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ARTICLES

The β3 Chain Short Arm of Laminin-332 (Laminin-5) Induces Matrix Assembly and Cell Adhesion Activity of Laminin-511 (Laminin-10)

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Abstract The basement membrane (BM) protein laminin-332 (Lm332) (laminin-5) has unique activity and structure as compared with other laminins: it strongly promotes cellular adhesion and migration, and its α3, β3, and γ2 chains are all truncated in their N-terminal regions (short arms). In the present study, we investigated the biological function of the laminin β3 chain. When the β3 chain short arm (β3SA) was overexpressed in HEK293 cells (β3SA-HEK), they deposited a large amount of β3SA and a small amount of laminin-511 (Lm511) (laminin-10) on culture plates. Control HEK293 cells secreted Lm511 but failed to deposit it. The extracellular matrix (ECM) deposited by β3SA-HEK cells strongly promoted cell attachment and spreading. The β3SA-HEK ECM did not directly bind Lm511, but it stimulated control HEK293 cells to deposit Lm511 on the culture plates. Although purified β3SA did not support cell adhesion by itself, it enhanced the cell adhesion activity of Lm511. Experiments with anti-integrin antibodies also suggested that the strong cell adhesion activity of the β3SA-HEK ECM was derived from the synergistic action of β3SA and Lm511. It has previously been found that β3SA binds an unknown cell surface receptor. Taken together, the present study suggests that the short arm of the laminin β3 chain enhances the matrix assembly of Lm511 and its cell adhesion activity by interacting with its receptor. J. Cell. Biochem. 100: 545–556, 2007.

Key words: extracellular matrix; cell adhesion; laminin-332 (laminin-5); laminin-511 (laminin-10); matrix assembly

Laminins are major extracellular matrix (ECM) components of basement membranes (BMs), which play essential roles in both the BM construction and the regulation of cellular functions [Miner and Yurchenco, 2004]. Laminins are large heterotrimers of α, β, and γ chains, linked together with disulfide bonds. Five types of α chain (α1-5), three types of β chain (β1-3) and three types of γ chain (γ1-3) have been identified so far in mouse and human, and different combinations of one of each chain produce more than 15 recognized laminin isoforms (laminin-1 through laminin-15). These laminin isoforms are differently distributed in specific tissues and specific developmental stages. In a recently proposed nomenclature, which is based on the types of α, β, and γ chains, laminin-1 (α1β1γ1), laminin-5 (α3β3γ2), and laminin-10 (α5β1γ1) are referred to as laminin-111, laminin-332 (Lm332), and laminin-511

Abbreviations used: BM, basement membrane; BSA, bovine serum albumin; ECM, extracellular matrix; FCS, fetal calf serum; Lm332, laminin-332; Lm511, laminin-511; mAb, monoclonal antibody; β3SA, a recombinant protein of laminin β3 chain short arm; WT-Lm5, Lm332 with the full-length β3 chain; ΔLN-Lm5, Lm332 with a β3 chain lacking the LN domain; ΔSA-Lm5, Lm5 with a β3 chain lacking the whole short arm; β3SA-ECM, ECM deposited by β3SA-HEK cells; WT-Lm5-ECM, ECM deposited by WT-Lm5 cells.

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(Lm511), respectively [Aumailley et al., 2005]. Each chain of laminins contains many functional domains. In general, the N-terminal regions of all laminin chains, often called "short arms," are required for the matrix assembly of laminins. The C-terminal region of laminin α chains has five globular domains that interact with cell surface receptors, such as integrins, α-dystroglycans, and syndecans, to express the biological activities of laminins [Kariya et al., 2003; Miner and Yurchenco, 2004].

