Characterization of Laminin 5B and NH$_2$-terminal Proteolytic Fragment of Its $\alpha$3B Chain

**PROMOTION OF CELLULAR ADHESION, MIGRATION, AND PROLIFERATION**

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Various laminin isoforms have specific biological functions depending on their structures. Laminin 5A, which consists of the three truncated chains $\alpha$3A, $\beta$3, and $\gamma$2, is known to have strong activity to promote cell adhesion and migration, whereas a laminin 5 variant consisting of a full-sized $\alpha$3 chain ($\alpha$3B) and the $\beta$3 and $\gamma$2 chains, laminin 5B, has not been characterized yet. In the present study, we for the first time cloned a full-length human laminin $\alpha$3B cDNA and isolated the human laminin 5B protein. The molecular size of the mature $\alpha$3B chain (335 kDa) was approximately twice as large as the mature $\alpha$3A chain in laminin 5A. Laminin 5B had significantly higher cell adhesion and cell migration activities than laminin 5A. In addition, laminin 5B potently stimulated cell proliferation when added into the culture medium directly. Furthermore, we found that the $\alpha$3B chain undergoes proteolytic cleavage releasing a 190-kDa NH$_2$-terminal fragment. The 190-kDa fragment had activities to promote cellular adhesion, migration, and proliferation through its interaction with integrin $\alpha$6$\beta$1. These activities of the NH$_2$-terminal structure of the $\alpha$3B chain seem to contribute to the prominent biological activities and the physiological functions of laminin 5B.

Laminins are an important family of extracellular matrix proteins, which are mainly localized in the basement membranes of various tissues and regulate various cellular functions including cell adhesion and migration (1, 2). The different combinations of five $\alpha$, three $\beta$, and three $\gamma$ chains give rise to at least 15 laminin heterotrimers with the well-known cross-shaped structure (1). Of the three laminin subunits, the $\alpha$ chain is most important in determining the tissue-specific distribution and specific biological activity of the laminin isoforms (3). The laminin $\alpha$ chains are classified into two groups, “full-sized” ($\alpha$1, $\alpha$2, $\alpha$3B, and $\alpha$5) and “truncated” ($\alpha$3A and $\alpha$4) (1). The amino-terminal region of the full-sized $\alpha$ chains contains three globular domains (VI, IVb, and IVa) and three rodlike domains (V, IIIb, and IIIa) consisting of laminin type epidermal growth factor repeats. The laminin NH$_2$-terminal domain VI, which is completely absent in the truncated $\alpha$ chains (3A and 4), is thought to be essential for the self-assembly and copolymerization of laminins into large noncovalent networks in the basement membranes and have several cell-binding sites (1). On the other hand, all $\alpha$ chains contain a globular (G) domain consisting of five laminin G modules (G1 to G5) at the COOH terminus. The COOH-terminal G domain of the $\alpha$ chains plays major roles in the interaction with cell surface receptors such as integrins, syndecans, and dystroglycan (4–6).

Typical laminins are produced by the assembly of different $\alpha$ chains with the laminin $\beta$1 or $\beta$2 and $\gamma$1 chains, whereas laminin 5 (LN5)$^1$ is produced by the combination of $\alpha$3A, $\beta$3, and $\gamma$2 chains. The $\beta$3 and $\gamma$2 chains are found only in LN5, and all the three LN5 subunits are truncated in the short arms (NH$_2$-terminal regions). Consistent with the unique structure, LN5 has unique biological activities. LN5 strongly promotes cellular scattering, adhesion, and migration of various types of cells compared with other laminins and extracellular matrix proteins (7–9). These activities are mediated by the interaction with integrin receptors, $\alpha$6$\beta$1, $\alpha$4$\beta$1, and $\alpha$4$\beta$6 (10, 11). In the skin, the association of LN5 with integrin $\alpha$4$\beta$6 is critical to form the hemidesmosome structure, which supports the stable adhesion of basal keratinocytes to the underlying connective tissues (12–14). Therefore, functional defects of LN5 cause a lethal skin disease, Herlitz’s junctional epidermolysis bullosa (15, 16). On the other hand, the cell migration-promoting activity of LN5 is thought to contribute to wound healing (17, 18) and tumor invasion (19).

Cloning of the laminin $\alpha$3 chain cDNA has revealed two distinct mRNA transcripts encoding the truncated, $\alpha$3A chain ($\alpha$3EpA) and the full-sized, $\alpha$3B chain ($\alpha$3EpB) (17, 20). The expression of two transcripts is regulated by alternative splicing and distinct promoters (21). The complete sequence of the $\alpha$3B chain has recently been deduced from the sequences of partial cDNA clones and genomic DNA clones in mice (3, 20, 22) and humans (23, 24). However, neither complete cDNA clones

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The nucleotide sequence(s) reported in this paper has been submitted to the GenBank™/EBI Data Bank with accession number(s) AB107369.

