J. Biochem. **138**, 539–552 (2005)
doi:10.1093/jb/mvi153

Regulation of Cell Adhesion and Type VII Collagen Binding by the β3 Chain Short Arm of Laminin-5: Effect of Its Proteolytic Cleavage

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Received June 13, 2005; accepted July 31, 2005

The basement membrane protein laminin-5 (Lm5), a heterotrimer of α3 (or α3A), β3, and γ2 chains, regulates cellular adhesion and motility. Here we examined the proteolysis and biological function of the laminin β3 chain. First, we found that the β3 chain of Lm5 is cleaved at its N-terminal, short arm by an endogenous proteinase(s) in normal human keratinocytes and some other cell lines. To examine the effect of β3 chain cleavage, we expressed a wild-type Lm5 and two Lm5 mutants with partially deleted β3 chains in HEK293 cells. Experiments with the purified Lm5 forms demonstrated that the deletion of the β3 short arm or its N-terminal domain LN decreases the cell adhesion activity of Lm5, but does not significantly affect the motility activity. A recombinant β3 short arm protein enhanced integrin-mediated cell adhesion to Lm5 by binding to an unidentified cell receptor. It was also found that the laminin EGF-like domain of the β3 short arm is a binding site for type VII collagen. These results suggest that the β3 short arm is involved not only in the matrix assembly of Lm5, but also in its cell adhesion activity. The proteolytic cleavage of the β3 chain may modulate these functions of Lm5 *in vivo*.

Key words: cell adhesion, cell migration, laminin-5, laminin β3 chain, proteolytic cleavage.

Abbreviations: CBB, Coomassie Brilliant Blue R-250; FCS, fetal calf serum; Lm5, laminin-5; LN, laminin N-terminal domain; LE, laminin epidermal growth factor–like domain; mAb, monoclonal antibody; MMP, matrix metralloproteinase; NHK, normal human keratinocyte; WT-Lm5, Lm5 with the full-length β3 chain; ΔLN-Lm5, Lm5 with a β3 chain lacking the LN domain; ΔSA-Lm, Lm5 with a β3 chain lacking the whole short arm; β3SA, a recombinant protein of the β3 chain short arm.

Laminins are major basement membrane proteins consisting of α, β, and γ chains linked by disulfied bonds. To date, five α (α1–α5), four β (β1–β4), and three γ (γ1–γ3) chains have been identified, and at least 15 laminin isoforms with different combinations of these chains have been reported (*1*, *2*). These laminin isoforms are expressed differently at various developmental stages and in adult tissues (*1*, *3*). Therefore, each laminin isoform is thought to play a different biological role. In epithelial tissues, the adhesion of epithelial cells to laminins is crucial for the maintenance of tissue architecture and the regulation of cellular functions such as cell migration, differentiation, growth and survival. These biological activities of laminins are mediated by many functional domains distributed in the three laminin chains. It is known that the N-terminal regions of the three chains are required for matrix assembly (*1*), while the C-terminal, LG domain of the α chains are essential for the interaction with the cell surface receptor integrins (*4*, *5*).

Much attention has been focused on the structure-function relationship of laminin-5 (Lm5), which has a unique structure and unique functions compared to other laminins. Lm5 is composed of N-terminally truncated forms of three laminin chains, *i.e.* α3, β3, and γ2, and is mainly expressed in the basement membranes of the skin, lung, breast and kidney *in vivo* (*3*, *6*, *7*). In the skin, Lm5 contributes to the stable, epidermal and dermal connection by interacting with integrin α6β4 in the hemidesmosome structures (*8*, *9*). Lm5 also interacts with type VII collagen (*10*) and laminin-6/7 (α3, β1/2, and γ1) (*11*), forming anchoring filaments. Therefore, gene defects or mutations in any of the three chains of Lm5 cause a lethal blistering skin disorder, Herlitz junctional epidermolysis bullosa (*12*, *13*). On the other hand, in culture, Lm5 efficiently promotes cellular adhesion, migration and scattering by interacting with integrins α3β1, α6β1, and α6β4 (*14–16*). These activities of Lm5 are thought to contribute to wound healing (*17*, *18*) and tumor invasion (*19*, *20*). It has long been known that both the α3 and γ2 chains of Lm5 are proteolytically processed after secretion (*21*). The 190-kDa, precursor α3 chain is cleaved to the 160-kDa mature form, and partly to the 145-kDa form (*21*, *22*). The γ2 chain is processed from the 150-kDa precursor form to the 105-kDa mature form (*21*). Past studies have shown that these cleavages modulate the biological activities of Lm5 (*23–25*). However, since Lm5, as well as other laminins, has a large, complex structure and is difficult to purify, the biological effects of the proteolytic processing of Lm5 have not been

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clearly shown. Recently, we established stable expression systems for human recombinant Lm5 (*4*, *26*), laminin-6 (*27*), laminin-5B (*28*) and their mutant proteins. Using these recombinant proteins, we identified the active sites of the α3 chain that are responsible for the cell adhesion and cell motility activities of Lm5 (*4*, *5*). These studies also showed that cleavage of the γ2 chain at the N-terminal region, namely the short arm, decreases the cell adhesion activity of Lm5 but increases its cell migration activity (*29*), while cleavage of the α3 chain increases both activities (*30*). In addition, it has been reported that the LG3 domain of the α3 chain is the major site of interaction with integrins (*4*, *5*, *31*). In contrast to the laminin α3 and γ2 chains, there have been few studies about the laminin β3 chain, especially its proteolytic processing and the biological function of its short arm region. Like the laminin γ2 chain, the laminin β3 chain is found only in Lm5. Our previous finding that laminin 6 (α3β1γ1) has far lower cell migration and cell scattering activities than Lm5 suggests that not only the α3 and γ2 chains, but also the β3 chain is required for the unique activities of Lm5 (*27*). In the present study, we investigated the proteolytic processing and biological function of the short arm of the laminin β3 chain of Lm5.

MATERIALS AND METHODS

Antibodies—Mouse monoclonal antibodies (mAbs) against the human laminin α3 chain (LSα3, clone 4) (*7*), the human laminin γ2 chain (D4B5) (*7*), and the human laminin β3 chain (clone 29E) (*32*) were prepared in our laboratory as described previously. Two mouse mAbs that recognize the LE domain of the human laminin β3 chain (β3LE, clones 8A and 12C) were prepared using a recombinant protein of the β3 chain short arm as the antigen. Another mAb against the human laminin β3 chain, Kalinin B1, was purchased from Transduction Laboratories Inc. (Lexington, KY, USA). Other antibodies used and their sources were a mouse mAb against human type VII collagen (LH 7.2) from Sigma (St Louis, Mo, USA), an anti–histidine-tag (anti-His) antibody from Invitrogen (Carlsbad, CA, USA), mouse mAbs against human integrin α2 (P1E6), integrin α3 (P1B5), integrin α5 (P1D6) and integrin β1 (6S6) from Chemicon (Temecula, CA, USA), and a mouse mAb against human integrin α6 (G_0H3) from Pharmingen (San Diego, CA, USA).

Cell Culture—The human embryonic kidney cell line HEK293 (CRL-1573) was purchased from American Type Culture Collection (ATCC) (Rockville, MD, USA). The buffalo rat liver-derived epithelial cell line BRL was used in previous studies (*14*). A spontaneously immortalized human keratinocyte cell line HaCaT and human gastric adenocarcinoma cell line STKM-1 were generous gifts from Dr. N.E. Fusenig (Deutsches Krebsforschungszentrum, Heidelberg, Germany) and Dr. Yanoma (Kanagawa Cancer Center, Yokohama), respectively. Oral epidermoid carcinoma KB, gastric adenocarcinoma MKN-45, cervix epidermoid carcinoma C-4I and tongue squamous adenocarcinoma HSC-4 were obtained from the Japanese Cancer Resources Bank (JCRB). These cell lines were maintained in DMEM/F12 medium (Invitrogen) supplemented with 10% fetal calf serum (FCS) (JRH Biosciences, Lenexa, KS, USA), penicillin and streptomycin sulfate. Human mammary epithelial MCF10A cells were obtained from ATCC, and cultured in DMEM/F12 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. Normal human keratinocyte (NHK) from neonatal foreskin was obtained from Cascade Biologics (Portland, OR, USA) and cultured in Humedia-KG2 medium (Kurabo, Osaka), which was composed of keratinocyte basal medium, 0.1 ng/ml human EGF, 0.4% (v/v) bovine pituitary extract (BPE), 10 μg/ml insulin, 500 ng/ml hydrocortisone, 50 μg/ml gentamicin, and 50 ng/ml amphotencin B. Cells after 2 or 3 passages were used.

Construction of cDNA Expression Vectors for Laminin β3 Chain Deletion mMutants—Human laminin β3 chain cDNA was prepared from the human gastric carcinoma cell line STKM-1 by RT-PCR (*26*). To obtain cDNAs for laminin β3 chain deletion mutants, a full-length β3 chain cDNA cloned in pGEM3Zf (+) was used as a template for PCR with Ex Taq polymerase (Takara Shuzo, Tokyo) and the primer sets shown in Table 1. The cDNA fragments coding for the N-terminal regions of the β3 chains shown in Fig. 1A were amplified by PCR with the following primer sets: primers 1 and 2 for β3WT encoding the full-sized β3 chain, primers 3 and 4 for β3ΔLN encoding the β3 chain without laminin N-terminal domain (LN), and primers 5 and 6 for β3ΔSA encoding the β3 chain without the short arm. The cDNA fragment coding for the C-terminal region of the β3 chains (β3WT, β3ΔLN and β3ΔSA) was amplified by PCR with primers 7 and 8. To generate a β3SA cDNA encoding only the β3 short arm [LN plus laminin EGF-like (LE) domains], a cDNA fragment encoding a C-terminal part of the LE domain and containing a *Bst*XI site was amplified using primers 9 and 10. Each PCR product was cloned into the pGEM T-Easy vector (Promega, Madison, WI), and all sequences were verified by DNA sequencing. To construct the full-length β3WT (nucleotide number 52–3519, amino acid residues 18–1173), β3ΔLN (nucleotide number 688–3519, amino acid residues 230–1173) and β3ΔSA (nucleotide number 1735–3519, amino acid residues 579–1173) cDNAs, their cloned N-terminal cDNA fragments were inserted into the corresponding regions of the full-length β3 chain cDNA cloned in pGEM3Zf (+). The *Kpn*I–*Eco*RV fragments released from those cDNA vectors were ligated into the *Kpn*I–*Eco*RV site of the pSecTag2A mammalian expression vector containing a C-terminal 6× Histidine Tag epitope (Invitrogen). To prepare the expression vector of β3SA (nucleotide number 52–1740, amino acid residues 18–580), the *Bst*XI–*Eco*RV fragment released from the cloned cDNA fragment for the C-terminal region of LE was ligated into the corresponding site of β3WT cloned in pGEM3Zf (+). Finally, the *Kpn*I–*Eco*RV fragment released from the cDNA vector was ligated into the *Kpn*I–*Eco*RV site of the pSecTag2A mammalian expression vector.