Laminin-332 (abbreviated Lm332), formerly termed laminin-5, is a major component in the epidermal BM and also localized in the BMs of many epithelial tissues such as the esophagus, lung, breast, colon, and kidney [Carter et al., 1991; Mizushima et al., 1998]. In the BM of the skin, Lm332 interacts with integrin α6β4 of basal keratinocytes to form hemidesmosomes, while it interacts with type VII collagen, laminin-311 (laminin-6), and laminin-321 (laminin-7), forming anchoring filaments [Rousselle et al., 1991; Champliaud et al., 1996; Chen et al., 1997]. Thus, Lm332 plays an essential role in the tight binding of epidermis to dermis [Pulkkinen et al., 1995; Ryan et al., 1999; Muhle et al., 2005]. Consistent with these functions of Lm332 in vivo, it promotes cellular adhesion and migration much more strongly than other laminins by interacting with integrins α3β1, α6β1, and α6β4 in vitro [Miyazaki, 2006]. The β3 and γ2 chains of Lm332, which are found only in this laminin, are truncated and lack some domains found in the short arms of typical β and γ chains such as β1, β2, and γ1 [Kallunki et al., 1992; Gerecke et al., 1994]. Therefore, it is thought that Lm332 is incorporated into ECMs in a different manner from other laminins [Yurchenco and Cheng, 1993; Cheng et al., 1997]. In addition, the unique structures of the β3 and γ2 chains are expected to contribute to the strong biological activity of Lm332.

To clarify biological functions of laminin β3 chain, we previously expressed in HEK293 cells a recombinant Lm332 with the full-length β3 chain and its variants with a β3 chain lacking the N-terminal domain LN or the whole short arm. Using the purified Lm332 proteins, we identified a binding site for type VII collagen at the laminin EGF-like domain (LE) in the β3 chain short arm [Nakashima et al., 2005]. It was also found that the β3 chain short arm, especially the LN domain, is essential for the strong cell adhesion activity of Lm332. In the present study, we investigated the functional interaction of the β3 short arm with Lm511, formerly termed laminin-10. Our results demonstrate that the β3 short arm promotes the matrix assembly of Lm511 and enhances Lm511-dependent cell adhesion and spreading.

MATERIALS AND METHODS

Antibodies and Other Proteins

Monoclonal antibodies (mAbs) against human laminin-β3-chain (29E, β3LE-8A, and -12C) were prepared previously [Kagesato et al., 2001; Nakashima et al., 2005]. Other antibodies used were mouse anti-laminin-α5-chain mAb (4C7) from Life Technologies (Gaithersburg, MD), rat anti-laminin-β1-chain mAb (LT3), and mouse anti-laminin-γ1-chain mAb (2E8) from Chemicon (Temecula, CA). A mouse mAb against human laminin-α5-chain, (15H5), was a generous gift from Dr. K. Sekiguchi (Institute for Protein Research, Osaka University, Osaka, Japan). Function-blocking antibodies against human integrins used were anti-integrin-α2 mAb (P1E6), anti-integrin-α3 mAb (P1B5), anti-integrin-α5 mAb (P1D6), and anti-integrin-β1 mAb (6S6) from Chemicon and anti-integrin-α6 mAb (G_0H3) from Pharmingen (San Diego, CA). Recombinant Lm332 proteins were purified using mAb 29E as described before [Nakashima et al., 2005]. Human Lm511/521 was purchased from Sigma (St. Louis, MO).

Cell Lines and Culture Conditions

The human embryonic kidney cell line HEK293 (CRL-1573) was purchased from American Type Culture Collection (ATCC). Four kinds of HEK293 transfectants, WT-Lm5-HEK, ΔLN-Lm5-HEK, ΔSA-Lm5-HEK, and β3SA-HEK were previously established [Nakashima et al., 2005]. The human bladder carcinoma cell line EJ-1 was obtained from Japanese Cancer Resources Bank (JCRB, Tokyo). A spontaneously immortalized human keratinocyte line, HaCaT, was a generous gift from Dr. N. E. Fusenig (Deutsches Krebsforschungszentrum, Heidelberg, Germany). These cell lines were maintained in DMEM/F12 medium (Invitrogen, Carlsbad, CA) supplemented with 10% fetal calf serum (FCS) (JRH

Biosciences, Lenexa, KS), penicillin, and streptomycin sulfate.