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$^1$ The abbreviations used are: LN5, laminin 5; LN5A, laminin 5A; LN5B, laminin 5B; BSA, bovine serum albumin; CBB, Coomassie Brilliant Blue; FCS, fetal calf serum; DMEM, Dulbecco’s modified Eagle’s medium; PBS, phosphate-buffered saline; EGF, epidermal growth factor; HEK, human embryonic kidney; CHAPS, 3-(3-cholamidopropyl)dimethylammonio)-1-propanesulfonic acid.
for the α3B chain nor the α3B-containing laminins have been isolated so far. The α3B chain is expected to associate with the laminin β3 and γ2 chains and with the laminin β1/β2 and γ1 chains to produce laminin 5B (LN5B) and laminin 6B/7B, respectively, because the α3A and α3B chains share the common I/II domain, which is essential for the coiled-coil structure of the three subunits. LN5B consists of the full-sized α3 chain (α3B) and the truncated β3 and γ2 chains, and thereby is expected to have a long rodlike structure. This type of laminin isoform is not known apart from LN5B. Because the laminin α3B chain is more widely and more intensively expressed than the α3A chain in mouse and human tissues (20, 24), LN5B seems to be a major LN5 isoform in adult tissues.

In the present study, we cloned a human laminin α3 chain cDNA and for the first time isolated and characterized the human LN5B protein from HEK293 cells, which had been transfected with the cDNAs for the three LN5B subunits. The results demonstrate unique biological activities of this novel LN5 isoform and an NH2-terminal, proteolytic fragment of the α3B chain. To distinguish the two forms of LN5, we designate the well characterized LN5 consisting of the α3A, β3, and γ2 chains as LN5A in this report.

EXPERIMENTAL PROCEDURES

Materials—Human laminin 10/11 was purchased from Invitrogen (Carlsbad, CA). Mouse EHS laminin 1, human laminin 2/4 (merosin), and human fibronectin were purchased from Chemicon (Temecula, CA). Human recombinant LN5A was purified as described previously (27). Mouse monoclonal antibodies against the human laminin β3 chain (kalinin B1) was purchased from Transduction Laboratories (Lexington, KY). Function-blocking anti-integrin antibodies used were the anti-α2-integrin antibody (P1E6), the anti-α5-integrin antibody (P1B5), the anti-α6-integrin antibody (P1D6), and the anti-β3-integrin antibody (6S6) from Chemicon, and the anti-α4-integrin antibody (GoH3) from Pharmingen (San Diego, CA).

Cells and Culture—The human embryonic kidney cell line HEK293 (ATCC CRL-1573) was purchased from American Type Culture Collection (ATCC) (Rockville, MD). The human bladder carcinoma cell line EJ-1 and the Buffalo rat liver-derived epithelial cell line BRL have been used in previous studies (26). A spontaneously immortalized human keratinocyte cell line, HaCaT, was a generous gift from D. N. E. Fusing (Deutsches Krebsforschungszentrum, Heidelberg, Germany). These four cell types were maintained in DMEM/F-12 medium (Invitrogen) supplemented with 10% fetal calf serum (FCS), penicillin, and streptomycin sulfate. The human mammary epithelial cell line MCF-10A (ATCC CRL-10317) was obtained from ATCC, and cultured in DMEM/F-12 supplemented with 20 ng/ml epidermal growth factor (EGF), 100 ng/ml cholera toxin, 0.01 mg/ml insulin, 500 ng/ml hydrocortisone, and 5% horse serum.

Expression Vector Constructions—The expression vectors of human laminin α3, β3, and γ2 chains have been described previously (25). A human laminin α3B chain cDNA was constructed as follows. Because approximately half of the α3B sequence is identical to the α3A sequence, we used the α3A cDNA LS/CX encoding the COOH-terminal sequence of the α3B chain (28). The remaining α3B cDNA sequence was deduced from a previously reported partial sequence (GenBank™ accession no. AF005258) and the sequences of two genomic clones, RP1-609K12 and RP1-666022 (GenBank™ accession nos. AC067796 and AC090366, respectively). To obtain overlapping cDNA clones, a human lung 5′-Stretch Plus cDNA library (Clontech, CA) was screened and amplified by the ECL direct nucleic acid labeling/detection system and PCR with Ex Taq polymerase (Takara, Tokyo, Japan), respectively. A cDNA fragment of 900 bp in the 5′ region was synthesized as three oligonucleotides and amplified by PCR. All PCR-derived cDNA fragments were cloned into the pGEM T-Easy vector (Promega, Madison, WI), and their sequences were verified. All primers used for PCR procedures are listed in Table I. The different cDNA fragments that overlap each other were ligated. The full-length laminin α3B cDNA consisted of 10,002 bp and had an open reading frame encoding 3,333 amino acids (GenBank™ accession no. AB107369). To prepare an expression vector, we constructed a truncated α3B cDNA of 8,832 bp that had an open reading frame encoding 2,944 amino acid residues without the COOH-terminal G4-G5 domain. This truncated α3B cDNA was inserted into the ECoV sites of pcDNA3.1/Hygro (+) mammalian expression vector (Invitrogen) in correct orientation and used as LN3B3#3 pcDNA3.1 Hygro (+). Expression and Purification of LN5B—HEK293 cells were serially transfected with the expression vectors of the laminin γ2 chain and laminin β3 chain using the LipofectAMINE PLUS (Invitrogen) as described previously (25). A HEK293 clone highly expressing both β3 and γ2 chains, β3-γ2-HEK, was finally transfected with the LN5B3#3 pcDNA3.1 Hygro (+), and stable transfectants were selected with 100 μg/ml hygromycin (Wako, Osaka). Three HEK293 cell clones highly expressing the three LN5B chains were selected, and clone 9 was used as LN5B-HEK unless otherwise indicated.