Expression of Recombinant Lm5 Forms with Partially Deleted β3 Chains and a Recombinant β3 Chain Short Arm—The HEK293 cell line Lm5-HEK, which had been transfected with the cDNAs for the laminin α3, β3 and γ2 chains and overexpresses human recombinant Lm5, has previously been established (*26*). In the same way, we introduced expression vectors for the α3 chain and then the γ2 chain into HEK293 cells and selected a stable clone, α3γ2-HEK, that overexpresses both the α3 and γ2 chains at high levels. To establish a HEK293 clone expressing

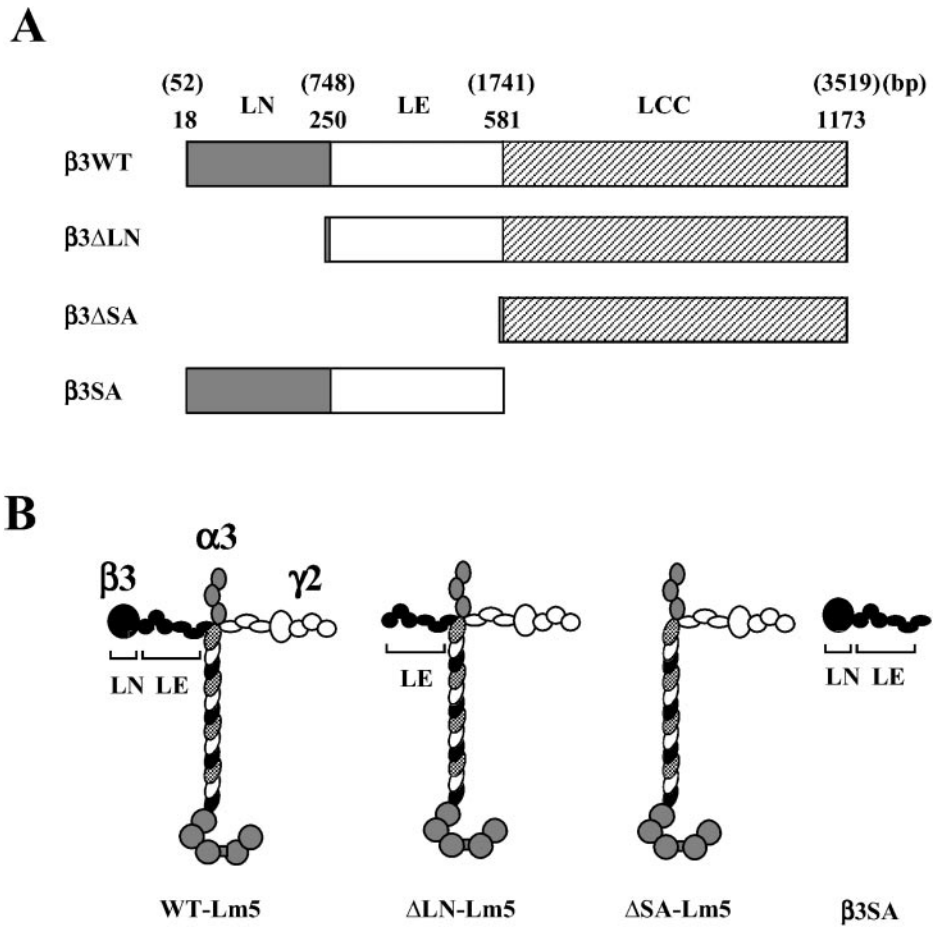

Fig. 1. **Schematic structures of wild-type and mutant forms of the laminin β3 chain and Lm5 used in this study.** (A) Four forms of laminin β3 chain. The cDNAs encoding four forms of laminin β3 chain were constructed: the wild-type of the β3 chain (β3WT), one lacking the laminin N-terminal domain LN (or domain VI) (β3ΔLN), one lacking the whole short arm (both LN and the laminin EGF-like domain LE (or domain III/V)) (β3ΔSA), and the short arm of β3 chain (β3SA). LCC, laminin coiled-coil domain (or domains I/II). The numbers indicate amino acid residue numbers, and those in parentheses indicate nucleotide numbers in the cDNA. (B) Three forms of recombinant Lm5 and a β3 chain short arm. The expression vectors of β3WT, β3ΔLN and β3ΔSA were separately introduced into a HEK293 cell clone overexpressing the laminin α3 and γ2 chains to produce WT-Lm5, ΔLN-Lm5 and ΔSA-Lm5, respectively. The expression vector for β3SA was introduced into a parent HEK293 cell clone to produce the β3 chain short arm protein (β3SA).

Table 1. **Primers used for the construction of full-length and deleted forms of laminin β3 chain.**

Primer name	Nucleotide sequence	Residues
1) β3-WTnt-5′	5′-AAGGTACCTCAACAAGCCTGCTCCCGTGG-3′	(nucleotides 52–71, sence)
2) β3-WTnt-3′	5′-GCAGTCGGCAGCCAGGTACTG-3′	(nucleotides 439–459, antisence)
3) β3-ΔLNnt-5′	5′-AAGGTACCTAGGGGCTACCACCCTCCC-3′	(nucleotides 688–705, sence)
4) β3-ΔLNnt-3′	5′-GCTCACAGTTCTTGCCTTCG-3′	(nucleotides 1050–1069, antisence)
5) β3-ΔSAnt-5′	5′-AAGGTACCTGTGGCCTGCCACCCTTG-3′	(nucleotides 1735–1751, sence)
6) β3-ΔSAnt-3′	5′-GGCTGCTGAGAACTGCTCGG-3′	(nucleotides 1902–1921, antisence)
7) β3-ct-5′	5′-CAGCTGGGTGACTTCTGGAC-3′	(nucleotides 3103–3122, sence)
8) β3-ct-3′	5′-CCGATATCTTGCAGGTGGCATAGTAG-3′	(nucleotides 3498–3516, antisence)
9) β3SAct-5′	5′-CGGGATCCGCCTACTATGCTGTGTCCCAG-3′	(nucleotides 709–729, sence)
10) β3SAct-3′	5′-TAGATATCGAGGCCACGCACACCGGGTAG-3′	(nucleotides 1725–1740, antisence)

Underlining indicates the *Kpn*I restriction site in primers 1, 3, 5, and the *Eco*RV restriction site in primers 8 and 10.

wild-type Lm5 (WT-Lm5) or two Lm5 variants with partially deleted β3 chains, α3γ2-HEK cells were separately transfected with the β3 chain expression vector for β3WT-, β3ΔLN- or β3ΔSA-pSecTag2A Zeo (+). To establish a HEK293 clone overexpressing only the β3 chain short arm, the β3SA-pSecTag2A Zeo (+) vector was transfected into parent HEK293 cells. These HEK293 transfectants were selected with 300 μg/ml Zeocin (Invitrogen), and stable clones producing the recombinant proteins at high levels were isolated from the respective transfectants.

Preparation of Conditioned Media—To obtain conditioned media from Lm5-producing human cell lines, the cells were plated on 90-mm cell culture dishes and grown to confluence in serum-containing medium. The cultures were washed several times with PBS and incubated in serum-free medium for 2 days. The resultant conditioned media (10 ml) were collected and dialyzed against pure water, and freeze-dried. The dried protein from one dish was dissolved in 0.2 ml of 20 mM Tris-HCl (pH 7.5) buffer containing 0.1% (w/v) CHAPS to achieve a 50-fold concentration. Two to ten microliters of the conditioned

media from NHK or other cell lines were subjected to immunoblotting analysis.

Purification of Recombinant Lm5 Forms and β3SA—Recombinant Lm5 proteins were purified by the previously reported method (*20*). HEK293 transfectants were grown to confluence in DMEM/F12 medium (Sigma) supplemented with 5% FCS in roller bottles, and then incubated in serum-free DMEM/F12 medium. The serum-free conditioned medium was harvested from each culture every 2 days and concentrated by protein precipitation with 80%-saturated ammonium sulfate. The precipitated protein was dissolved in a small volume of 20 mM Tris-HCl (pH 7.5) buffer containing 0.5 M NaCl, 0.01% (w/v) Brij 35, and 0.1% (w/v) CHAPS, and dialyzed against the same buffer. The concentrated conditioned medium was subjected to molecular-sieve chromatography on a Sepharose CL-4B column (Amersham Biosciences, Piscataway, NJ, USA). Fractions containing Lm5 were pooled and applied to a gelatin-Sepharose 4B column to remove fibronectin, and the resultant flow-through fraction was subjected to immunoaffinity chromatography with the anti-laminin β3 chain mAb (clone 29E). Bound proteins were eluted from the affinity column with 0.05% (v/v) trifluoroacetic acid and immediately neutralized with a small volume of 1 M Tris-HCl (pH 8.0). These purified proteins were stored in the presence of 0.005% Brij35 and 0.1% CHAPS at 4°C. For the purification of β3SA, the conditioned medium from β3SA-HEK was dissolved in and dialyzed against 50 mM Tris-HCl (pH 7.8) and 0.3 M NaCl. The concentrated conditioned medium was applied to a Chelating Sepharose FF nickel column (Amersham Biosciences) pre-treated with 100 mM $NiSO_4$. Bound proteins were eluted from the nickel column with 50 mM Tris-HCl (pH 7.0) buffer containing 0.5 M imidazole and 0.3 M NaCl. To remove imidazole and contaminating proteins, the β3SA protein was further purified by molecular sieve chromatography on a Superdex 200 HPLC-column (Amersham Biosciences). Protein concentrations were determined using a Bio-Rad protein assay kit (Hercules, CA, USA) with bovine serum albumin as a standard.