Preparation of Extracellular Matrices (ECMs) and Condtioned Media of HEK293 Cell Lines

ECMs deposited by various HEK293 cell lines on plastic surface were collected as follows and used as ECM. Cells were plated to culture dishes (2×10^6 cells/90-mm dish) or plates (2×10^4 cells/96-well plate) containing the growth medium (DMEM/F12 plus 10% FCS) and incubated at 37°C for 6 days unless otherwise noted. The cell culture medium was replaced with fresh medium everyday. To prepare plates with deposited ECM, cells were detached by incubation with 10 mM EDTA in PBS, and then the plates were washed three times with PBS. The ECM remaining on the plate was used for the assay of biological activity. For SDS–PAGE analysis, the deposited ECM proteins were dissolved in a SDS sample buffer. Conditioned media of HEK293 and β3SA-HEK cell lines were prepared as reported previously [Nakashima et al., 2005]. Briefly, cells were grown to confluence in 90-mm cell culture dishes with 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, 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. Ten microliters of the conditioned media were subjected to protein analysis. In some experiments, conditioned media from confluent cultures in serum-containing medium were used without concentration.

SDS–PAGE and Immunoblotting

Protein samples were analyzed by SDS–PAGE in 4%–7.5% or 5%–20% gradient polyacrylamide gels under reducing conditions. Separated proteins were stained with Coomassie Brilliant Blue R-250 (CBB). For immunoblotting analysis, separated proteins were transferred to nitrocellulose membranes (Schleicher & Schuell, NH). The membranes were blocked with skim milk and successively treated with the first antibody and the second antibody labeled with horseradish peroxidase (GE Healthcare Bio-Sciences, Piscataway, NJ). Immunoreactive signals were visualized by the Enhanced Chemiluminescence (ECL) detection method (GE Healthcare Bio-Sciences).

Cell Attachment Activity of Deposited ECMs and Coated Proteins

Each well of 96-well plates (Coster, Cambridge, MA) with deposited ECM was blocked with 200 μl of 1.2% (w/v) bovine serum albumin (BSA) (Sigma) in PBS for 1 h. One hundred microliters of EJ-1 cell suspension (2×10^5 cells/ml in DMEM/F12) was inoculated into each well and incubated at 37°C for 20 min. After 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 fluorescent intensity of each well was measured using a CytoFluor 2350 fluorometer (Millipore, Bedford, MA). For the assay of purified proteins, each well of 96-well plates was coated with 50 μl of a purified protein solution at 4°C overnight and then blocked with BSA. Cells were inoculated as described above. To identify integrins responsible for cell attachment, the cell suspension was previously incubated with 10 μg/ml of function blocking, anti-integrin antibodies at room temperature for 20 min. After the treatment, the cell attachment to test substrates was assayed as described above.

Immunofluorescent Staining of ECM

To stain the short arm of β3 chain (β3SA) and Lm511 in ECM deposited by β3SA-HEK cells, the cells were inoculated into wells of Lab-Tek 8-well chamber slides (Nunc, Naperville, IL) and cultured for 3 days in DMEM/F12 containing 10% FCS. Then, the cells were removed from the wells as described above. The resultant chambers were blocked with 1.2% BSA in PBS at 37°C for 1 h, and incubated with the anti-laminin-α5-chain mAb 4C7 as a primary antibody for 1 h. Goat anti-mouse-IgG antibody conjugated with tetramethylrhodamine (Invitrogen), as a secondary antibody, was used for the detection of the immunoreactivity. To detect β3SA, the anti-laminin-β3-chain mAb LE-12C was coupled with FITC (Dojindo Laboratories, Kumamoto, Japan) and applied to the ECM without a second antibody. Fluorescence images were obtained using a fluorescence microscope (BX50-FLA; Olympus, Tokyo, Japan) equipped with a 100×/1.35 Uplan-Apochromat oil immersion objective.

RESULTS

Biological Activity of ECMs Deposited by HEK293 Cell Lines Secreting Lm332 Forms with a Wild-Type or a Truncated β3 Chain, or One Secreting the β3 Chain Short Arm Alone