For purification of LN5B, the serum-free conditioned media from β3-γ2-HEK293 cells and LN5B-HEK293 cells were collected in roller bottles, which had been precoated with poly-L-lysine, and proteins were precipitated by 80% saturated ammonium sulfate. The precipitate was dissolved in and dialyzed against 20 mM Tris-HCl buffer (pH 7.5) containing 0.5 M NaCl, 0.005% (w/v) BriJ35, and 0.1% (w/v) CHAPS, and then applied to molecular sieve chromatography on a Sepharose 4B column (Amersham Biosciences) pre-equilibrated with the same buffer. Fractions containing LN5B were pooled and applied to a gelatin-Sepharose 4B column to remove fibronectin. LN5B in the unbound fractions from the gelatin column was purified by immunoaffinity chromatography with the anti-laminin α3 monoclonal antibody Ls3c3. Bound proteins were eluted from the affinity column with 0.05% (v/v) trifluoroacetic acid and immediately neutralized to pH 7.0 to 7.5 with a small volume of 1 M Tris-HCl (pH 8.0). The LN5B protein thus purified was stored in the presence of 0.005% BriJ35 and 0.1% CHAPS. Protein concentrations were determined using a Bio-Rad protein assay kit with bovine serum albumin (BSA) as a standard.

Purification of 190-Kd NH2-terminal Fragment of Laminin α3B Chain—The serum-free conditioned medium from LN5B-HEK293 cells was fractionated by molecular-sieve chromatography on a Sepharose 4B column as described above. Fractions containing the 190-Kd, proteolytic fragment of laminin α3B chain were pooled, dialyzed against 20
Immunoﬂuorescence Microscopy—Two hundred microliters of the cell suspension (2 × 10⁵ cells/ml) in DMEM/F-12 medium supplemented with 10% FCS was inoculated per well of Lab-Tek 8-well chamber slides (Nunc, Naperville, IL). After incubation for 2 days, the cells were washed with PBS (−) and then ﬁxed in 3.7% (w/v) formaldehyde in PBS (−) for 15 min. For permeabilization, the cells were treated with 0.2% (v/v) Triton X-100 in PBS (−). The ﬁxed cells were blocked with 1.2% BSA in PBS (−) for 1 h and then incubated with a primary antibody diluted in the BSA-containing buffer for 1 h. A ﬂuorescein isothiocyanate-coupled secondary antibody (Vector Laboratories, Burlingame, CA) was used for detection. F-actin was stained using rhodamine phalloidin (Molecular Probes). Fluorescence images were obtained using a fluorescence microscope (BX50-FLA; Olympus, Tokyo, Japan) equipped with 100 ×/1.35 UPlan-Apochromat oil immersion objectives.

Cell Adhesion Assay—Cell adhesion assay was performed as described previously (26). Brieﬂy, each well of 96-well enzyme-linked immunosorbent assay plates (Costar, Cambridge, MA) was coated with a substrate protein and then blocked with 1% BSA. Cells (2 × 10⁴ cells) were inoculated per each well containing serum-free DMEM/F-12 medium, and incubated at 37 °C for 1 h. After nonadherent cells were removed, adherent cells were ﬁxed and stained with Hoechst 33432. The ﬂuorescent intensity of each well of the plates was measured using a CytoFluor 2350 ﬂuorometer (Millipore, Bedford, MA). For inhibition assay, the cell suspension was incubated with function-blocking anti-integrin antibodies for 20 min at room temperature before inoculation.

Assays of Cell Scattering and Migration—The cell-scattering and cell migration activities of LN5A and LN5B proteins were assayed as reported previously (26). BRL cells were suspended in DMEM/F-12 plus 1% FCS and inoculated into each well of 24-well plates at a density of 7 × 10⁴ cells/well. Each test sample was directly added to the cultures and incubated at 37 °C for approximately 40 h. For the cell migration assay, EJ-1 cells (2.0 × 10⁶ cells in DMEM/F-12 plus 1% FCS) were inoculated per well of 24-well plates precoated with a test protein. After the preincubation for 1.5 h at 37 °C, cell movement was monitored using a time-lapse video equipment for 8 h.

RESULTS

Secretion of LN5B by β3γ2-HEK Cells—The laminin α3B chain has been reported to be expressed in HEK293 cells (24). We detected none of the laminin α3B, β3, and γ2 chains in the culture medium of HEK293 cells by immunoblotting analysis (data not shown). However, when HEK293 cells were transfected with the cDNA expression vectors for the β3 and γ2 chains, the conditioned medium of the HEK293 transfectant (β3γ2-HEK) showed a weak cell-scattering activity toward BRL cells, suggesting that the heterotrimer of LN5 or LN5B was secreted to the culture medium (data not shown). We puriﬁed this cell-scattering factor from the serum-free conditioned medium of β3γ2-HEK cells by immunoaffinity chromatography with a monoclonal antibody against the laminin α3 chain. The puriﬁed protein had activities to promote cell adhesion, migration, and scattering (data not shown). SDS-PAGE under nonreducing conditions resolved the puriﬁed protein into four major bands at ~630, 580, 435, and 385 kDa (Fig. 1A, lane 2), of which the latter two bands were similar in size to the two LN5A forms (Fig. 1A, lane 1). Under reducing conditions, the puriﬁed protein was resolved into ﬁve major bands of 335, 150, 145, 135, and 105 kDa (Fig. 1A, lane 4). These bands were reactive to one of the antibodies against the three LN5 subunits in immuno-
Characterization of Laminin 5B and Its Proteolytic Fragment

