesmosome structures is essential for stable attachment of basal cells to their substrate, polarization of the epithelial sheet, and stabilization of cellular architecture through the intermediate filament (2, 3).

LN5 is synthesized and secreted in a precursor form containing a 190-kDa α3, a 140-kDa β3, and a 150-kDa γ2 chains, but the α3 and γ2 chains undergo proteolytic processing to smaller species of 160 kDa and 105 kDa, respectively (8). Recent studies suggest that post-translational processing of LN5 molecules modulates their function. Proteolytic cleavage of the γ2 chain by MMP-2 (gelatinase A) or MT1-MMP increases the cell motility activity of LN5 (9, 10) but decreases the cell adhesion activity (11). Similarly, it has been reported that the processing of the 190-kDa α3 chain to the 160-kDa one by plasmin converts the precursor LN5 from a migration ligand to an anchorage-dependent cell attachment ligand (12). However, the mature LN5 with the processed α3 chain has been shown to strongly promote cell motility or cell scattering via integrin αβ1 binding sequence (15). Taken together, these past studies indicate that the G3-1 domain of the α3 chain is the primary site

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1 The abbreviations used are: LNx, laminin-x; HSPG, heparansulfate proteoglycan; mat-LN5, mature LN5 without G4-5 domain of laminin α3 chain; LN6ΔGx, LN6 lacking Gx domain of laminin α3 chain; α3G4-5, a G4-5 fragment of laminin α3 chain.
to bind cell surface receptors, but the short arms of the y2 and possibly 3 chains are likely to affect the biological activity of LN5.

To clarify the structure and function relationship of LN5, it seems important to characterize LN6, because these two laminins share the common a3 subunit and are often produced by the same cell types. LN6 was first identified as k-laminin in culture media of human keratinocytes and a squamous cell carcinoma line (8, 16). In human amnion, about half-amount of LN5 is expressed as LN6 or LN7. The relative shadowed image analysis of these laminin and immunoblot analysis suggests that LN5 binds with LN6 or LN7 through the interaction of their short arms. It is assumed that the complex formation allows stable association of LN5 with the basement membrane in the amnion (17). However, no previous studies have shown the biological activity of LN6 or LN7 presumably because of the difficulty in isolating these laminins in LN5-free forms. In the present study, we isolated LN6 as an unprocessed single protein and as truncated forms lacking the G4-5, G3-5, or G5 domain of the chain. This report describes the biological activities of the four forms of LN6, as well as their differences from LN5.

EXPERIMENTAL PROCEDURES

Cell Culture—Human fibrosarcoma cell line HT1080 was obtained from Japanese Cancer Research Bank (Tokyo, Japan). Four transfectants of HT1080 cells, HT1080/WT, HT1080/G5, HT1080/G4-5, and HT1080/G5-3 were isolated in our recent study (14). HT1080/WT cells have been stably transfected with a full-length cDNA of human laminin a3 subunit, and HT1080/G5, HT1080/G4-5, and HT1080/G5-3 with mutant cDNAs for the laminin a3 chains lacking the G5, G4-5, and G3-5 domains, respectively. Buffalo rat liver-derived epithelial cell line BRL has been described in our previous studies (5, 14). These cell lines were maintained in 1:1 mixture of Dulbecco’s modified Eagle’s medium and Ham’s F12 medium (Dulbecco’s modified Eagle’s medium/F12) (Invitrogen) supplemented with 10% fetal bovine serum, penicillin, and streptomycin sulfate. A spontaneously immortalized human keratinocyte cell line, HaCaT (18), was a generous gift from Dr. N. Fusenig (Deutsches Krebsforschungszentrum, Heidelberg, Germany). HaCaT cells were maintained in Dulbecco’s modified Eagle’s medium.

Antibodies and Other Materials—Mouse monoclonal antibodies against human laminin a3 chain (LS34c4) and y2 chain (D4B5) were previously established and characterized (14, 19). A rabbit polyclonal antibody against the G4 domain of laminin was previously established and characterized (14, 19). A rabbit polyclonal antibody against human laminin b1 chain (VNR147) and mouse monoclonal antibodies against human laminin y1 chain (2E8) and against human integrin a2 (P1D6) and b3 (6E6) subunits from Chemicon International Inc. (Temecula, CA); mouse monoclonal antibodies against integrin a1 (P1E6) and a3 (P1B5) subunits from Invitrogen; and a rat monoclonal antibody against human integrin a(v) (GOH3) from BD PharMingen (San Diego, CA). Mouse EHS-laminin (laminin-1, LN1), human laminin-10/11 (LN10/11), and human plasma fibronectin were purchased from Transduction Laboratories, Invitrogen, and Asahi Technoglass (Tokyo, Japan), respectively.