Lm332 is a heterotrimer of α3, β3, and γ2 chains (Fig. 1A). In many types of human cell culture, the α3 chain (190 kDa) and the γ2 chain (150 kDa) are cleaved by endogenous proteinases to produce the 160-kDa α3 chain and the 105-kDa γ2 chain, respectively [Miyazaki, 2006]. The β3 chain (135 kDa) consists of the laminin N-terminal domain LN, the laminin EGF-like domain LE, and the coiled-coil domain [Aumailley et al., 2005]. We recently found that in some types of human cell cultures, the β3 chain is also cleaved at the short arm to produce 80–125 kDa β3 chains [Nakashima et al., 2005]. In the previous study, we established HEK293 cell lines expressing Lm332 with a wild-type β3 chain (WT-Lm5-HEK), with a β3 chain lacking the LN domain (ΔLN-Lm5-HEK), or with a β3 chain lacking the whole short arm (LN plus LE domains) (ΔSA-Lm5-HEK) (Fig. 1A). At the same time, a HEK293 cell line expressing the short arm of the β3 chain alone (β3SA-HEK) was also established (Fig. 1B). Experiments with the recombinant proteins purified from the HEK293 transfectants showed that the deletion of the LN domain decreases the cell adhesion activity of Lm332 and the LE domain of the β3 chain is a binding site for type VII collagen. These results suggest that the β3 chain short arm is important for both cell adhesion activity and matrix assembly of Lm332. It was also found that a recombinant β3 chain short arm protein (β3SA) binds to an unidentified membrane receptor.

To clarify the role of the β3 short arm in the matrix assembly of Lm332, first we analyzed the deposition of the recombinant Lm332 proteins and β3SA secreted by the HEK293 transfectants on culture plates. Extracellular matrices deposited on the plates, designated ECMs, were prepared and analyzed by SDS–PAGE (Fig. 2). WT-Lm5-HEK, ΔLN-Lm5-HEK, and ΔSA-Lm5-HEK cell lines deposited almost the same levels of Lm332 proteins (Fig. 2A). β3SA-HEK cells also efficiently deposited β3SA protein on the plate (Fig. 2B).

Next, we examined cell adhesion activity of the HEK293-derived ECMs using the human bladder carcinoma cell line EJ-1. This cell line

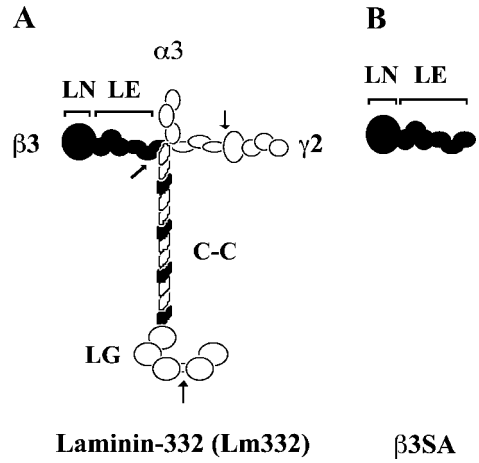

Fig. 1. Structural models of Lm332 and the short arm of β3 chain. **A**: Lm332 is composed of laminin α3, β3, and γ2 chains linked by disulfide bonds. The β3 chain consists of the laminin N-terminal (LN) domain, the laminin EGF-like (LE) domain, and the laminin coiled-coil domain. **B**: The short arm of β3 chain contains the LN and LE domains. In a previous study, HEK293 cell lines overexpressing Lm332 with the full-length β3 chain (amino acid no. 18–1173) (WT-Lm5), one with a β3 chain lacking domain LN (amino acid no. 230–1173) (ΔLN-Lm5), or one with a β3 chain lacking both LN and LE (amino acid no. 579–1173) (ΔSA-Lm5), and a cell line overexpressing the short arm of β3 chain (amino acid no. 18–580) (β3SA) were established [Nakashima et al., 2005]. C-C, coiled-coil domain; LG, laminin globular domain. Arrows indicate main proteolytic cleavage sites observed in Lm332-producing cell lines.