Fig. 2. Comparison of domain structures among laminin α3B, α3A, α1, and α5 chains. Three globular domains (VI, IVb, and IVa) in the NH₂-terminal region of the α chains are indicated by ovals, and three rodlike EGF repeats (V, IIIb, and IIIa) are shown as vertical rectangles (1). The COOH-terminal globular domain consists of five globular modules (G1-G5). Domain II/I is the coiled coil-forming domain. The NH₂-terminal amino acid sequences for the 335-kDa α3B chain (ATARDPGA), the 190-kDa fragment chain (ATARDPGA), and the 145-kDa α3B and α3A chains (DSSPAEE) were determined by amino acid sequencing and are indicated with bold letters. The last amino acid residue number in each domain is indicated above the schematic diagram of the α3B chain. Arrows indicate proteolytic cleavage sites of the α3B and α3A chains to produce the 145-kDa α3B/A chains. Arrowheads indicate the cleavage sites within the G domain.

 blotting analysis, and the three smaller protein bands were also found in LN5A (data not shown). Because the 335-kDa band was recognized by the anti-laminin α3 antibody, the purified protein was thought to be LN5B. However, the yield of LN5B was too low for further characterization.

Cloning of Human Laminin α3B Chain cDNA—We previously established the LN5A-producing HEK293 cell line, LN5-HEK, which had been transfected with the laminin α3A, γ2, and β3 expression vectors (25). To express recombinant LN5B in HEK293 cells, a cDNA encoding the human laminin α3B chain was cloned as described under “Experimental Procedures.” The deduced full-length cDNA sequence consisting of 10,002 base pairs had an open reading frame encoding 3,333 amino acids (GenBank™ accession no. AB107369) (Fig. 2). Like the other full-length α chains, the short arm of the human laminin α3B chain can be separated into six domains (VI, V, IVb, IIIb, IVa, and IIIa), and the total molecular size is larger than the α1 and α2 chains but smaller than the α5 chain. Because the LN5B secreted from β3γ2-HEK cells lacked the G4-G5 domain of the α3 chain as a result of the proteolytic processing as in the case of LN5A (29), we constructed a truncated cDNA of 8,832 base pairs, which has an open reading frame encoding the α3B sequence (2,944 amino acid residues) without the G4-G5 domain (Fig. 2). This expression vector, named LNa3B#3 pcDNA3.1 Hygro (+), was used to express a recombinant LN5B in HEK293 cells.

Establishment and Characterization of HEK293 Cells Overexpressing LN5B—To establish LN5B-producing HEK293 cells, HEK293 cells were sequentially transfected with the laminin α3B chain cDNA of 8,832 base pairs, which has an open reading frame encoding the 335-kDa α3B chain (ATARDPGA), the 190-kDa fragment chain (ATARDPGA), and the 145-kDa α3B and α3A chains (DSSPAEE). Domain II/I is the coiled coil-forming domain. The NH₂-terminal amino acid sequences for the 335-kDa α3B chain (ATARDPGA), the 190-kDa fragment chain (ATARDPGA), and the 145-kDa α3B and α3A chains (DSSPAEE) were determined by amino acid sequencing and are indicated with bold letters. The last amino acid residue number in each domain is indicated above the schematic diagram of the α3B chain. Arrows indicate proteolytic cleavage sites of the α3B and α3A chains to produce the 145-kDa α3B/A chains. Arrowheads indicate the cleavage sites within the G domain.

When the serum-free conditioned medium of LN5B-HEK cells was analyzed by nonreducing SDS-PAGE, the two major bands of 630 and 580 kDa and a trace of the 435- and 385-kDa bands were directly detected by the CBB staining in a high molecular mass region (Fig. 1B, lane 2). On reducing SDS-PAGE, the major protein bands found in the LN5B purified from β3γ2-HEK cells (Fig. 1A, lane 4) were all detected in the conditioned medium (Fig. 1B, lane 4). In addition, the conditioned medium contained a 190-kDa protein, which was not found in the purified LN5B. These results indicated that LN5B was a major secreted protein in LN5B-HEK cells.

Deposition of the LN5 isoforms was compared between LN5B-HEK and LN5-HEK cell lines. When the extracellular matrix proteins deposited by LN5B-HEK cells were analyzed by reducing SDS-PAGE, the LN5B subunits found in the conditioned medium, but not the 190-kDa protein, were also detected as major bands by the CBB staining (Fig. 1B, lane 7). There was no apparent difference in the efficiency of the LN5B/5A deposition between LN5B-HEK and LN5-HEK cell lines. When LN5B and LN5A were subjected to SDS-PAGE under nonreducing conditions, these proteins did not migrate from the top of the gel, suggesting that they formed large protein complexes in the matrix (data not shown).