Purification of LN5 and LN6—Various recombinant LN5 forms have been isolated from the conditioned media of HT1080 transfectants (14). In the present study, LN5 and three forms of LN6 were purified by basically the same method as before. Briefly, the serum-free conditioned medium of HT1080/WT cells was fractionated by molecular-sieve chromatography on a Sepharose-4B column (Amersham Biosciences) and then eluted with a heparin-Sepharose column (Amersham Biosciences). Proteins bound to the heparin column were eluted with 0.5 M NaCl and then with 1.0 M NaCl. Each fraction was passed through a gelatin-Sepharose column to remove fibronectin, and then subjected to two kinds of immunoaffinity chromatographies. LN5 was purified from the 0.5 M NaCl eluate using a LS34c4-Sepharose (anti-a3 chain antibody) column and a D4B5-Sepharose (anti-y2 chain antibody) column as reported previously (14). To isolate LN6 from the NaCl eluate from the heparin column, the NaCl concentration was 10-fold to decrease the NaCl concentration, and then applied to a Q-Sepharose HPLC column (Amersham Biosciences). The materials eluted between 0.3 and 0.4 M NaCl were passed through the D4B5-Sepharose column to remove LN5. Finally, the unbound materials were applied to the LS34c4-Sepharose immunoaffinity column. Bound LN6 was eluted with 0.05% trifluoroacetic acid and immediately neutralized. LN6 mutants without the G5 domain of laminin a3 chain (LN6aG5), without the G4-5 domain (LN6aG4-5), and without the G3-5 domain (LN6aG3-5) were purified from the conditioned media of HT1080/G5, HT1080/G4-5, and HT1080/G3-5 cells, respectively, by essentially the same procedure as above.

Purification of LN6—Laminin a3 Chain—The G5-4 fragment of laminin a3 chain (a3G4-5) was isolated according to the recently published procedure with some modifications (13). Briefly, the concentrated conditioned medium of HT1080/WT cells was fractionated on a Sepharose 4B column and then on a heparin-Sepharose column. Proteins bound to the heparin column at 0.1 M NaCl were eluted with 0.4 M NaCl. A blank value corresponding to an empty well was subtracted. After incubation at 37°C, adherent cells were fixed with 2.5% glutaraldehyde and stained with 0.005% Hoechst 33342 and 0.001% Triton X-100 for 1.5 h. The fluorescent intensity of each well of the plates was measured using a CytoFluor 2350 fluorometer (Millipore, Bedford, MA). A blank value corresponding to an empty well was automatically subtracted.

Assay of Cell Attachment—The cell attachment assay was performed as described previously (14). 96-well microtiter plates (Corning Costar, Acton, MA) were coated with various substrates in Ca2+- and Mg2+-free saline at 4 °C overnight, and then blocked with 1% gelatin in PBS at 37°C for 1.5 h. Cells were plated at a density of 24,000 cells/well. The plates were incubated at 37°C, adherent cells were fixed with 2.5% glutaraldehyde and stained with 0.005% Hoechst 33342 and 0.001% Triton X-100. The fluorescent intensity of each well of the plates was measured using a CytoFluor 2350 fluorometer (Millipore, Bedford, MA). A blank value corresponding to an empty well was automatically subtracted.

Assay of Cell Scattering and Cell Migration—Cell scattering activity of various forms of laminins toward BRL cells was assayed as reported previously (14). Cells were plated at a density of 7,000 cells/well onto 24-well plates (Sumibe Medical, Tokyo, Japan) containing 0.5 ml/well of Dulbecco’s modified Eagle’s medium/F12 plus 1% fetal bovine serum. In the standard assay, test samples were directly added into the culture and incubated at 37°C for 24 h. After 2 days, cell scattering was judged by microscopic observation. Alternatively, test samples were coated on 24-well plates as described above, and the plates were used for assays of cell scattering and cell migration. Cell migration on these substrates was monitored at 37°C with a time-lapse video. The length of cell migration was measured with a video micrometer (VM-30; Olympus, Tokyo, Japan).

RESULTS

Unprocessed and Processed Forms of LN6—It was previously found that when a cDNA for human laminin a3 chain is transfected into human fibrosarcoma cell line HT1080, the exogenous a3 chain is assembled with the endogenous b3 and y2 chains to produce the LN5 hetrotrimer of a3b3y2 (14). In that study, the conditioned medium of the HT1080 transfectant (HT1080/WT) contained both the 190-kDa and 160-kDa a3 chains, but only the LN5 with the 160-kDa a3 chain, which had been proteolytically cleaved between the G3 and G4 domains, was purified from the conditioned medium. Antibodies to the laminin b3 chain and to the y2 chain precipitated the 160-kDa a3 chain but not the 190-kDa one from the conditioned medium, suggesting the presence of a3-containing laminin(s) other than LN5. In the present study, the conditioned medium of HT1080/WT cells was analyzed by two-dimensional SDS-PAGE and the following immunoblotting with the antibodies to the laminin a3, y2, b3, and y1 chains. On the first dimensional non-reducing SDS-PAGE, the a3 chain was separated into at
least four different molecular sizes, over 1,000 kDa (the top of gel), 600, 450, and 400 kDa (Fig. 1 A). The 450- and 400-kDa a3 chains were associated with the y2 chain, showing that they were the LN5 forms with the 150- and 160-kDa y2 chains, respectively (Fig. 1 B). This demonstrated that the 190-kDa a3 chain had completely been converted to the 160-kDa mature form in LN5 molecule. On the other hand, the a3 chain in the 600-kDa complex was not associated with the y2 chain (Fig. 1 B). On the second dimensional reducing SDS-PAGE, the a3 chain at this position was separated into a 190-kDa major spot and a 160-kDa minor one. Laminin a1 and y1 chains were separated as broad bands, showing that they existed as different complexes including the a1-y1 heterodimer (Fig. 1, C and D). The a1 and y1 chains were found at the position of the 600-kDa a3 complexes. These results suggested that the 190-kDa a3 chain might exist as LN6 (a2β1γ1). The results also imply that the proteolytic processing of the laminin a3 chain occurs preferentially in LN5 in the HT1080/WT cells. The high-molecular weight aggregate of the a3 chain on the top of gel was not further investigated in this study.