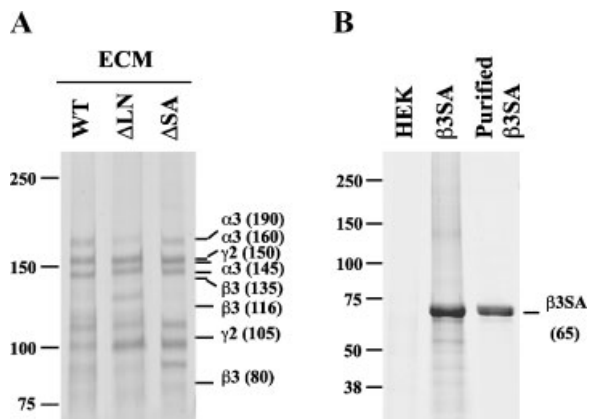

Fig. 2. Electrophoretic analyses of ECM proteins deposited by HEK293 cell lines. Parent HEK293 cells or HEK293 transfectants were inoculated into dishes and cultured for 6 days. From the resultant cultures, ECM proteins deposited by the cells were collected and analyzed by SDS–PAGE on a 4%–7.5% gradient gel (**A**) or a 5%–20% gradient gel (**B**) under reducing condition. Purified β3SA was also analyzed (B). Proteins were stained with Coomassie Brilliant Blue R-250 (CBB). WT, ΔLN, ΔSA, HEK, and β3SA indicate the ECMs from WT-Lm5-HEK, ΔLN-Lm5-HEK, ΔSA-Lm5-HEK, parent HEK293, and β3SA-HEK cell lines, respectively. Ordinates indicate molecular sizes in kDa of marker proteins, and laminin chains and their approximate molecular sizes. Other experiment conditions are described in Materials and Methods.

does not express Lm332, but effectively adheres to and migrates on the Lm332 substrate [Kariya et al., 2003]. When EJ-1 cells were plated on the ECMs of HEK293 cell lines, the ECMs from the three Lm5-HEK cell lines supported efficient attachment of EJ-1 cells compared with that of control HEK293 cells (Fig. 3A). The ECMs from two HEK293 cell lines expressing Lm332 mutants (ΔLN-Lm5-HEK and ΔSA-Lm5-HEK) showed slightly lower cell attachment activity than that of WT-Lm5-HEK cells (WT-Lm5-ECM). This is consistent with our previous finding that the partial deletion of the β3 chain short arm decreases the cell adhesion activity of Lm332. Unexpectedly, the ECM of β3SA-HEK cells, designated β3SA-ECM, showed high cell attachment activity, although the purified β3SA did not support cell attachment at all. Morphological analysis showed that EJ-1 cells were well spread on β3SA-ECM, as well as WT-Lm5-ECM, whereas they poorly spread on the ECMs of ΔLN-Lm5-HEK and ΔSA-Lm5-HEK (Fig. 3B). EJ-1 cells did not spread on the ECM of HEK293 cells or on β3SA-coated plates.

Furthermore, we compared the migration speeds of EJ-1 cells on three ECM substrates. The cell migration was monitored by time-lapse video microscopy as reported previously [Nakashima et al., 2005]. The cell migration speed was 7.49 μm/h on the control HEK293 ECM, 6.26 μm/h on WT-Lm5-ECM, and 41.2 μm/h on β3SA-ECM. This indicated that β3SA-ECM supported rapid cell migration as compared with the ECMs of control HEK293 and WT-Lm5-HEK cells.

The high cell-spreading activity of β3SA-ECM was also observed with the immortalized human keratinocyte cell line HaCaT, which secreted endogenous Lm332 (Fig. 3C). The keratinocytes showed the most spread morphology on β3SA-ECM among five substrates tested. The cell spreading was again more prominent on WT-Lm5-ECM than on the ECMs of ΔLN-Lm5-HEK and ΔSA-Lm5-HEK cells.