Digestion of Recombinant Lm5 Forms by Trypsin—To conform that the recombinant Lm5 proteins have the proper structures, they were subjected to trypsin digestion. The purified Lm5 proteins were boiled at 95°C for 5 min. Two hundred nanograms of the heat-denatured proteins and native proteins were then incubated with 2 μg/ml trypsin-TPCK (Worthington Biochemical, Freehold, NJ, USA) in 20 μl of 30 mM Tris-HCl (pH 7.5) buffer at room temperature for 60 min, and then separated by reducing SDS-PAGE in a 4–7.5% gradient gel.

SDS-PAGE and Immunoblotting—Purified proteins were analyzed by SDS-PAGE in 5 or 6% gels or 4–7.5 or 5–20% gradient polyacrylamide gels under non-reducing or reducing conditions. Separated proteins were stained with Coomassie Brilliant Blue R-250 (CBB). For immunoblotting analyses, separated proteins were transferred to nitrocellulose membranes. The blotted proteins were detected with ECL detection reagents (Amersham Biosciences). The molecular weight markers used were Precision Plus Protein Standards (Bio-Rad).

Cell Adhesion Assay—Each well of 96-well plates (Coster, Cambridge, MA) was coated with 50 μl of a purified protein solution at 4°C overnight and then blocked with 200 μl of 1.2% (w/v) bovine serum albumin (Sigma) in PBS for 1 h. One hundred microliters of NHK cell suspension (2×10^5 cells/ml in Humedia-KG2) was inoculated into each well and incubated at 37°C for 30 min. After the non-adherent cells were removed by gentle vibration, adherent cells were fixed with 2.5% (v/v) glutaraldehyde and stained with Hoechst 33342 in 0.001% (w/v) Triton X-100. The fluorescence intensity of each well was measured using a CytoFluor 2350 fluorometer (Millipore, Bedford, MA, USA). To identify Lm5 receptors, the cell suspension was incubated with function-blocking anti-integrin antibodies for 20 min at room temperature before inoculation.

Assay of Cell Scattering and Migration—The scattering of BRL cells was assayed as reported previously (*14*). Briefly, 500 μl of cell suspension [1.4×10^4 cells/ml in DMEM/F12 plus 1% (v/v) FCS] was inoculated into each well of 24-well plates (Sumibe Medical, Tokyo). Test samples were added directly to each culture and incubated at 37°C for 40 h. The cultures were fixed and stained with Giemsa. Total cells and scattered, single cells were counted in three randomly selected microscope fields. The degree of cell scattering was expressed as the percentage of single cells in each field. For the cell migration assay, 24-well plates were coated with substrate proteins to be assayed. NHK cells (2.5×10^4 cells) were inoculated into each well of the 24-well plates. After incubation at 37°C for 1.5 h, cell movement was monitored for 3 h by time-lapse video microscopy, and the migration distance was determined by measuring the total length of the random path covered by each cell.

Preparation of β3SA-Bound Beads—Dynabeads M-280 conjugated with sheep anti-mouse IgG were purchased from Dynal Biotech (Oslo, Norway). The Dynabeads were sequentially bound with an anti-His or anti–laminin-β3-chain mAb and then with the purified β3SA protein according to the manufacturer's instructions. Briefly, the anti-His mAb (8 μg) or anti–laminin-β3-chain mAb (β3LE-12C) (16 μg) was incubated with the anti–mouse IgG Dynabeads M-280 (10^7 beads) at room temperature for 30 min with slow rotation, followed by washing three times with 0.1% bovine serum albumin in PBS. The resultant antibody-bound beads were further bound to the purified β3SA protein (3 μg) as described above. The resultant β3SA-bound beads (β3SA beads) were washed and used.

Analysis of the Interaction between Lm5 and Type VII Collagen—Anti–mouse-IgG–conjugated Sepharose 4B (MP Biomedicals, CA, USA) was incubated with 20 μg of the anti–laminin-α3-chain mAb LSα3 at 4°C overnight. Each Lm5 protein (3 μg in PBS containing 0.05% Tween 20) was rotated with the anti–α3-antibody–bound beads at 4°C for 4 h to obtain Lm5-bound beads. The resultant Lm5-bound beads were incubated with 50-fold concentrated, serum-free conditioned medium of KB cells containing type VII collagen, at 4°C overnight with slow rotation. The beads were collected by centrifugation, washed three times with PBS/Tween, and resuspended in a small volume of SDS-sample buffer containing 2-mercaptoethanol. The beads were boiled for 5 min, and the eluted type VII collagen was analyzed by immunoblotting with the anti-collagen antibody LH 7.2. To obtain β3SA-bound beads, the purified β3SA (3 μg in PBS containing 0.05% Tween 20) was bound to Chelating Sepharose FF beads

(Amersham Biosciences) pre-treated with 100 mM $NiSO_4$. The β3SA-bound beads were used as described above.

RESULTS

Proteolytic Cleavage of the Laminin β3 Chain of Lm5—It has been believed that, unlike the laminin α3 and γ2 chains of Lm5, the laminin β3 chain is resistant to proteolytic cleavage in culture. However, a recent study has shown that the laminin β3 chain of Lm5 can be cleaved by membrane-type 1 matrix metalloproteinase (MT1-MMP), but in a cell-free system (*33*). In the present study, we examined whether the laminin β3 chain is cleaved in cultures of Lm5-producing human cell lines. Serum-free conditioned media were collected from two squamous cell carcinoma lines (C-4I, HSC-4), two gastric carcinoma cell lines (MKN-45, STKM-1), two immortalized epithelial cell lines (MCF10A, HaCaT) and normal human keratinocytes (NHK). The Lm5 in the conditioned media was immunoprecipitated with an anti–laminin-α3-chain antibody and then detected by immunoblotting with the anti–laminin-β3-chain antibody (Kalinin B1), which recognizes the C-terminal, coiled-coil region (Fig. 2). This analysis detected a major 135-kDa β3 chain in all cell lines tested, and some proteolytic products in C-4I, MCF10A and NHK cells. The β3 chains with apparent sizes of 80, 110 and 125 kDa were relatively dominant among the proteolytic products.

Lm5 in the conditioned medium of NHK cells was also analyzed by immunoblotting with another anti–laminin-β3-chain antibody (β3LE-8A), which was raised against the short arm of the β3 chain in this study. β3LE-8A recognized a recombinant β3 chain protein lacking the LN domain (β3ΔLN), but not one lacking the whole short arm (β3ΔSA), indicating that the epitope of this antibody is located in the laminin EGF-like (LE) domain (Fig. 2B) (see Fig. 1). The 80-kDa fragment of the β3 chain, as well as some other fragments, in the conditioned medium of NHK cells was detected with the Kalinin B1 antibody but not with the β3LE-8A antibody, indicating that the 80-kDa fragment had lost the short arm. These results indicate that the β3 chain of Lm5 is cleaved at its short arm by an endogenous proteinase(s) in some cell lines.

To characterize proteinases that cleave the β3 chain of Lm5, we cultured NHK cells in the presence of a proteinase mixture (0.1 mM AEBSF, 80 nM aprotinin, 5 μM bestatin, 1.5 μM E-64, 2 μM leupeptin and 1 μM pepstatin A) or the matrix metalloproetinase (MMP) inhibitor TAPI (20 μM). These proteinase inhibitors did not affect the proteolytic cleavages of the α3, β3 and γ2 chains, suggesting that a common enzyme may cleave the three laminin chains (data not shown).

Production of Recombinant Lm5 Forms with Partially Deleted β3 Chains and a Recombinant Short Arm Fragment of the β3 Chain—To investigate the effect of β3 chain cleavage on the biological activity of Lm5, we tried to express recombinant Lm5s with normal or N-terminally deleted β3 chains (Fig. 1B). In a previous study, we established an efficient expression system for human recombinant Lm5 by introducing full-length cDNAs encoding the human laminin α3, β3 and γ2 chains into the human embryonic kidney cell line HEK293 (*26*). In this study, the same expression system was utilized to produce Lm5 variants. First, we established a HEK293 cell clone expressing the laminin α3 and γ2 chains at high levels, named α3γ2-HEK. The α3γ2-HEK cells were separately transfected with expression vectors of the full-length β3 chain (β3WT), the β3 chain without the laminin N-terminal domain LN (β3ΔLN), and one lacking the whole short arm (LN plus LE domains) (β3ΔSA) (Fig. 1A). Thus, HEK293 cell transfectants producing the Lm5