To characterize the 600-kDa complexes containing the laminin a3 chain, we attempted to isolate the laminin isoforms from the HT1080/WT conditioned medium. The conditioned medium was fractionated by the molecular-sieve chromatography, followed by the heparin affinity chromatography. Both the 190- and the 160-kDa a3 chains bound to the heparin column, but the former was eluted at 1.0 M NaCl, whereas the latter was mainly eluted at 0.5 M NaCl (data not shown). The difference in the affinity to heparin was consistent with our previous observation that the G4-5 fragment of the a3 chain tightly binds to a heparin column (13). The eluted 190-kDa a3 chain was further purified by an anion-exchange HPLC and then passed through the anti-laminin γ2 antibody column to remove LN5. Finally, the material was bound to an immunoadfinity column conjugated with the anti-laminin a3 antibody and eluted therefrom.

The purified material was analyzed by SDS-PAGE and immunoblotting with five kinds of antibodies. The isolated protein migrated as a single band of about 600 kDa slightly beneath mouse LN1 under non-reducing conditions (Fig. 2A). Reducing SDS-PAGE resolved the 600-kDa laminin into three bands with molecular masses of 220, 210, and 190 kDa (Fig. 2B), and these bands were identified as the laminin a1, y1, and a3 chains, respectively (Fig. 2C). Neither anti-β nor anti-γ2 antibody reacted with any of the three bands (Fig. 2C). These results indicate that the purified 600-kDa laminin is LN6. The LN5 purified by the immunoadfinity chromatography with the anti-laminin γ2 antibody contained only the 160-kDa form of the a3 chain, in addition to the 135-kDa β3 chain and the 150/105-kDa γ2 chain (hereafter referred as mature LN5; mat-LN5) (Fig. 2, B and C).

We also purified a small amount of LN6 with the 160-kDa a3 chain from the 0.5 M NaCl eluate from the heparin column. The 160-kDa a3 chain in this preparation was not reactive with the antibody against the G4 domain (data not shown). This indicated that the 160-kDa a3 chain had been cleaved between the G3 and G4 domains just like the 160-kDa a3 chain in mat-LN5. Therefore, we decided to purify this laminin at a higher yield from the conditioned medium of HT1080/G4-5 cells, which had been transfected with the cDNA for the laminin a3 chain lacking both G4 and G5 domains (14). By the same purification procedure as above, a LN6 mutant, which lacked the G4 and G5 domains, was purified. This preparation, named LN6ΔG4-5, contained a 220-kDa β1 chain, a 210-kDa γ1 chain, and a 160-kDa a3 chain as analyzed by SDS-PAGE under reducing conditions (Fig. 2, B and C). Furthermore, we prepared a LN6 mutant, which lacked the G3, G4, and G5 domains, named LN6ΔG3-5, from the HT1080/GΔG3-5 cells (14).

Cell Attachment and Spreading Activities of LN6—To characterize LN6, we compared some biological activities of the three forms of LN6 (LN6, LN6ΔG4-5, and LN6ΔG3-5), mat-LN5 and some other matrix proteins using three different cell lines: human keratinocyte cell line HaCaT, human fibrosarcoma cell line HT1080, and the rat liver cell line BRL. First, attachment of HaCaT cells was examined by inoculating these cells onto plastic plates precoated with different concentrations

![Fig. 1. Analysis of laminin α3, γ2, β1, and γ1 subunits secreted from HT1080/WT cells by two-dimensional SDS-PAGE. Serum-free conditioned medium of HT1080/WT cells was concentrated and analyzed by two-dimensional SDS-PAGE (non-reducing and reducing SDS-PAGE), followed by immunoblotting with an antibody to the laminin α3 chain (LS03E4; panel A), the γ2 chain (D4B5; panel B), the β1 chain (VNR147; panel C), or the γ1 chain (2E8; panel D). Arrowsheads indicate estimated molecular sizes in kDa of protein spots in the first dimension (abscissa) and in the second dimension (ordinate). Molecular sizes in kDa of marker proteins are shown at the left side.](image1)