The deposition of LN5B and LN5A on the matrix was further analyzed by immunofluorescent microscopy using the anti-γ2 antibody (Fig. 3B). LN5B-HEK cells and LN5-HEK cells...
showed distinct distribution of the LN5 isoforms. LN5A was highly deposited at peripheral regions of cell bodies forming treelike patterns. LN5A was also deposited on cell-free spaces. In contrast, LN5B was deposited under cell bodies producing cloudlike high density spots, which were co-localized with the marginal actin accumulation. LN5B was scarcely deposited on cell-free spaces.

We also found that expression of LN5B in HEK293 cells has a striking effect on the cell growth (Fig. 3C). Parent HEK293 cells very poorly grew in a standard culture medium containing 10% FCS, whereas LN5B-HEK cells rapidly grew in the same conditions. LN5-HEK cells also grew well but at a slightly lower rate than LN5B-HEK cells. The two other clones, 2 and 7 of LN5B-HEK cells, also showed high growth rates similar to...
that of clone 9 (Fig. 3D). Unexpectedly, the growth rate of β3γ2-HEK cells was comparable with that of LN5B-HEK cells (Fig. 3D), although the two cell types showed different morphology (Fig. 3A). These results suggested that the secreted LN5B and LN5A stimulated the proliferation of the producing cells.

The LN5B production in β3γ2-HEK cells was estimated to be less than 1% of that in LN5B-HEK cells. Therefore, it is considered that a high LN5B production is required for the morphological change, but a very low level of LN5B is enough for the growth stimulation.

**Purification of Recombinant LN5B**—To purify recombinant LN5B, the conditioned medium of LN5B-HEK was applied to molecular sieve chromatography with the anti-laminin α3 monoclonal antibody. The purified LN5B was analyzed by SDS-PAGE under both reducing and nonreducing conditions (Fig. 4). The purified LN5B was separated into two major bands of 630 and 580 kDa and two minor bands of 435 and 385 kDa under nonreducing conditions (Fig. 4A, lane 1). The relative weight ratio of the 435/385-kDa LN5B to the 630/580-kDa form was estimated to be approximately 1/4. Under reducing conditions, it was separated into five major bands of 335, 150, 145, 135, and 105 kDa (Fig. 4A, lane 3). These electrophoretic profiles were essentially the same as those of the LN5B purified from β3γ2-HEK cells (Fig. 1A) and similar to the CBB staining profile of the conditioned medium from LN5B-HEK cells (Fig. 1B). Immunoblotting analysis identified the 335- and 145-kDa bands as the α3B chain, the 135-kDa band as the β3 chain, and the 150- and 105-kDa bands as the γ2 chain (Fig. 4B).

As shown above, LN5B contained the 335- and 145-kDa α3B chains, whereas LN5A contained the 160- and 145-kDa α3A chains (Fig. 4B, lanes 1 and 2). It has been reported that the 190- or 160-kDa α3A chain in LN5A is converted to the 145-kDa form by a proteolytic cleavage at the NH2-terminal region (30). Therefore, it was expected that the 335-kDa α3B band in LN5B was the unprocessed α3B chain (without G4-G5 domain) and the 145-kDa band was a cleaved form that had lost the NH2-terminal region of the α3B chain. Both LN5B and LN5A preparations contained the 150-kDa, native γ2 chain and the 105-kDa, processed γ2 chain. It is known that the 150-kDa γ2 chain produces the LN5 heterotrimer of approximately 450 kDa, whereas the 105-kDa γ2 chain produces the heterotrimer of approximately 400 kDa (see Fig. 4A, lanes 2 and 4) (30). Therefore, it seems very likely that the 630- and 580-kDa LN5B proteins in the nonreducing SDS-PAGE contain the 335-kDa α3B chain, whereas the 435- and 385-kDa proteins contain the 145-kDa, processed α3B chain (Fig. 4A, lane 1). It is also expected that the 630- and 435-kDa LN5B proteins contain the 150-kDa γ2 chain, whereas the 580- and 385-kDa LN5B proteins contain the 105-kDa γ2 chain.

To confirm the conversion of the 335-kDa α3B chain to a 145-kDa chain, we purified the 190-kDa protein found in the conditioned medium of LN5B-HEK cells to a single protein as described under “Experimental Procedures” (Fig. 4A, lane 5; also see Fig. 1B, lane 4). We analyzed the NH2-terminal amino acid sequences of the 190-kDa protein, the 335- and 145-kDa α3B bands, and the 145-kDa α3A band (Fig. 2). Both NH2-terminal amino acid sequences of the 335- and 145-kDa proteins were determined to be ATARPDGA (amino acid numbers 34–41), indicating that the 190-kDa protein was an NH2-terminal fragment of the α3B chain and they had been cleaved after the signal peptide sequence. Both 145-kDa α3B and α3A bands had an NH2-terminal amino acid sequence of DSSPAAEE (amino acid numbers 1812–1818), which was a common sequence between LN5A and LN5B. These results indicate that the 335-kDa α3B chain, which does not contain the COOH-terminal G4-G5 domain, is further converted to the 145-kDa...
Experimental Procedures.