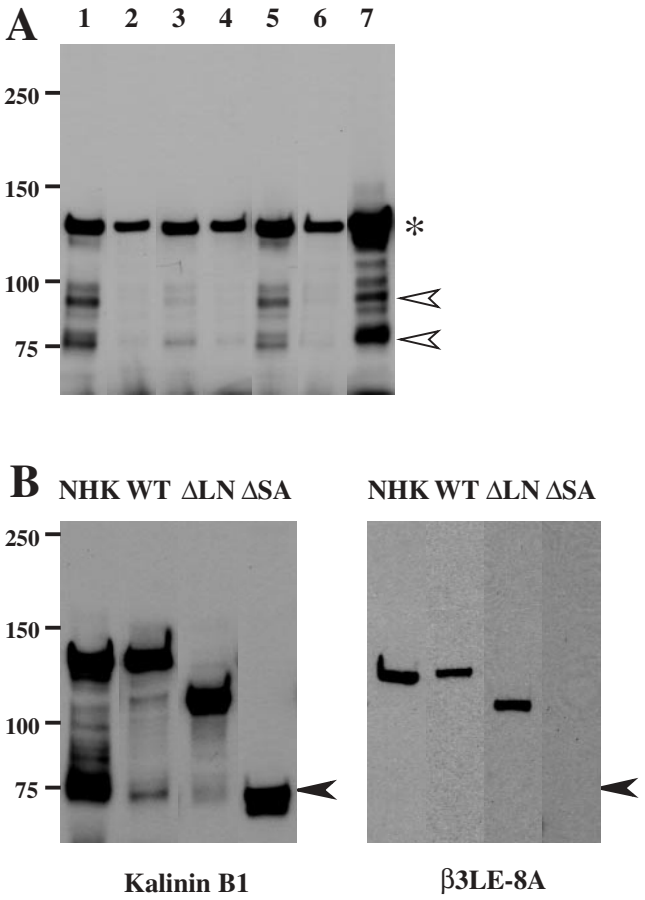

Fig. 2. **Proteolytic cleavage of laminin β3 chain in cultures of Lm5-producing human cell lines.** (A) Lm5 present in the serum-free conditioned medium of 7 human cell lines was immunoprecipitated with the anti–laminin-α3-chain antibody LSα3 to separate it from laminin chain monomers. The resultant precipitates were analyzed by reducing SDS-PAGE followed by immunoblotting with the anti–laminin-β3-chain antibody Kalinin B1, which recognizes a C-terminal, coiled-coil region (LCC). Lanes 1, C-4I; 2, HSC-4; 3, MKN-45; 4, STKM-1; 5, MCF10A; 6, HaCaT; 7, NHK. The asterisk and arrowheads indicate the full-length and cleaved forms of the β3 chain, respectively. Bars show molecular sizes in kDa of the marker proteins. (B) The conditioned medium of NHK cells was immunoprecipitated with the anti–laminin-α3-chain antibody. The resultant precipitate (NHK) and purified three recombinant Lm5 forms (WT, WT-Lm5; ΔLN, ΔLN-Lm5; ΔSA, ΔSA-Lm5), which are described in detail below, were analyzed by immunoblotting with the two anti–laminin-β3-chain antibodies: Kalinin B1 (left panel) and β3LE-8A (right panel), which recognizes the LE domain of the β3 short arm. Note that the 80-kDa proteolytic fragment (arrowhead) in the NHK conditioned medium and the β3 chain of ΔSA-Lm5, which lacks the whole short arm, were detected with Kalinin B1 but not with β3LE-8A. Other experimental conditions are described in "MATERIALS AND METHODS."

forms with the full-length β3 chain (WT-Lm5), the β3 chain lacking LN (ΔLN-Lm5), and that lacking both LN and LE (ΔSA-Lm5) were established. In addition, we constructed a cDNA encoding only the β3 chain short arm (β3SA). The expression vector of β3SA was transfected into parent HEK293 cells. Immunoblotting analyses of the conditioned media from these transfectants verified high expression levels of WT-Lm5, ΔLN-Lm5, ΔSA-Lm5 and β3SA (data not shown).

To purify these recombinant proteins, conditioned media were collected from the serum-free cultures of WT-Lm5-HEK, ΔLN-Lm5-HEK and ΔSA-Lm5-HEK cells. The three forms of Lm5 were purified from the conditioned media by molecular-sieve chromatography, gelatin-Sepharose 4B column chromatography, and then immunoaffinity chromatography with the anti-laminin β3 antibody. The purified proteins, WT-Lm5, ΔLN-Lm5 and ΔSA-Lm5, were analyzed by SDS-PAGE and immunoblotting (Fig. 3, A and B). These analyses showed that the recombinant Lm5s were highly purified, but had heterogeneity due to proteolytic processing. The β3 chains of WT-, ΔLN- and ΔSA-Lm5 had molecular sizes of 135 kDa, 116 kDa and 80 kDa, respectively, as expected from the amino acid sequences (Fig. 3A, left panel and 3B, center panel). No proteolytic cleavage was observed in the HEK293 transfectants. In addition, nonreducing SDS-PAGE showed that all Lm5 forms had the expected heterotrimeric structures with molecular sizes of 345 to 450 kDa (Fig. 3A, right panel). To confirm further that the Lm5 mutants have proper structures, we examined the susceptibility of the Lm5 forms to trypsin digestion. The three Lm5 proteins were incubated with 2 μg/ml trypsin-TPCK for 1 h before or after heat treatment, and then analyzed by SDS-PAGE (Fig. 3C). All of the heat-denatured Lm5 forms were mostly degraded by trypsin during the incubation, but the native ΔLN- and ΔSA-Lm5s, as well as WT-Lm5, remained intact after the trypsin treatment. This suggests that the two deletion mutants of Lm5 have the proper three-dimensional structures.

It is well known that the 190-kDa precursor α3 chain of Lm5 is processed to the 160-kDa mature form and then sometimes to the 145-kDa form, while the 150-kDa γ2

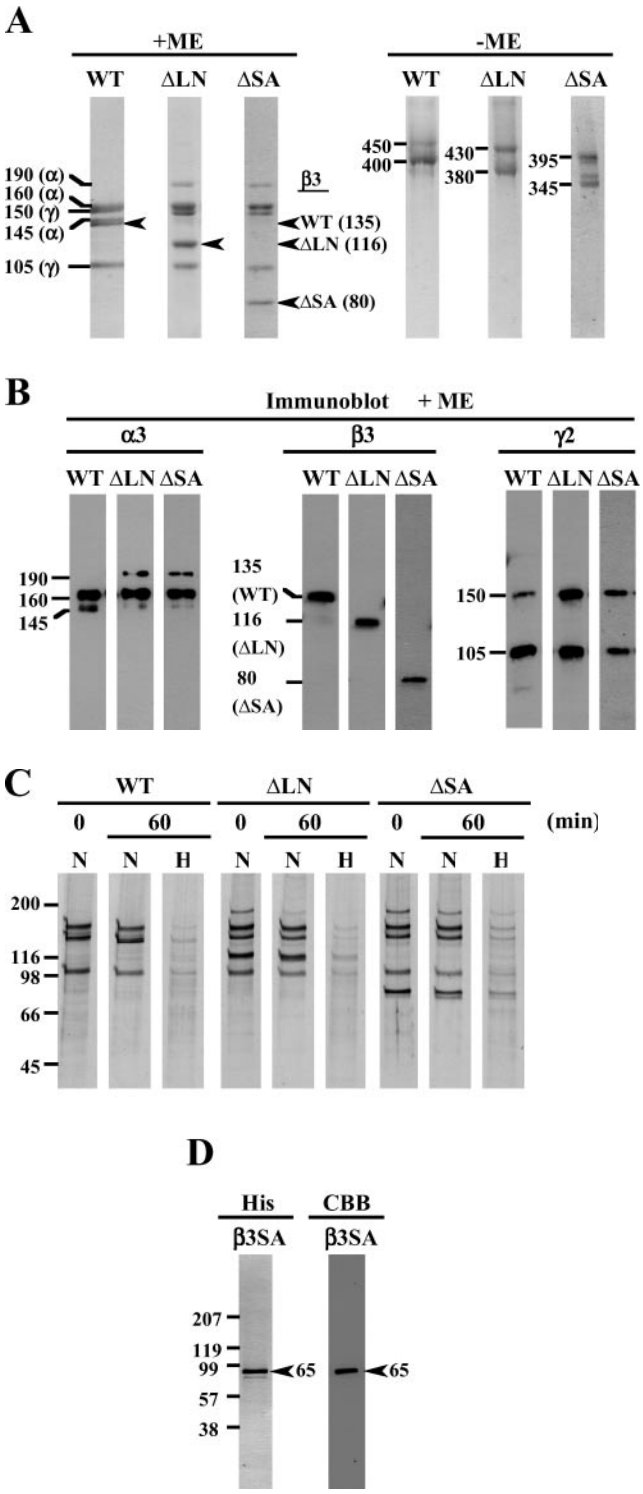

Fig. 3. **Electrophoretic analyses of purified Lm5 proteins and β3SA.** (A) Three purified Lm5 proteins (WT, WT-Lm5; ΔLN, ΔLN-Lm5; ΔSA, ΔSA-Lm5) were analyzed in a 6% SDS-PAGE gel under reducing conditions (left panel) or a 5% gel under non-reducing conditions (right panel). The separated proteins were stained with CBB. Arrows indicate the β3 chain and its apparent molecular size in kDa in each Lm5 form, and bars in the left panel indicate the α3 and γ2 chains and their molecular sizes in kDa. Bars in the right panel indicate Lm5 heterotrimers and their molecular sizes in kDa. Lower bands represent Lm5 forms with partially cleaved laminin chains. (B) The purified three Lm5 forms were analyzed in 6% SDS-PAGE gels under reducing conditions and transferred to nitrocellulose membranes. The blotted proteins were detected by immunoblotting with the anti–laminin-α3-chain (LSα3) (left panel), anti–laminin-β3-chain (Kalinin B1) (center panel) or anti–laminin-γ2-chain antibody (D4B5) (right panel). In the left panel, the 190-, 160- and 145-kDa α3 chain bands correspond to the precursor form, the mature form and a further cleaved form, respectively. In the right panel, the 150-and 105-kDa γ2 chain bands correspond to the unprocessed form and the processed form, respectively. (C) Aliquots of the purified Lm5 proteins were boiled at 95°C for 5 min. Two hundred nanograms of the heat-denatured proteins (H) and native proteins (N) were digested with 2 μg/ml trypsin-TPCK for 60 min, and then separated by reducing SDS-PAGE in a 4–7.5% gradient gel, as described in "MATERIALS AND METHODS." Proteins were stained with silver. (D) Purified β3SA was separated by reducing SDS-PAGE in a 5–20% gradient gel, and the separated proteins were immunoblotted with the anti-His antibody (left lane) or stained with CBB (right lane). Arrowheads indicate the β3SA band of 65 kDa.

chain is often processed to the 105-kDa form (*21*). These proteolytic cleavages modulate the biological activities of Lm5 (*23–25*). Unexpectedly we found that the partial deletion of the β3 chain short arm affected the proteolytic processing of the α3 and γ2 chains. WT-Lm5 contained the 160-kDa α3 chain and a small amount of the 145-kDa form but no 190-kDa form at all, whereas ΔLN- and ΔSA-Lm5s contained the 190-kDa α3 chain in addition to the 160-kDa mature form (Fig. 3A, left panel and 3B, left panel). On the other hand, WT-Lm5 contained a trace amount of the 150-kDa precursor γ2 chain and a high level of the 105-kDa mature form, whereas the relative amounts of the two forms were comparable in ΔLN- and ΔSA-Lm5s (Fig. 3A, left panel and 3B, right panel). The differential ratio of the processed to unprocessed Lm5 was also seen when the three Lm5s were analyzed by non-reducing SDS-PAGE (Fig. 3A, right panel). These results demonstrate that the partial deletion of the β3 chain short arm suppresses the proteolytic processing of the α3 and γ2 chains catalyzed by an endogenous proteinase(s).