![Fig. 2. SDS-PAGE and immunoblotting analyses of purified mat-LN5, LN6, and LN6ΔG4-5. Panel A, non-reducing SDS-PAGE of mat-LN5 (lane 1), LN6 (lane 2), and mouse LN1 (lane 3) on a 5% gel followed with silver staining. Panel B, reducing SDS-PAGE of mat-LN5 (lane 1), LN6 (lane 2), and LN6ΔG4-5 (lane 3) on a 6% gel and silver staining. Panel C, immunoblotting after reducing SDS-PAGE of mat-LN5 (lane 1), LN6 (lane 2), and LN6ΔG4-5 (lane 3) with monoclonal antibodies specific for laminin α3, β1, γ1, β3, and γ2 chains from the left to the right. Ordinate, molecular size in kDa.](image2)
of the protein substrates (Fig. 3A). mat-LN5, LN6, and LN6ΔG4-5 efficiently supported the cell attachment, but LN6ΔG3-5 did hardly. Compared on a molar basis assuming a similar coating efficiency, the cell attachment efficiencies of mat-LN5, LN6, and LN6ΔG4-5 were comparable, but higher than those of LN1, LN10/11, and fibronectin. The marked difference of the cell attachment activity between LN6ΔG4-5 and LN6ΔG3-5 suggests that the G3 domain is essential for the high affinity binding of LN6 to integrins. Essentially the same dose-response curves were obtained with HT1080 cells (data not shown). In the case of the BRL cells, LN6ΔG3-5 showed a cell attachment activity similar to those of LN1 and LN10/11 (Fig. 3B).

Morphology of HaCaT cells on the different substrates was examined (Fig. 4, a–d). Despite the apparently similar cell attachment activity of mat-LN5, LN6, and LN6ΔG4-5, there was a marked morphological difference between the two LN6 forms. Rapid and extensive spreading of HaCaT cells was induced on mat-LN5 or LN6ΔG4-5, whereas on the LN-6 substrate they barely spread but exhibited some small spikes (or projections). HaCaT cells displayed more flattened morphology on LN10/11 than on mat-LN5 or LN6ΔG4-5. Poor cell spreading on LN6 was reproduced in HT1080 cells (Fig. 4e–h) and BRL cells (data not shown). These results demonstrate that the proteolytic processing of lamin a3 chain converts LN6 from the inactive form to the active form regarding its cell spreading activity.

To compare integrin requirement of mat-LN5 and two forms of LN6 (LN6 and LN6ΔG4-5), effects of function-blocking anti-integrin antibodies on the attachment of HaCaT cells were examined. The cell attachment activity of LN6 was completely blocked by the anti-integrin α6 or the anti-integrin β1 antibody (Fig. 5A). Almost the same results were obtained for LN6ΔG4-5 and mat-LN5 (data not shown). HaCaT cells are known to express both integrins α6β1 and α6β2 (21). Therefore, our results imply that in HaCaT cells integrin α6β1 is the primary receptor for these laminins, and integrin α6β2 is not functional at least as the initial receptor. On the other hand, attachment of HT1080 cells to LN6 was partially blocked by the anti-integrin α6 antibody (P1E6), anti-α6 antibody (P1B5), anti-α6 antibody (P1D6), anti-α6 antibody (G0H3), or anti-β1 antibody (656) in suspension at room temperature for 15 min, plated onto 96-well plates precoated with 1 μg/ml LN6, and incubated for 30 min in the presence of the antibodies. The cell attachment in the presence of control mouse IgG was taken 100%. Each bar represents the mean and S.D. for triplicate assays. Other experimental conditions are the same as described in the legend to Fig. 3.

Characteristics of Laminin-6

FIG. 3. Cell attachment activity of LN6, LN6ΔG4-5, LN6ΔG3-5, mat-LN5, and three other proteins toward HaCaT and BRL cells. 96-well plates were coated with the indicated concentrations of each substrate and incubated with HaCaT (panel A) and BRL (panel B) cells in serum-free medium at 37 °C for 1 h. After the incubation, relative numbers of cells attached to the substrates were determined by measuring fluorescent intensity. Each point represents the mean ± S.D. for triplicate determinations. Other experimental conditions are described under “Experimental Procedures.” LN6 (open circle), LN6ΔG4-5 (open square), LN6ΔG3-5 (open diamond), mat-LN5 (open triangle), mouse LN1 (filled triangle), LN10/11 (filled circle), fibronectin (filled square).

FIG. 4. Morphology of HaCaT and HT1080 cells on mat-LN5, LN6, LN6ΔG4-5, and LN10/11. Plastic plates were coated with 0.5 μg/ml mat-LN5 (a and e), 1 μg/ml LN6 (b and f), 1 μg/ml LN6ΔG4-5 (c and g), or 4 μg/ml LN10/11 (d and h). HaCaT cells (a–d) and HT1080 cells (e–h) were plated on each substrate and incubated for 60 min. Other experimental conditions are the same as described in the legend to Fig. 3. Scale bar, 40 μm.