**triplicate assays.** Other experimental conditions are described under determined by fluorescent intensity. Each concentrations of purified LN5A (open circles) or LN5B (closed squares) and placed into each well of 96-well plates precoated with the indicated HaCaT cells (open circles) were suspended in serum-free DMEM/F-12 medium derived epithelial cell line BRL and the human keratinocyte this, their activities were compared using the Buffalo rat liver—

Cell Adhesive Activity of LN5B and Receptor Identification—LN5A strongly promotes adhesion of a variety of cultured cell lines (8, 9). Because the α3β chain of LN5B contains almost the whole sequence of the α3A chain in LN5A, both LN5 isoforms are expected to have similar cell adhesion activities. To confirm this, their activities were compared using the Buffalo rat liver—

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**Biological Activities of 190-kDa NH2-terminal Fragment (α3Bnt)**—The functional difference between LN5B and LN5A seems to depend on the long NH2-terminus sequence of the α3B chain. Therefore, we also purified the 190-kDa NH2-terminal fragment of the α3B chain, named α3Bnt (Fig. 4A, lane 5), and examined for its biological activity. To avoid the contamination of α3Bnt with LN5B, the purified protein was subjected to the anti-γ2 antibody (D4B5) affinity column, which bound LN5B but not the fragment. When α3Bnt was applied to a heparin column pre-equilibrated with a buffer containing 0.15 M NaCl, it could scarcely bind to the column, suggesting that the fragment had a very low affinity to heparin if any.

LN5B and LN5A, when precoated at 2 μg/ml on culture plates, supported rapid attachment and spreading of EJ-1 cells and HT1080 cells (Fig. 9A). α3Bnt supported attachment of both EJ-1 and HT1080 cell lines to the plates at concentrations higher than 5 μg/ml, although it could not support cell spreading (Fig. 9, A and B). The cell attachment activity of α3Bnt was effectively blocked by pretreating the cells with the anti-integrin α3 or β1 antibody (Fig. 9C). The anti-integrin α3 antibody weakly inhibited the cell attachment. EDTA inhibited the cell attachment activity of α3Bnt, but not heparin at all. These results indicate that α3Bnt supports weak cell adhesion through the interaction with integrin α3β1 or α6β1. α3Bnt seems not to interact with heparan sulfate proteoglycans on the cell surface.

We also examined whether or not α3Bnt promotes the migration of EJ-1 cells. α3Bnt significantly promoted the cell

**Experimental Procedures.**

**form by releasing the NH2-terminal 190-kDa fragment.** This conversion is thought to be mediated by a metalloprotease of the BMP-1 family, which selectively cleaves X-D (Asp) peptide bonds (31).

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**Effect of LN5B on Cellular Scattering, Migration, and Proliferation—**LN5A is known to promote both cell scattering and cell migration (7, 8). We compared these activities between LN5A and LN5B. The cell-scattering activity was assayed by adding LN5A or LN5B directly to the culture of BRL cells in the presence of 1% FCS. LN5A and LN5B efficiently scattered BRL cells (Fig. 7A), and the ED50 was −3 ng/ml for both laminins (Fig. 7B).

Next, the cell migration activity was compared between LN5B and other cell adhesion proteins (Fig. 7C). As reported before (32), when coated on plastic plates, LN5A and laminin 10/11 strongly promoted the migration of EJ-1 cells with laminin 1, laminin 2/4, and fibronectin. LN5B promoted the cell migration more than LN5A and laminin 10/11.

Because LN5B-HEK cells grew much faster than the parent HEK293 cells (Fig. 3C), LN5B and two other laminins were examined for growth-stimulatory activity toward the parent HEK293 cells. When directly added to a culture medium containing 1% FCS, LN5B strongly stimulated the growth of HEK293 cells. LN5A and laminin 1 appeared to stimulate the cell growth but at far lower levels than LN5B (Fig. 8, A and B). When the LN5A-producing, nontransformed mammary epithelial cell line H9251 was used, LN5B also stimulated the cell growth most effectively. In contrast, laminin 1 and LN5A showed little growth stimulation toward this cell line (Fig. 8C). This suggests that soluble LN5B has a growth factor-like activity.

**Biological Activities of 190-kDa NH2-terminal Fragment (α3Bnt)**—The functional difference between LN5B and LN5A seems to depend on the long NH2-terminus sequence of the α3B chain. Therefore, we also purified the 190-kDa NH2-terminal fragment of the α3B chain, named α3Bnt (Fig. 4A, lane 5), and examined for its biological activity. To avoid the contamination of α3Bnt with LN5B, the purified protein was subjected to the anti-γ2 antibody (D4B5) affinity column, which bound LN5B but not the fragment. When α3Bnt was applied to a heparin column pre-equilibrated with a buffer containing 0.15 M NaCl, it could scarcely bind to the column, suggesting that the fragment had a very low affinity to heparin if any.