In addition to the three forms of Lm5, we expressed a short arm fragment of the β3 chain, named β3SA, in HEK293 cells. The β3SA protein, which consists of the LN and LE domains and a C-terminal, 6× histidine tag, was purified from the conditioned medium of β3SA-HEK cells by a nickel column and HPLC gel filtration chromatographies. The purified protein was detected as a 65-kDa band by CBB staining after reducing SDS-PAGE (Fig. 3D, right panel) and immunoblotting with the anti-His-tag antibody (Fig. 3D, left panel). N-terminal amino acid sequencing also verified that the 65-kDa protein is β3SA. About 0.5 mg of Lm5 and about 1 mg of β3SA were purified from one liter of the respective conditioned media.

Cell Adhesion and Spreading Activities of Recombinant Lm5 Mutants and β3 Chain Short Arm Fragment—Lm5 has activities to promote cellular adhesion, spreading and scattering (*14–16*). To examine possible roles of the β3 chain short arm in the expression of Lm5 activities, we compared these activities among WT-Lm5, ΔLN-Lm5, ΔSA-Lm5 and β3SA. To assay the cell adhesion activity, NHK cells were plated in serum-free medium on plates pre-coated with various concentrations of WT-Lm5, ΔLN-Lm5, ΔSA-Lm5 and β3SA and incubated for 30 min, followed by quantification of attached cells. As shown in Fig. 4A, ΔLN-Lm5 and ΔSA-Lm5 supported cell adhesion at higher concentrations than the WT-Lm5 substrate. The effective concentration for half-maximal adhesion activity (ED_{50}) was determined to be 0.8 μg/ml (2 μM) for WT-Lm5, 1.5 μg/ml (3.9 μM) for ΔLN-Lm5 and 1.5 μg/ml (4.0 μM) for ΔSA-Lm5, indicating that the two deletion mutants have about half the activity of WT-Lm5.

The differential cell adhesion activities of the Lm5 forms are reflected in their cell spreading activities. NHK cells spread on 1.25 μg/ml WT-Lm5, whereas only a portion of the cells could spread even on 5 μg/ml ΔLN-Lm5 or ΔSA-Lm5 (Fig. 4B). Thus, the deletion of the β3 chain short arm also decreased the cell spreading activity of Lm5. On the other hand, β3SA showed neither cell adhesion nor spreading activity toward NHK cells, even at a coating concentration of 10 μg/ml (Fig. 4, A and B). These results indicate that the β3 chain short arm supports the cell adhesion and spreading activities of Lm5, although it is not essential. Lower cell adhesion activity of the mutant Lm5 forms was also observed with the Buffalo rat liver cell line BRL (data not shown).

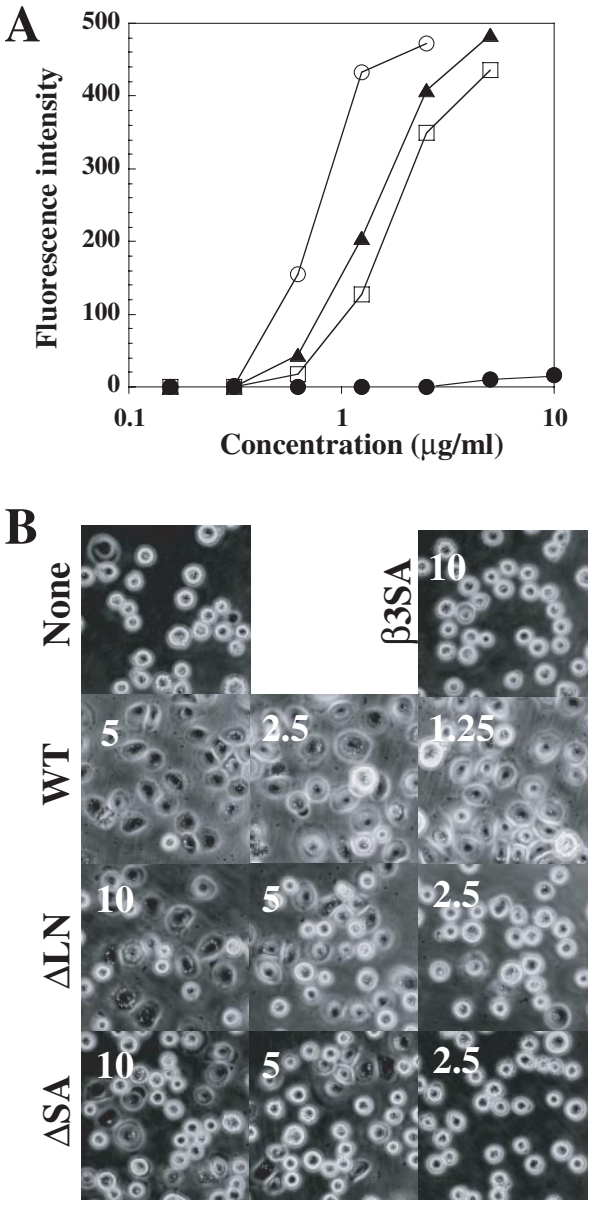

Fig. 4. **Cell attachment and spreading on three Lm5 proteins and β3SA.** (A) NHK cells were inoculated into the wells of 96-well plates precoated with the indicated concentrations of each substrate protein. After incubation for 30 min, the relative numbers of adherent cells were determined by measuring fluorescence intensity. Each point represents the average of duplicate assays. Open circles, WT-Lm5; closed triangles, ΔLN-Lm5; open squares, ΔSA-Lm5; closed circles, β3SA. (B) The morphology of NHK cells incubated on the indicated concentrations of the substrates for 30 min was observed under a phase-contrast microscope. WT, WT-Lm5; ΔLN, ΔLN-Lm5; ΔSA, ΔSA-Lm5. Original magnification is ×300. Other experimental conditions are described in "MATERIALS AND METHODS."

The major activities of Lm5 are mediated by the interaction between the C-terminal LG1-3 domain of the α3 chain and integrins α3β1, α6β1 and α6β4 as cell surface receptors (*4*). Especially, integrin α3β1 plays an important

role in cell adhesion to Lm5 (*5*). In the present study, we also tested whether the deletion of β3 chain short arm affects the integrin requirement, using function-blocking antibodies against integrins. As shown in Fig. 5, the anti-α3 integrin and anti-β1 integrin antibodies almost completely blocked the adhesion of NHK cells to both WT-Lm5 and ΔSA-Lm5. Essentially the same result was obtained with ΔLN-Lm5 (data not shown). Other antibodies and heparin had no effect on cell adhesion. These results indicate that both the wild-type and mutated Lm5s recognize integrin α3β1 as a major cell adhesion receptor, and that the deletion of β3 chain short arm does not affect the integrin specificity of Lm5.

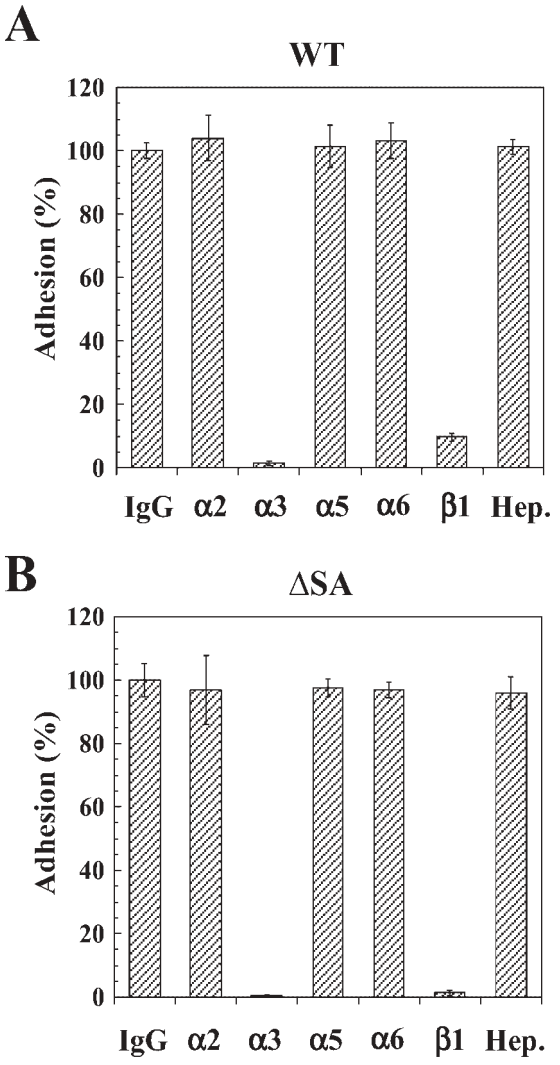

Fig. 5. **Effects of function-blocking antibodies against various integrin subunits and heparin on cell attachment to WT-Lm5 and ΔSA-Lm5.** NHK cells were incubated with function-blocking integrin antibodies (10 μg/ml) or heparin (Hep., 100 μg/ml) at room temperature for 20 min. After treatment, the cells were inoculated into wells pre-coated with 2.5 μg/ml of WT-Lm5 (A) or ΔSA-Lm5 (B). Cell attachment in the presence of a control mouse IgG was taken 100%. Each bar represents the mean and SD of triplicate assays. Other experimental conditions are the same as described in the legend to Fig. 4. Almost the same result was obtained with ΔLN-Lm5 as with ΔSA-Lm5.