FIG. 5. Effects of function-blocking antibodies against various integrin subunits on attachment of HaCaT (panel A) or HT1080 (panel B) cells to LN6 substrate. The cells were preincubated with anti-α6 antibody (P1E6), anti-α6 antibody (P1B5), anti-α6 antibody (P1D6), anti-α6 antibody (G0H3), or anti-β1 antibody (656) in suspension at room temperature for 15 min, plated onto 96-well plates precoated with 1 μg/ml LN6, and incubated for 30 min in the presence of the antibodies. The cell attachment in the presence of control mouse IgG was taken 100%. Each bar represents the mean and S.D. for triplicate assays. Other experimental conditions are the same as described in the legend to Fig. 3.

Cell Motility Activity of LN6—LN5 promotes not only cell adhesion but also cell scattering and migration (5, 6). Both cell
scattering and migration on LN5 are though to reflect the enhanced cellular motility. Although most of adherent cultured cell lines efficiently adhere to LN5, a limited number of cell lines are responsive to the cell-scattering and cell migration activities of LN5 (5). Because HaCaT and HT1080 were poorly responsive to the cell motility activity of LN5, BRL cells were used to compare this activity between LN5 and LN6. When each laminin was directly added into the culture of BRL cells in a medium containing 1% fetal bovine serum, the typical cell morphology. BRL cells (7 × 10^3 cells/well) were incubated in culture medium supplemented without (a) or with 50 ng/ml of mat-LN5 (b), LN6 (c), or LN6ΔG4-5 (d) in the presence of 1% fetal bovine serum on 24-well plates. The pictures were taken under a phase-contrast microscope after 2 days of incubation. Panel A, cell scattering activity of soluble laminins. mat-LN5 (open square), LN6 (open circle), and LN6ΔG4-5 (filled triangle) were directly added at the indicated final concentrations into the culture medium as shown in panel A, and scattered cells were counted after 2 days of incubation. Panel C, cell scattering activity of insoluble laminins. Each well of 24-well plates was coated with the indicated concentrations of mat-LN5 (open square), LN6 (open circle), or LN6ΔG4-5 (filled triangle). After BRL cells were incubated on the plates for 2 days, scattered cells were counted. Panel D, cell migration on insoluble laminins. Each well of plastic plates was coated with the indicated concentrations of mat-LN5 (dotted bar), LN6 (hatched bar), or LN6ΔG4-5 (black bar). BRL cells were incubated on non-coated wells (clear bar), and the laminin-coated wells in the medium containing 1% fetal bovine serum. The migration of BRL cells was monitored by video microscopy for 10 h, and the migration distance was quantified. Each bar represents the mean migration speed and S.D. for optional 10 cells.

When the cell scattering activity was assayed on the plastic plates precoated with various concentrations of each laminin, mat-LN5 again promoted prominent cell scattering (Fig. 6C). LN6ΔG4-5 induced weak cell scattering, but scarcely LN6 (Fig. 6C). We also analyzed the migration of BRL cells on the laminin-coated plates by the video-microscopy (Fig. 6D). mat-LN5 supported the highest cell migration, whereas LN6 did not stimulate the cell migration. LN6ΔG4-5 stimulated the cell migration dose-dependently, but to a lesser extent than mat-LN5. These results demonstrate that LN6ΔG4-5 has a lower cell motility activity than mat-LN5 though they share the common α3 subunit. The results also imply that a relatively low cell motility activity is acquired in LN6 by the loss of the G4-5 domain. The complete lack of the cell motility activity in LN6 may be related with its inability to support cell spreading.

Roles of α3G4-5 Domain and Its Released Fragment—It has been reported that bacterially expressed recombinant G4 and G5 domains of laminin α3 chain show cell adhesion activity that is inhibited by heparin (15, 24). LN5-producing cell lines secrete the G4-5 fragment of α3 chain (α3G4-5) into the culture medium (13). In the culture of HT1080/WT cells, α3G4-5 was seen in the extracellular matrix fraction, as well as in the conditioned medium (data not shown). It indicates that a part of the released G4-5 fragment is assembled into the matrix. This was true in the culture of HaCaT cells.