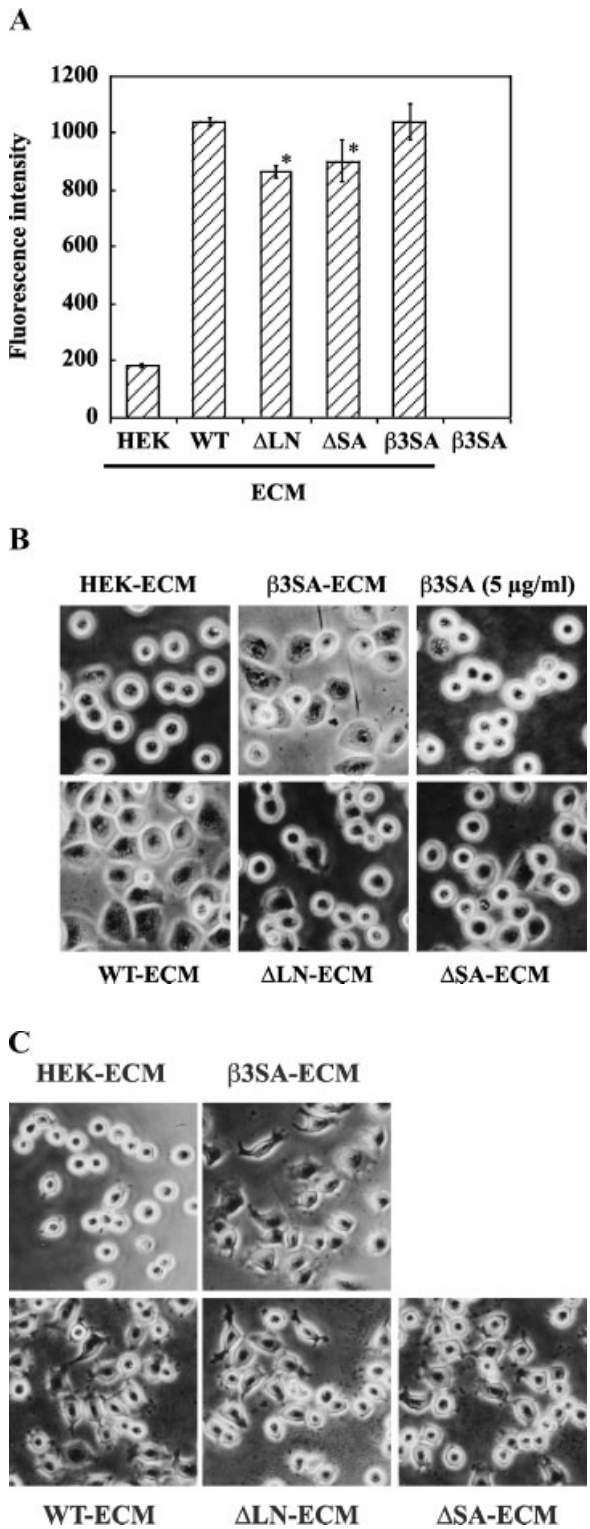

Fig. 3. Cell attachment and cell spreading on deposited ECMs. **A**: EJ-1 cells were inoculated into wells with ECM deposited by each HEK293 cell line or wells pre-coated with purified β3SA (5 μg/ml) and incubated for 20 min. The relative numbers of adherent cells were determined by measuring fluorescence intensity. Each point represents the mean ± SD (bar) from four separate wells. HEK, WT, ΔLN, ΔSA, and β3SA indicate the ECMs from parent HEK293, WT-Lm5-HEK, ΔLN-Lm5-HEK, ΔSA-Lm5-HEK, and β3SA-HEK cell lines, respectively. The 6th column (β3SA) indicates the wells coated with purified β3SA. *, $P < 0.01$. **B**: EJ-1 cells were incubated as described above, and cell morphology was examined under a phase-contrast microscope. Original magnification, 300×. **C**: HaCaT cells were incubated on the five kinds of ECM substrates for 30 min. Original magnification, 200×.

Deposition of Lm511 by β3SA-HEK Cells

As described above, β3SA-ECM efficiently promoted both cell attachment and spreading, while purified β3SA did not show any activity even at a high concentration (5 μg/ml). We expected that some cell adhesive proteins were deposited on β3SA-ECM. First, we compared ECM proteins deposited by control HEK293 and β3SA-HEK cells. However, we could not see clear difference except for the strong protein band of β3SA (Fig. 2B). To identify a molecule responsible for the high cell adhesion activity of β3SA-ECM, next we used function-blocking, anti-integrin antibodies (Fig. 4). When EJ-1 cells were pretreated with anti-integrin-α3 or -β1 antibody, the cell attachment to the β3SA-ECM was partially blocked (Fig. 4A). A combination of anti-integrin-α3 and -α6 antibodies more strongly inhibited the cell attachment to β3SA-ECM. In contrast, anti-integrin-α2, -α5, and -α6 antibodies, and heparin had no inhibitory effect. The inhibitory activity of the anti-integrin antibodies was confirmed when their effects on cell spreading were examined (Fig. 4B). The cell spreading was effectively inhibited by the combination of the integrin-α3 and -α6 antibodies and partially by the integrin α3 or β1 antibody alone. These results suggested that some cell adhesion molecule, which is