LN5B and LN5A, when precoated at 2 μg/ml on culture plates, supported rapid attachment and spreading of EJ-1 cells and HT1080 cells (Fig. 9A). α3Bnt supported attachment of both EJ-1 and HT1080 cell lines to the plates at concentrations higher than 5 μg/ml, although it could not support cell spreading (Fig. 9, A and B). The cell attachment activity of α3Bnt was effectively blocked by pretreating the cells with the anti-integrin α3 or β1 antibody (Fig. 9C). The anti-integrin α3 antibody weakly inhibited the cell attachment. EDTA inhibited the cell attachment activity of α3Bnt, but not heparin at all. These results indicate that α3Bnt supports weak cell adhesion through the interaction with integrin α3β1 or α6β1. α3Bnt seems not to interact with heparan sulfate proteoglycans on the cell surface.

We also examined whether or not α3Bnt promotes the migration of EJ-1 cells. α3Bnt significantly promoted the cell
characterization of laminin 5B and its proteolytic fragment

Figure 6. Inhibitory effects of various integrin antibodies, heparin, and EDTA on adhesion of EJ-1 cells to LN5A or LN5B substrate. EJ-1 cells, which had been pretreated with the indicated, function-blocking integrin antibodies, heparin (Hep.), or EDTA for 20 min, were incubated for 1 h on 96-well plates precoated with 50 μl of 2 μg/ml LN5A (A) or LN5B (B). The relative number of adherent cells in the presence of mouse IgG was taken 100%. Each point represents the mean ± S.D. for triplicate assays.

Discussion

In the present study, we for the first time cloned a full-length human laminin α3B cDNA and characterized human LN5B consisting of the α3B, β3, and γ2 chains. The molecular size of the mature α3B chain (without G4-G5 domain) was ~335 kDa, approximately twice as large as the mature α3A chain (160 kDa) (Fig. 2). Comparison of the biological activities of the two LN5 isoforms demonstrated that LN5B had significantly higher cell adhesion and cell migration activities than LN5A. In addition, LN5B potently stimulated cell proliferation when added to the culture medium directly. It has been generally accepted that integrin-mediated signals cooperate with growth factor signals (33). Indeed, there are some reports showing that laminin-derived signals support cell proliferation (34, 35). However, there is little information about growth stimulation by soluble laminin. In the present study, LN5B stimulated cell growth as well as cell migration in soluble conditions. Recently, we found that LN5A, but not other laminins, stimulates cell migration as a soluble factor.2 The association of soluble LN5A with integrin α3β1 on the apical cell surface induces protein kinase C-mediated growth signal. Such growth-factor-like activities of LN5B and LN5A suggest their contribution to the cellular growth and migration in some physiological and pathological conditions. This was supported by the findings that LN5B-HEK cells showed highly motile cell morphology and a very high growth rate as compared with the parent HEK cells.

Laminins are large complex molecules, which have many functional domains in their three subunits (1, 2). They interact with cell surface receptors such as integrins, syndecans, and dystroglycan and matrix proteins such as nidogen, collagens, and perlecan through different domains. It has been generally accepted that the NH2-terminal regions of the three subunits are essential for the assembly to the matrix, whereas the COOH-terminal G domain of α chains mediates the interaction with cell surface receptors including integrins (1). In LN5A, the G1–G3 domain of the α3A chain, especially G3, is critical for the expression of the biological activity (6, 26, 36). The COOH-terminal G4-G5 fragment of the α3A chain, which is proteolytically released from the immature LN5A after secretion (29), supports weak cell adhesion by interacting with cell surface syndecans (37). Thus, the G (G1–G5) domain of α3 chain plays a primary role in the interaction with cell surface receptors and the resulting regulation of cellular functions. In some full-sized laminin α chains, however, their NH2-terminal regions are known to contain binding sites to integrins and heparan sulfate proteoglycans. The recombinant domain VI-V proteins of the α1 chain (38–40) and the α2 chain (41) mediate cell adhesion through the interaction with integrins α1β1 and α6β1. Both integrin-binding site and heparin-binding site are localized in domain VI but not domain V. A recent study has shown that the domain VI-V of mouse laminin α5 chain exhibits a cell binding activity, which is mainly mediated by integrin α6β1 and heparan sulfate proteoglycans on the cell surface (42). This study also identified two amino acid sequences in domain VI, which are responsible for the cell binding. These sequences are highly conserved in the domain VI of human laminin α3B chain. In the present study, we found that a 190-kDa NH2-terminal fragment of the α3B chain, named α3Bnt, was released from LN5B presumably by the action of BMP-1-related metalloproteinases. Consistent with the studies about the recombinant domain VI-V of other laminin α chains, α3Bnt had activities to promote cell adhesion through the interaction with integrin α3β1. Unlike other α chains, however, the cell adhesion activity of α3Bnt was not inhibited by heparin, suggesting that cell surface heparan sulfate proteoglycans were not involved in the cell adhesion. In addition, α3Bnt stimulated cell migration and proliferation. These activities have not been reported in any NH2-terminal recombinant proteins of other α chains. It is interesting that α3Bnt promotes cell migration without spreading cells. These activities of the NH2-terminal structure of the α3B chain may contribute to the elevated biological activity of LN5B compared with LN5A, which lacks most part of the sequence of α3Bnt. Although the cell adhesion activity of α3Bnt was approximately one-fifth of those of LN5A and LN5B, its activity was close to that of laminin 1. Furthermore, the growth-stimulatory activity of α3Bnt toward HEK cells was rather higher than or comparable with those of laminin 1 and LN5A. It is also noted that the activity of α3Bnt was similar to