To analyze the biological activity of the G4-5 domain, we purified α3G4-5 from the conditioned medium of HT1080/WT cells. The purified α3G4-5 protein exhibited a single band of ~45 kDa on SDS-PAGE under both non-reducing and reducing conditions (Fig. 7A). Plastic plates precoated with α3G4-5 supported the attachment of HaCaT cells (Fig. 7B). The effective concentration for cell attachment was similar in a weight concentration (μg/ml) between α3G4-5 and LN6, indicating that the specific activity in a molar concentration of α3G4-5 was less
a polyclonal antibody against the G4 domain (H9251) toward HaCaT cells. In this experiment, cells were trypsinized, in LN6. cooperation with mat-LN5 or LN6/H9004 SDS-PAGE of purified 3G4-5 is in contrast to the predicted role of the G4-5 domain. We also investigated the cell surface receptors of LN6. 3G4-5 fragment supports cell adhesion independently or in presence (H9262) cells. 96-well plates were coated with 0.125 g/ml LN6 (Fig. 7). The attachment of HaCaT cells to G4-5 was almost completely inhibited by either EDTA or heparin (Fig. 8A), but not by the anti-integrin β1 antibody (data not shown). Pretreatment of HaCaT cells with heparitinase also inhibited their attachment to G4-5 (Fig. 8A). These results indicate that the cell attachment to G4-5 is mediated by heparin-like non-integrin receptors, presumably heparan sulfate proteoglycans (HSPGs). Some divalent cations seem to be required for the active structure of the receptors or G4-5. Furthermore, heating at 90 °C almost completely abolished the cell attachment activity of G4-5 (data not shown), indicating that it requires the heat-sensitive conformation.

To show the interaction of unprocessed LN6 with HSPGs, the inhibitory effects of heparin and heparitinase treatments were examined. The attachment of HaCaT cells to LN6 was blocked completely by EDTA but only slightly by the heparin or heparitinase treatment of cells (Fig. 8B). A similar weak inhibition of cell attachment by heparin was obtained when LN6G4-5 was used as the substrate (data not shown). When HT1080 cells were used, neither heparin nor heparitinase treatment inhibited the cell attachment to LN6 (Fig. 8C). These results demonstrate that the interaction between the G4-5 within LN6 and cell surface HSPGs is not involved in the cell attachment to LN6. We also found that the treatment of HaCaT cells with heparitinase or heparin did not induce cell spreading on LN6 (data not shown). This also implies that the impaired cell spreading on LN6 is not due to the interaction between its G4-5 domain and cell surface HSPGs.

As a reason for the impaired cell spreading on LN6, we supposed a mechanism in which G4-5 in LN6 might interfere with the interaction between G1-3 and cell surface integrins by a steric hindrance. To examine this possibility, we prepared a LN6 mutant without G5, named LN6G5, from the conditioned medium of HT1080/G5 cells (14), and compared the cell spreading activity toward HaCaT cells among the three LN6 forms (LN6, LN6G4-5, and LN6G5). LN6G5 promoted spreading of HaCaT cells at almost the same level as LN6G4-5 (Fig. 9A). In addition, there is no significant difference in the cell attachment activity toward HaCaT cells among the three LN6 forms (Fig. 9B). Similar results were obtained with HT1080 cells (data not shown). These results clearly indicated that the presence of G5 in LN6, rather than the heparin-binding domain G4, suppresses the cell spreading. LN6G5 showed essentially the same cell spreading activity as LN6G4-5 (data not shown).
Fig. 9. Cell adhesion activity of LN6ΔG5 toward HaCaT cells. Panel A, morphology of HaCaT cells on plastic plates precoated with 1 µg/ml each of LN6 (a and d), LN6ΔG5 (b and e), or LN6ΔG4-5 (c and f). Cells were plated on each substrate and incubated for 20 min (a–c) or 60 min (d–f). Panel B, attachment of HaCaT cells to plastic plates precoated with various concentrations of LN6 (open circle), LN6ΔG5 (filled triangle), or LN6ΔG4-5 (open square). The cell attachment was determined by measuring fluorescent intensity. Each point represents the mean ± S.D. Other experimental conditions are the same as described in the legend to Fig. 3.

Functional Interplay of LN5 and LN6—LN6 was originally found as the covalent complex with LN5 (17). Since these laminins are expected to cooperatively function in vivo, we examined cooperative effects of unprocessed LN6 and mat-LN5 on the adhesion of HaCaT cells (Fig. 10). LN6 did not support cell spreading by itself even at 1 µg/ml, but it stimulated cell spreading in the presence of a low concentration of mat-LN5 that did not support cell spreading. This suggests the possible cooperative action of LN6 and mat-LN5 in vivo. mat-LN5 at a concentration higher than 0.5 µg/ml supported cell spreading within 20 min by itself. An excess amount of LN6 did not have any significant effect on the spreading of HaCaT cells induced by mat-LN5 alone.

We further examined the direct interaction of LN6 and mat-LN5. Simple mixing of purified LN6 and mat-LN5 in solution did not lead to the formation of LN5-LN6 complex as analyzed by immunoprecipitation (data not shown). In addition, we could not identify the LN5-LN6 complex in the conditioned medium of the HT1080/WT cells.