Cell Motility Activities of Lm5 Mutants—Lm5 promotes not only cell adhesion and spreading but also cell migration and scattering. Both soluble and insoluble (or coated) forms of Lm5 are able to induce cell migration and scattering (*34*). First, the cell-scattering activity toward BRL cells was assayed for each Lm5 form and β3SA. BRL cells were inoculated into each well in medium containing 1% serum together with various concentrations of each Lm5 or β3SA, and incubated for 40 h. All Lm5 proteins efficiently scattered BRL cells (Fig. 6, A and B), and the effective doses for half-maximal scattering activity (ED_{50}) of WT-Lm5, ΔLN-Lm5 and ΔSA-Lm5 were approximately 9 ng/ml (23 nM), 13 ng/ml (34 nM) and 20 ng/ml (50 nM), respectively. This indicates that the partial deletion of the β3 short arm weakly reduces the cell-scattering activity of Lm5. On the other hand, β3SA did not induce cell scattering even at a final concentration of 1.6 μg/ml (25 μM).

Next, the cell migration activity toward NHK cells was assayed on plates pre-coated with each of the three Lm5 forms. At the concentration required for sufficient cell adhesion, 1 μg/ml for WT-Lm5 and 2.5 μg/ml for

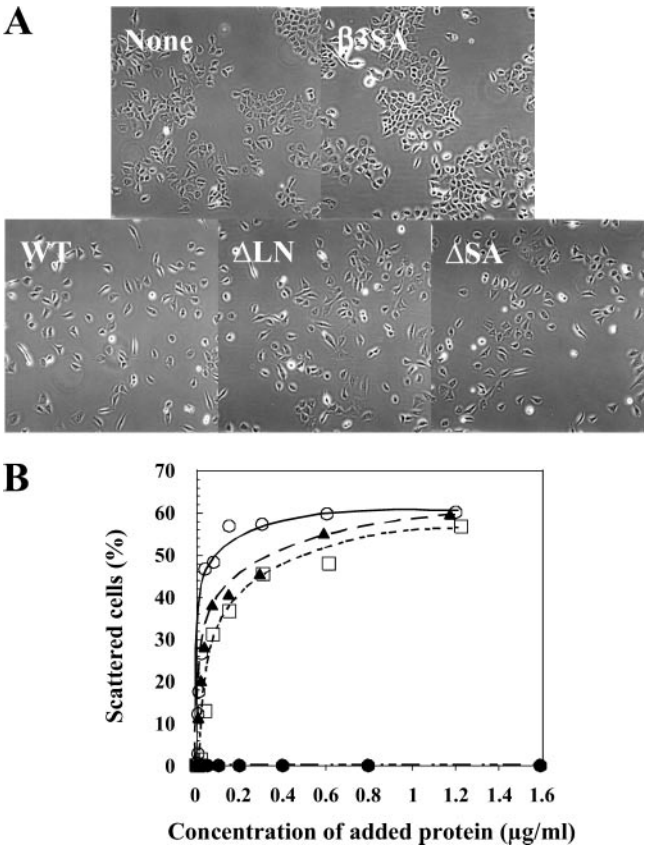

Fig. 6. **Cell scattering activity of three Lm5 forms and β3SA.** (A) BRL cells were inoculated and incubated in a 1% FCS-containing medium supplemented with WT-Lm5 (WT, 60 ng/ml), ΔLN-Lm5 (ΔLN, 60 ng/ml), ΔSA-Lm5 (ΔSA, 60 ng/ml) or β3SA (1.6 μg/ml) for 40 h. After incubation, cell morphology was observed under a phase-contrast microscope. (B) BRL cells were incubated in the presence of the indicated concentrations of the substrate proteins as described above. Cell scattering was determined as described in "MATERIALS AND METHODS." Open circles, WT-Lm5; closed triangles, ΔLN-Lm5; open squares, ΔSA-Lm5; closed circles, β3SA.

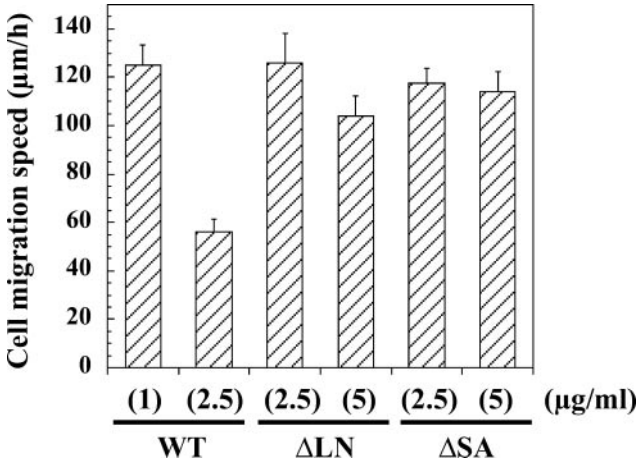

Fig. 7. **Cell migration on three Lm5 forms.** NHK cells were inoculated onto plates precoated with the indicated concentrations of WT-Lm5, ΔLN-Lm5, or ΔSA-Lm5 and incubated for 1.5 h. After the incubation, cell migration was monitored by a time-lapse microscope for 3 h, and the migration distance was quantified. Each bar represents the mean ± SD of the migration speeds of optional 7 cells.

ΔLN-Lm5 and ΔSA-Lm5, the cell migration speed was around 120 μm/h on each substrate (Fig. 7). However, increasing the WT-Lm5 concentration to 2.5 μg/ml diminished the cell migration speed to about 60 μm/h, possibly due to excess cell adhesion strength. In contrast, cell migration on ΔLN-Lm5 and ΔSA-Lm5 was not affected as their concentrations were increased to 5 μg/ml. These results suggest that the partial deletion of the β3 chain short arm does not significantly affect the cell motility activity of Lm5. The slight decrease in the cell-scattering activity in the Lm5 mutants appears to be caused by the decrease in cell adhesion activity.

Synergistic Stimulation of Cell Adhesion by the β3 Chain Short Arm Fragment and ΔSA-Lm5—As shown above, the β3 chain short arm does not support cell adhesion by itself, but its truncation results in a lower cell adhesion activity of Lm5. To demostrate the role of the β3 chain short arm in the cell adhesion and spreading activities of Lm5, we examined whether β3SA cooperates with ΔSA-Lm5 in the promotion of cell adhesion and spreading. When culture plates were co-coated with increasing concentrations of β3SA and a constant concentration (1.0 or 1.5 μg/ml) of ΔSA-Lm5, the adhesion of NHK cells to the plates was significantly increased compared to that on ΔSA-Lm5 alone (Fig. 8A). The cell adhesion activity of β3SA was more evident at the lower concentration of ΔSA-Lm5. β3SA also promoted the spreading of NHK cells in a dose-dependent manner on the ΔSA-Lm5 substrate (Fig. 8B). These results indicate that β3SA promotes cell adhesion synergistically with ΔSA-Lm5, although the effective molar concentration of β3SA (1 μg/ml, 15 μM) is at least about 5-times higher than that of ΔSA-Lm5 (1 μg/ml, 2.9 μM).

Next, we examined the synergistic effect of a soluble form of β3SA with ΔSA-Lm5 on cell adhesion. NHK cells were preincubated with a soluble form of β3SA and then plated on ΔSA-Lm5–coated plates. In contrast to the effect of co-coating with β3SA, pretreatment with soluble β3SA produced a dose-dependent inhibition of cell adhesion to ΔSA-Lm5 (Fig. 8C). This suggests that the synergistic effect of β3SA might be exerted only when it exists in close vicinity to ΔSA-Lm5 ligand or integrins.

We also examined the synergistic cell adhesion activity of the insoluble (or coated) form of β3SA with ΔSA-Lm5, using the Lm5-nonproducing cell line HEK293. When β3SA was co-coated with ΔSA-Lm5, it efficiently promoted the adhesion of HEK293 cells (Fig. 8D). The synergistic cell adhesion activity of the insoluble β3SA was more evident in HEK293 cells than NHK cells (Fig. 8, A and D).

The activities of the soluble and insoluble forms of β3SA suggest that it might act on some cell surface receptors. To test this possibility, we prepared β3SA-conjugated beads and applied them to NHK cells, which express a high level of endogenous Lm5 (Fig. 9, A and B). β3SA-conjugated beads bound to the apical surface of NHK cells, but control beads bound only slightly. When the β3SA-conjugated beads were applied to HEK293 cells, which express neither the laminin β3 chain nor Lm5, the β3SA beads bound markedly to the cell surface compared to the control beads (Fig. 9, C and D). These results suggest that β3SA binds to an unidentified cell surface receptor(s). The lower binding capacity of the β3SA beads to NHK cells seems to be due to the occupation of β3SA receptors by endogenous Lm5.

To characterize further β3SA binding to the cell surface, we examined the integrin requirement for the adhesion of NHK cells to mixed substrates of ΔSA-Lm5 and β3SA using function-blocking antibodies against integrin α3 and β1 in Fig. 10. Although β3SA produced a dose-dependent increase in both cell attachment (Fig. 10A) and spreading (Fig. 10B), both were almost completely blocked by the anti–integrin-α3 antibody at all doses tested. The anti–integrin-β1 antibody also efficiently blocked cell adhesion regardless of the presence or absence of β3SA. These results suggest that β3SA might enhance the interaction between ΔSA-Lm5 and integrin α3β1.

Localization of a Type VII Collagen–Binding Domain in the β3 Chain Short Arm—The interaction of Lm5 with type VII collagen, a major component of anchoring fibrils, is important for the dermal–epidermal junction in the skin. Past reports have shown that Lm5 binds to type VII collagen through the short arms of the laminin β3 chain and possibly the γ2 chain (*10*, *35*). Since the binding site in the β3 chain short arm has not been identified, we examined the ability of β3SA and the three forms of Lm5 to bind type VII collagen using Sepharose beads conjugated with each of these proteins. As HEK293 cells do not secrete type VII collagen, the conditioned medium of the human oral epidermal carcinoma cell line KB was used as a source of the collagen. As shown in Fig. 11A, β3SA-conjugated beads, but not control beads, bound type VII collagen, indicating that β3SA indeed contains a binding site for type VII collagen. A similar pull-down assay showed that WT-Lm5 and ΔLN-Lm5 bind collagen, but ΔSA-Lm5 does not (Fig. 11B). These results imply that the EGF-like domain LE is the binding site for type VII collagen, and that the γ2 chain is not required for the interaction of Lm5 with type VII collagen.