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or higher than the reported activities of the recombinant NH₂-terminal domains of mouse laminin α1, α2, and α5 chains (40–42). Taken together, it is expected that the biological activity of α3Bnt through the interaction with integrin α3β1 has some biological significance. It seems possible that the NH₂-terminal structure of the α3B chain cooperates with its COOH-terminal structure to regulate cellular functions. In vivo, the released α3Bnt may cooperate with laminins and other matrix proteins to regulate cellular adhesion and migration. On the other hand, the proteolytic processing of the α3B chain converts LN5B to the LN5A with the 145-kDa α3 chain. The functional conversion of LN5B seems to have some physiological significance.

Laminin polymerization is an important process to produce...
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**Fig. 9. Properties of the 190-kDa α3B NH$_2$-terminal fragment α3Bnt.** A, morphology of EJ-1 cells and HT1080 cells adhered to α3Bnt, LN5A, and LN5B. EJ-1 cells in serum-free medium were placed on plates precoated without (None) or with 15 μg/ml α3Bnt, 2 μg/ml LN5A, or 2 μg/ml LN5B. After 1 h of incubation, the cell morphology was examined under a phase-contrast microscope. Original magnification, ×300. B, effect of various concentrations of α3Bnt on attachment of EJ-1 cells. EJ-1 cells were incubated on plates precoated with the indicated concentrations of α3Bnt for 1 h. Other experimental conditions are described in Fig. 5 and under "Results." C, inhibitory effect of various integrin antibodies, heparin, and EDTA on attachment of EJ-1 cells to α3Bnt. The experiment was done as described for Fig. 6 using plates pretreated with 50 μl of 15 μg/ml α3Bnt. The relative number of adherent cells in the presence of mouse IgG was taken as 100%. Each point represents the mean ± S.D. for triplicate assays.

The NH$_2$-terminal domains of the full-sized α chains, as well as those of laminin β1, β2, and γ1 chains, are involved in the self-assembly and co-assembly of laminins (43, 44). It is known that the genetic deletion of the NH$_2$-terminal domain in the α2 chain causes muscular dystrophy (45). Because LN5A lacks the NH$_2$-terminal domains in the three subunits, it is assembled into the basement membrane scaffold through making a complex with laminin 6/7 (α3, β1/β2, γ1) (14, 46). A recent report has shown that a recombinant NH$_2$-terminal fragment (domain VI/V) of mouse laminin α3B chain has a strong activity to bind to domain VI/V of laminin α1 and γ1 chains and a moderate self-binding activity (22). This suggests that LN5B, unlike LN5A, may be assembled into laminin networks. The matrix assembly of LN5B may be very different from that of the full-sized laminins (α1/2/5-laminins), because LN5B has a very long rodlike structure with the NH$_2$-terminally truncated forms of β/γ chains (β3 and γ2). The NH$_2$-terminal domain VI can associate not only with other laminins and presumably other matrix proteins but also with integrins. Although we found no significant difference in the efficiency of matrix deposition of LN5B and LN5A, their deposition patterns were clearly different from each other as analyzed by immunofluorescent microscopy. This suggests that LN5B and LN5A interact with different extracellular matrix molecules and have different effects on the producing cells. It is conceivable that in vivo LN5B links epithelial cells on the basement membrane with the underlying stromal cells by binding the former cells through the COOH-terminal G domain of the α3B chain and the latter through the NH$_2$-terminal domain VI. Further studies are needed to clarify the structural and functional roles of LN5B in the basement membranes. It seems also important to characterize two unidentified full-sized laminins, laminin 6B/7B (α3Bβ1γ1 and α3Bβ2γ1), that are expected to form non-covalent networks by self-polymerization and co-polymerization with other full-sized laminins.

It has been reported that the laminin α3B chain is more widely and more intensively expressed than the α3A chain. For example, in human tissues α3B chain is exclusively expressed in the uterus, lung, liver, brain, and heart, whereas α3A is predominantly expressed in the placenta and salivary gland (24). In mouse skin, α3B and α3A display different distribution (20, 22). On the other hand, human cancer cell lines almost exclusively express the α3A transcript (28). The differential expression of α3B and α3A is regulated by distinct two promoters (21). Acute phase reactant consensus sequences and NF-IL-6 binding sites are present in the two α3B/α3A promoters. The present study demonstrates that LN5B has higher activities to stimulate cell migration and cell growth than LN5A and other laminins. Together with the past studies, our results suggest that LN5B may be involved in some pathological processes such as inflammation and wound healing. The differen-
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tial expression and biological activity between LN5A and LN5B demonstrate that they play differential physiological roles.

In conclusion, this study provides new information on the unique laminin isoform LN5B. The recombinant LN5B and the 190-kDa NH2-terminal fragment α3Bn1 produced in this study seem useful to elucidate their physiological and pathological roles.

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Characterization of Laminin 5B and NH$_2$-terminal Proteolytic Fragment of Its $\alpha_3B$ Chain: PROMOTION OF CELLULAR ADHESION, MIGRATION, AND PROLIFERATION

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