DISCUSSION

The laminin α3 chain forms the trimeric assemblies of α3β3γ2 (LN5), α3β1γ1 (LN6), α3β2γ1 (LN7), or α3β2γ3 (LN13). Of these laminins, LN5 is known to promote both cell adhesion and migration. The unique activities of LN5 are likely to be in large part mediated by the interaction of the carboxyl-terminal globular domain with cell surface receptors. This predicts that the other three α3-laminins (LN6, LN7, and LN13) may have a similar activity to LN5. In the present study, we found that the HT1080 cell line transfected with the laminin α3 chain cDNA (HT1080/WT) secreted LN6 with a 190-kDa α3 chain and a small amount of its processed form with a 160-kDa α3 chain lacking the G4-5 domain, as well as the mature LN5 with the 160-kDa α3 chain (mat-LN5). We isolated the unprocessed LN6 from HT1080/WT cells, as well as three recombinant LN6 forms lacking the G4-5 domain (LN6ΔG4-5), the G5 domain (LN6ΔG3-5), or the G5 domain (LN6ΔG5) from different cDNA transfectants of HT1080 cells. The complex analysis of the biological activities of these LN6 forms and mat-LN5 disclosed interesting differences. First, the unprocessed LN6 and LN6ΔG4-5 showed a marked difference in capability to promote cell spreading. LN6 and LN6ΔG4-5, as well as mat-LN5, showed an apparently similar and high cell attachment activity through integrins α1β1 and α5β1. This indicates that LN6 and LN5 have a common receptor-binding specificity, and that the presence of G4-5 domain has practically no effect on the integrin-binding specificity. However, morphological examination revealed that LN6ΔG4-5 and mat-LN5, both of which lack the G4-5 domain in the α3 chain, promoted cell spreading efficiently, but the unprocessed LN6 did not. This implies that the G4-5 domain of LN6 negatively regulates cell spreading. Second, there is a clear difference in stimulation of cell migration between LN6 and LN5. Especially when added into culture medium, the two forms of LN6 showed no cell scattering activity toward BRL cells, whereas mat-LN5 strongly stimulated cell scattering.

Recent studies have suggested that LN5 interacts with integrins through the G1-3 domain of the laminin α3 chain (14, 15). Especially, G3 seems to play an important role in regulating cell adhesion and migration (14, 25). In this study, we found that LN6ΔG4-5 promoted cell adhesion much more efficiently than LN6ΔG3-5, which lacked G3-5, in good agreement with our previous findings on the role of the G3 domain in LN5. On the other hand, the function of the G4-5 domain has not been understood even in LN5. Bacterially expressed recombinant G4 and G5 have been reported to show a weak cell attachment activity, presumably binding to cell surface HS PGs (15). More recently, the recombinant G4 protein of the α3 chain, but not G5, was shown to bind heparin and syndecans-2 and -4 (24). We have found that the recombinant G4 protein binds syndecans-1 and -4 extracted from the membrane fraction of HT1080 cells. Furthermore, the natural G4-5 fragment released from the precursor LN5 has a strong heparin binding activity and stimulates cell spreading in the presence of a low concentration of mat-LN5 (13). There are reports showing that the G4 or G5 domains of other laminin α chains contain binding sites for

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syndecans and α-dystroglycan, despite their relatively low sequence homology among different α chains (26–29).

In the present study, the secreted G4-5 fragment supported cell attachment by binding cell surface HSPGs, and in combination with a low concentration of LN6ΔG4-5 or mat-LN5 it promoted even cell spreading. It is very likely that the G4-5 fragment induces an intracellular signaling to promote cell adhesion through interaction with cell surface HSPGs such as syndecans. We also found that the G4-5 fragment was deposited on the matrix produced by the LN5-secreting cells. These results strongly suggest that in vivo the G4-5 fragment released from LN5 contributes to cellular adhesion, presumably cooperating with mat-LN5 and other cell adhesion molecules. This biological activity of the G4-5 fragment is apparently contradictory to the finding that the G4-5 domain in the LN6 molecule rather suppresses the cell spreading and motility activities of LN6. Although the cell adhesion to the G4-5 fragment was completely blocked by heparin or heparitinase treatment of the target cells, the impaired cell spreading activity of LN6 was not rescued by either treatment. We also found that not only LN6ΔG4-5 but also LN6ΔG5 supports cell spreading. The major heparin-binding site in the G domain of the α3 chain has been shown to be located in G4 but not G5 (24). All these facts clearly indicate that the lack of cell spreading activity in LN6 is not due to the interaction between G4-5 and HSPGs. The presence of the G5 domain of the α3 chain rather than the heparin-binding domain G4 is responsible for the impaired cell spreading activity of LN6. A recent study has demonstrated the crystal structure of the G4-5 domain of laminin α2 chain (30). Based on the analysis, a model of the entire G domain of laminin α chain has been proposed (31). This model predicts that the G1-3 domain of laminins has a shape of a cloverleaf in contact with the rod domain, and G5 is located closer to the G1-3 cloverleaf than G4. In this model, G5 is very likely to reduce or interfere with the interaction between the G1-3 domain and integrins by a steric hindrance. If this is true in LN6, deletion of G5 or G4-5 from the entire G domain should allow efficient G1-3 binding to integrins. Our experimental results agree well with this model, strongly suggesting that G4-5 in LN6 partially masks the integrin-binding site in the G1-3 domain of the α3 chain. It is also noted that the proteolytic processing of the α3 chain occurs in LN5 and LN6 with different efficiency. In HT1080/WT cells, the α3 chain of LN5 was completely processed to the 160-kDa form, whereas that of LN6 remained in large part intact. It is very likely that the cleavage site between G3 and G4 is exposed in LN5 but masked in LN6. This also suggests a special conformation of the G domain in LN6. On the other hand, it is well known that integrins are colocalized or associated with not only syndecans (32, 33) but also other membrane proteins including tetraspanins (transmembrane-4 superfamily proteins) (34, 35). We cannot exclude the possibility that the interaction of G5 with such integrin-associated proteins other than HSPGs suppresses the integrin-induced cytoskeletal changes.