DISCUSSION

In the present study, we examined the biological function of the short arm of the laminin β3 chain using

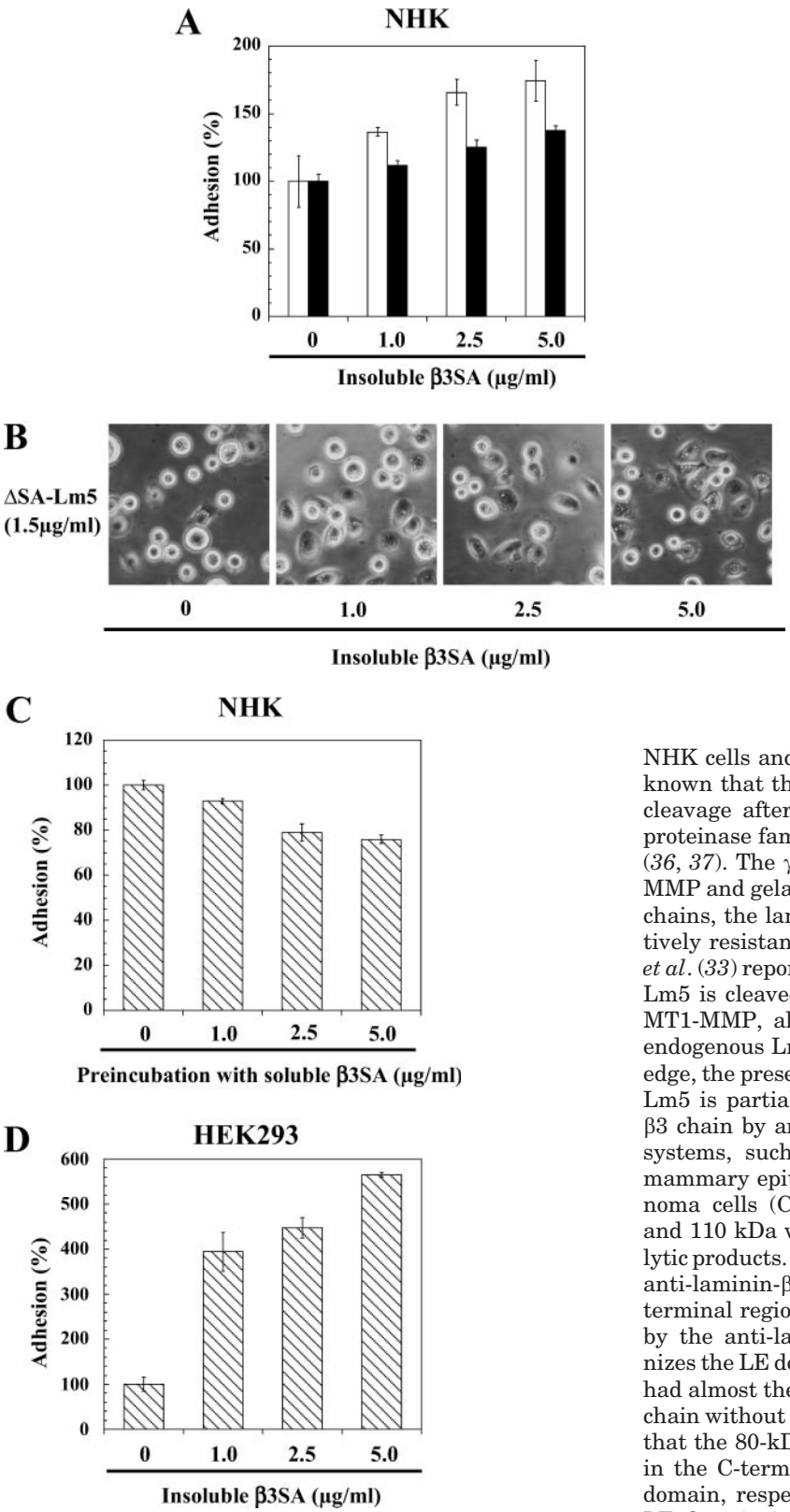

Fig. 8. **Effect of β3SA on cell adhesion of ΔSA-Lm5.** (A) The indicated concentrations of β3SA were co-coated with 1.0 μg/ml (open columns) or 1.5 μg/ml (closed columns) of ΔSA-Lm5 in 96-well plates. NHK cells were inoculated into each well and incubated for 30 min, and adherent cells were measured as described in the legend to Fig. 4. Cell attachment without β3SA was taken 100%. (B) The cell morphology of the above cultures (A) was observed under a phase-contrast microscope. (C) NHK cells were incubated with the indicated concentrations of β3SA at room temperature. After the incubation for 20 min, the cells were inoculated into wells precoated with ΔSA-Lm5 (1.5 μg/ml). Adherent cells were measured as above. (D) The indicated concentrations of β3SA were co-coated with 1.5 μg/ml of ΔSA-Lm5 on 96-well plates. HEK293 cells were incubated in the wells for 60 min, and adherent cells were measured as above. Cell attachment without β3SA was taken 100%.

recombinant Lm5 forms with partially deleted β3 chains and a short arm fragment of the β3 chain. First, we showed that the laminin β3 chain of Lm5 is cleaved at the N-terminal region by an endogenous proteinase(s) in NHK cells and some other Lm5-producing cells. It is well known that the α3 and γ2 chains of Lm5 undergo limited cleavage after secretion (*22*, *23*, *25*). The BMP-1/TLD-1 proteinase families are likely responsible for this cleavage (*36*, *37*). The γ2 chain of rat Lm5 is also cleaved by MT1-MMP and gelatinase A (MMP-2) (*24*). Unlike the α3 and γ2 chains, the laminin β3 chain has been thought to be relatively resistant to the proteolysis. Recently, Udayakumar *et al*. (*33*) reported that the β3 chain of human recombinant Lm5 is cleaved in the N-terminal region by recombinant MT1-MMP, although they did not show the cleavage of endogenous Lm5 in cell cultures or tissues. To our knowledge, the present study is the first to show that endogenous Lm5 is partially cleaved in the N-terminal region of the β3 chain by an endogenous proteinase(s) in some culture systems, such as normal human keratinocytes (NHK), mammary epithelial cells (MCF10A) and squamous carcinoma cells (C-4I). β3 chains with apparent sizes of 80 and 110 kDa were relatively dominant among the proteolytic products. These cleaved β3 chains were detected by the anti-laminin-β3 antibody Kalinin B1, which recognizes a C-terminal region of the coiled-coil domain, but not detected by the anti-laminin-β3 antibody β3LE-8A, which recognizes the LE domain (Figs. 1A and 2B). The 80-kDa product had almost the same molecular size as the recombinant β3 chain without the short arm (β3ΔSA). Therefore, it is likely that the 80-kDa and 110-kDa products have been cleaved in the C-terminal and the N-terminal regions of the LE domain, respectively. Similar proteolytic cleavage in the LE domain is known to occur in the γ2 chain (*24*). The 80-kDa, major cleaved product of the β3 chain in NHK cells is similar in size to the MT1-MMP-cleaved fragment reported by Udayakumar *et al*. (*33*). In our culture systems, however, BMP-1/mTLD proteinases are more likely candidates for the β3-chain–cleaving enzymes than

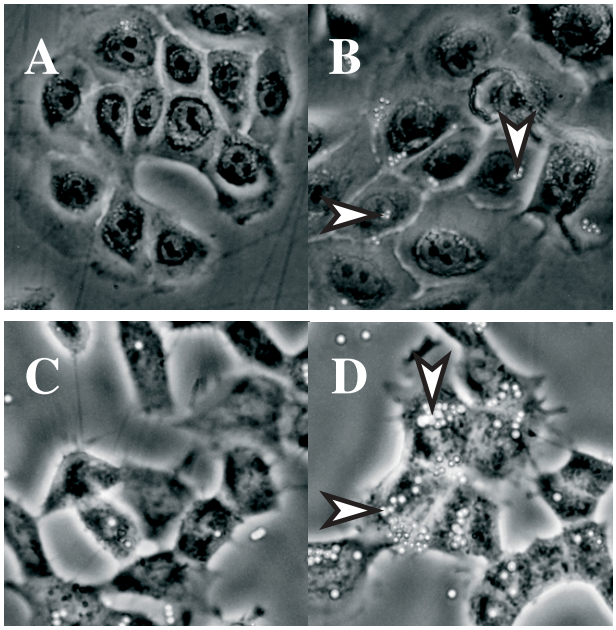

Fig. 9. **Binding of β3SA to NHK cells (A and B) and HEK293 cells (C and D).** (A and B) Anti–His-antibody–conjugated beads were bound with purified β3SA. The β3SA-bound beads (B) and the unbound, anti-His-antibody beads (A) were incubated for 1.5 h with NHK cells, which express a high level of Lm5, and then washed twice with PBS. The beads bound to the cells were observed directly under a phase-contrast microscope. (C and D) Beads conjugated with the anti–laminin-β3-chain antibody (β3LE-12C) were further bound with purified β3SA. The β3SA-bound beads (D) and the unbound, anti–laminin-β3-chain-antibody beads (C) were incubated for 18 h with HEK293 cells, which do not express endogenous Lm5, and then washed twice for microscopic observation. Arrowheads indicate beads bound to cells. Other experimental conditions are described in Materials and Methods. When NHK cells were treated with the anti–laminin-β3-chain-antibody beads, the beads bound densely to Lm5 deposited in the cell-free space and cell surface (data not shown). Therefore, the relatively poor binding of the β3SA-beads to NHK cells in (B) seems due to the occupation of the β3SA receptor with endogenous Lm5. Poor binding of the β3SA protein to the anti–His-antibody beads in (B) may be another reason for the less efficient binding of β3SA beads to NHK cells.