The present study also demonstrated that LN6 and LN5 have distinct biological activities though they share the common α3 subunit. The poor cell motility activity of LN6ΔG4-5 as compared with mat-LN5 indicates that the activity of mat-LN5 depends not only on the α3 chain but also on the other two chains y2 and β3. Indeed, it has been reported that a specific cleavage of the y2 chain of mat-LN5 by matrix metalloproteinases increases the ability of mat-LN5 to stimulate cell migration (9, 10). It was also reported that the mat-LN5 with the unprocessed y2 chain has a higher cell adhesion activity than that with the processed y2 chain (11). However, it remains to be clarified how the y2 and/or β3 chains affect the interaction of the G domain of the α3 chain with integrins. All of the LN5 subunits (α3, β3, and y2) have the truncated amino-terminal structures, and the β3 and y2 chains are found only in LN5. This unique amino-terminal structure of LN5 may be responsible for the high cell motility activity.

Physiological roles of LN6 are mostly unknown. This laminin isoform has been found in the cultures of keratinocytes and squamous carcinoma (8, 16), and in tissue extracts from the skin and the amnion (17). Approximately half of the LN5 extracted from the amnion and the skin is covalently associated with LN6 or LN7 (17). LN5 is thought to play an essential role in the epithelial-stromal attachment as a monomeric form and as a complex form with LN6 or LN7. Monomeric LN5 works as the primary bridge between integrin αEβ1 in the hemidesmosomes and type VII collagen in the stroma (36). LN6 cannot substitute for LN5 in stabilizing epithelial attachment because it does not bind to type VII collagen (36). This is also supported by the fact that genomic mutations in not only the laminin α3 but also the β3 or γ2 chain result in Herlitz’s junctional epidermolysis bullosa, which exhibits severe detachment of the epidermis from the dermis (37–39). On the other hand, LN6 and LN7 have a nidogen-binding site identified within the laminin γ1 chain (40), and the VI domains of both the β1 (or β2) and γ1 chains allow the assembly of the laminin network. Therefore, the formation of the LN5-LN6 or LN5-LN7 complex may be able to mediate the stable epithelial attachment and basement membrane assembly. In the complex, LN5 appears to associate with LN6 or LN7 through binding of the short arms from both laminins (17). In vivo the carboxyl-terminal globular domain of the LN5 α3 chain in the LN5-LN6 or LN5-LN7 complex is likely to bind to integrin αEβ1, or αEβ1, while that of the LN6 α3 chain or LN7 α3 chain may be free (36). This model appears to be consistent with our finding that the unprocessed LN6 itself has a very poor cell spreading activity. Although mat-LN5, LN6, and LN6ΔG4-5 recognize both integrins αEβ1, and αEβ1, the affinity of the unprocessed LN6 to integrins may be far lower than that of mat-LN5 due to the presence of G4-5. Therefore, we suppose a model that the G4-5 in LN6 may interact with HSPGs assembled into the matrix to stabilize the LN5-LN6 or LN5-LN7 complex. This model is supported by a recent study showing that in laminin-2 the G4-5 domain contributes to basement membrane assembly (41).

In the present study, we could not detect the LN5-LN6 complex in the conditioned medium of HT1080/WT cells. Even if the purified mat-LN5 and LN6 were mixed in solution, the LN5-LN6 complex was not detected, suggesting that they do not have a high affinity. Special microenvironment or conditions may be necessary for the complex formation. We also found that the monomeric LN6 poorly supported cell spreading by itself, but it promoted cell spreading in the presence of mat-LN5. This suggests a synergistic action of the two free laminins to support cell adhesion in vivo. On the other hand, the loss of G4-5 from LN6 by proteolytic processing leads to the production of the active LN6 and the G4-5 ligand. The processed monomeric LN6 (LN6ΔG4-5) stimulates epithelial cell adhesion and migration more effectively than the unprocessed LN6. Although it is unknown where and when LN6 undergoes the proteolytic processing of the α3 chain in vivo, this event may be important under some physiological and pathological conditions that induce expression of LN6 and LN7 (42).

Acknowledgments—We thank T. Izumi and K. Suzuki for technical assistance and Drs. H. Yasumitsu and S. Higashi for helpful discussions.

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