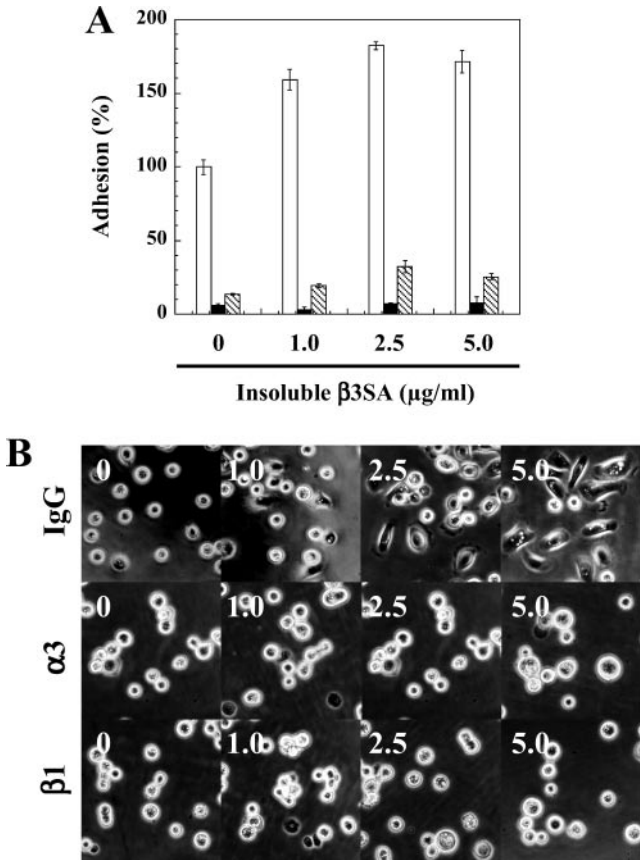

Fig. 10. **Integrin requirement for cell adhesion to mixed substrates of ΔSA-Lm5 and β3SA.** (A) β3SA at the indicated concentrations was co-coated with ΔSA-Lm5 (1.5 μg/ml) on 96-well plates. NHK cells were incubated with mouse IgG (open columns), the function-blocking anti–integrin-α3 antibody (closed columns), or the function-blocking anti–integrin-β1 antibody (striped columns) at room temperature for 20 min. The treated cells were inoculated into each well of the coated plates and incubated for 60 min; adherent cells were measured as described in Fig. 4. The attachment of the mouse-IgG–treated cells to ΔSA-Lm5 alone was taken 100%. (B) The cell morphology of the above cultures (A) was observed under a phase-contrast microscope. The numbers on the pictures indicate the concentrations (μM) of β3SA.

MT1-MMP, because an MMP inhibitor and serine proteinase inhibitors did not inhibit the cleavage.

Next, to show the biological consequence of the β3 chain cleavage of Lm5, we expressed two Lm5 mutants with N-terminally deleted β3 chains in HEK293 cells. ΔLN-Lm5 lacks the N-terminal globular domain LN of the β3 chain, and ΔSA-Lm5 lacks the whole sequence of the β3 chain short arm. Unexpectedly, the expression of the Lm5 mutants in HEK293 cells revealed that the N-terminal deletion of the β3 chain suppresses the proteolytic processing of the α3 and γ2 chains. This phenomenon suggests two possible mechanisms. First, the N-terminal deletion of the β3 chain might change the localization of Lm5, reducing the chance of interaction with processing enzymes. Second, the partial deletion of the β3 chain might induce conformational changes in the processing sites of the α3 and γ2 chains, reducing their susceptibility to proteinases. These possibilities should be examined in further studies.

Comparative analyses of the biological activities of the wild-type Lm5 (WT-Lm5) and two deletion mutants (ΔLN-Lm5 and ΔSA-Lm5) demonstrated that the deletion of the β3 short arm decreases the cell adhesion and spreading activities of Lm5, but has only a weak effect on the cell-scattering or migration activities. ΔLN-Lm5 and ΔSA-Lm5 show similar activities, indicating that the loss of the LN domain is responsible for the decreased activity. In addition, both the wild-type and mutant laminins showed the same integrin specificity. These results imply that the β3 short arm, especially its LN domain, is involved in the cell adhesion activity of Lm5. The differential cell adhesion activity seem not to depend on the differential coating efficiency of these Lm5 forms, because there was little difference in the coating efficiency (data not shown). In addition, it should be noted that the proteolytic processing of the γ2 and α3 chains affects Lm5 activity. The cleavage of the 150-kDa γ2 chain to the 105-kDa form decreases the cell adhesion activity of Lm5 but increases

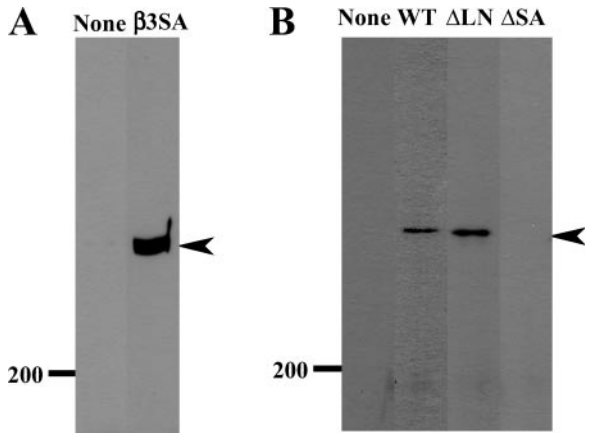

Fig. 11. **Interaction between type VII collagen and Lm5 forms with partially deleted β3 chains or β3SA.** β3SA, WT-Lm5 (WT), ΔLN-Lm5 (ΔLN) and ΔSA-Lm5 (ΔSA) were individually bound to Sepharose beads as described in "MATERIALS AND METHODS." The beads were incubated at 4°C overnight with the conditioned medium of KB cells, which contained type VII collagen but not Lm5. After the incubation, the beads were collected, washed and suspended in SDS-sample buffer containing 3.3% 2-mercaptoethanol. The released proteins were analyzed by SDS-PAGE and immunoblotting with the anti-type VII collagen antibody. None, incubation with control beads. (A) Binding of type VII collagen to β3SA-conjugated Sepharose beads. (B) Binding of type VII collagen to Sepharose beads pre-conjugated with WT-Lm5, ΔLN-Lm5, or ΔSA-Lm5 (ΔSA). Arrowheads indicate the 290-kDa band corresponding to type VII collagen. Other experimental conditions are described in "MATERIALS AND METHODS."

its cell motility activity (*29*). On the other hand, the cleavage of the 190-kDa α3 chain to the 160-kDa form increases both the cell adhesion and motility activities (*30*). As described above, the partial deletion of the β3 chain increases the proportions of the 150-kDa γ2 chain and the 190-kDa α3 chain compared to the wild-type Lm5 (Fig. 3B). It is expected that the increase in the proportion of the 150-kDa γ2 chain increases the cell adhesion activity of Lm5. Although the increase in the proportion of the 190-kDa α3 chain is expected to reduce the cell adhesion activity of Lm5, the relative amount of this Lm5 was estimated to be less than 30% that of the 160-kDa α3 chain. Therefore, it was concluded that the change in Lm5 activity results from the partial deletion of the β3 chain rather than the changes in the proteolytic processing of the α3 or γ2 chain.

We also found that a recombinant laminin β3 short arm protein (β3SA) promotes both the adhesion and spreading of NHK or HEK293 cells when co-coated with β3SA-deficient Lm5 (ΔSA-Lm5). This effect was observed only when excess concentrations of β3SA were used compared to that of ΔSA-Lm5. However, when these cells were pretreated with a soluble form of β3SA, the cell adhesion to ΔSA-Lm5 was significantly suppressed. These results suggest that β3SA interacts with some receptors on the cell surface. Indeed, the cell surface binding of β3SA was directly confirmed by the experiments with β3SA-conjugated beads. Based on these results, we speculate that the β3 short arm of Lm5, most likely its LN domain, binds to a cell surface receptor when the LG1-3 domain of the α3 chain binds to integrins. β3SA itself did not show cell adhesion activity, and the cell adhesion to the mixed substrates of β3SA and ΔSA-Lm5 was almost completely inhibited by either the anti–integrin-α3 or -β1 antibody. Therefore, it is assumed that the β3 receptor may be directly or indirectly associated with integrins, and that β3 binding to the receptor may enhance the Lm5-integrin interaction or the resultant integrin signaling. Thus, the β3 short arm can contribute to the efficient cell adhesion activity of Lm5. The pretreatment of cells with soluble β3SA might recruit the receptors with or without integrins to the apical cell surface, resulting in the suppression of the cell adhesion efficiency to Lm5. It is well known that laminin-binding integrins form complexes with tetraspanins, strengthening integrin affinities to laminins (*38*). Tetraspanins or other integrin-associated proteins are possible candidates for the β3 short arm receptors.

The N-terminal regions (short arms) of the three laminin chains contain functional domains that are required for matrix assembly (*39*). Although the β3 short arm lacks the L4 domain and some EGF-like repeats found in the full-sized β1 and β2 chains (*40*), it contains a site to interact with type VII collagen (*35*). In addition, Lm5 forms complexes with laminin-6 and laminin-7, presumably by interactions between the short arms of the two laminins (*11*). Thus, the β3 short arm plays important roles in the matrix assembly of Lm5. In the present study, we provide the first demonstration that Lm5 interacts with type VII collagen via the LE domain of the β3 chain short arm.

In summary, the present study reveals that the LN domain of the β3 chain short arm regulates the Lm5-integrin interaction through binding with an unidentified receptor, while its LE domain interacts with type VII collagen. The LN and LE domains in the β3 chain short arm are lost from Lm5 through the action of endogenous proteinases in cell cultures. The proteolytic cleavage of the β3 chain short arm is likely to modulate the matrix assembly and the cell adhesion activities of Lm5. Further studies are needed to determine whether the proteolytic cleavage of the β3 chain occurs in tissues under some physiological and pathological conditions, such as tissue repair, inflammation and tumor invasion.

We are grateful to Drs. H. Yasumitsu, S. Higashi, T. Ogawa and Y. Tsubota for helpful suggestions and technical support. This work was supported in part by Grants-in-Aid from the Ministry of Education, Culture, Sports, Science and Technology of Japan and from the Yokohama City Collaboration of Regional Entities for the Advancement of Technological Excellence, Japan Science and Technology Agency.

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