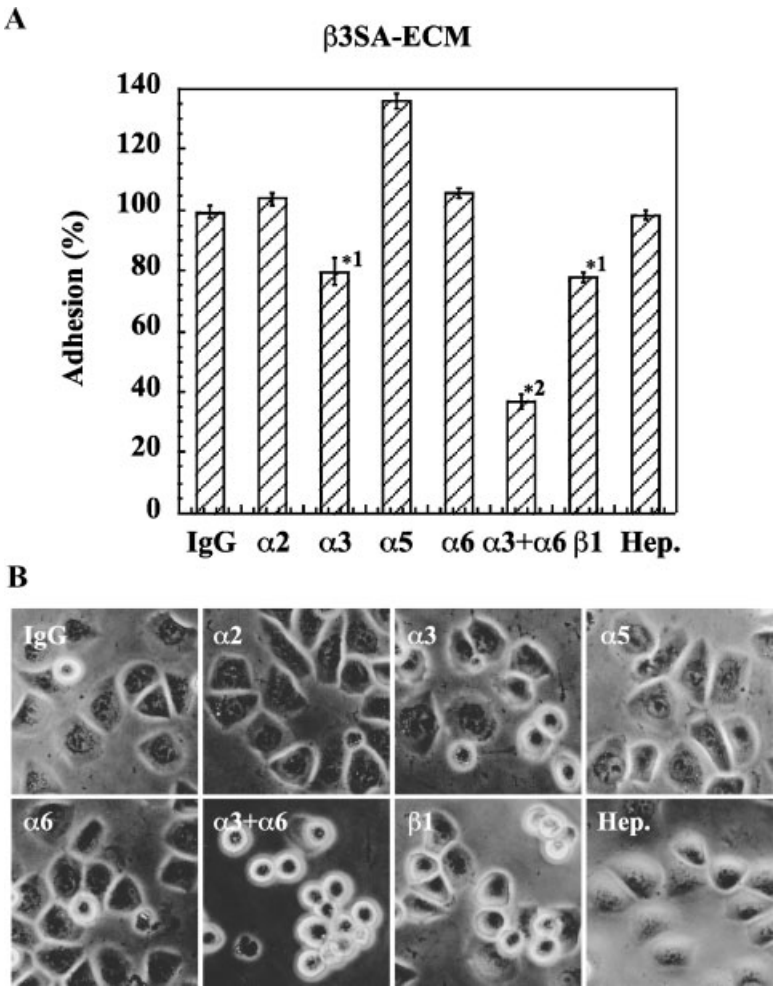

Fig. 4. Effects of function-blocking, anti-integrin antibodies, and heparin on cell attachment to β3SA-ECM. **A**: EJ-1 cells were incubated with the indicated function-blocking, anti-integrin antibodies (10 μg/ml), or heparin (Hep., 100 μg/ml) at room temperature for 20 min. After the treatment, the cells were plated on β3SA-ECM, which had been prepared from 6-day old cultures of β3SA-HEK cells, and incubated for 20 min. The relative numbers of adherent cells were determined as described in Figure 3A. Cell attachment in the presence of a control mouse IgG was taken 100%. Each point represents the mean ± SD (bar) for triplicate assays. *1, $P < 0.05$; *2, $P < 0.001$. **B**: The cell morphology in the above cultures was examined under a phase-contrast microscope.

recognized by the integrins α3β1, α6β1, and/or α6β4, possibly some laminin, is present in β3SA-ECM.

Many of laminin isoforms interact with integrin α3β1, α6β1, and α6β4 as receptors. Especially, Lm332 and Lm511/521 (a mixture of Lm511 and laminin-521) have high affinity to these integrins, and both show high cell adhesion activity [Rousselle and Aumailley, 1994; Kikkawa et al., 1998]. First, we checked the presence of laminin α3 chain in β3SA-ECM with anti-laminin-α3-chain antibody, but its immunoreactivity was not detected. This indicated that α3 laminins, that is, Lm332, laminin-311 (laminin-6), and laminin-321 (laminin-7), were not contained in β3SA-ECM. Then, we analyzed the presence of Lm511 in the ECMs of control HEK293 and β3SA-HEK by immunoblotting. This analysis revealed that β3SA-ECM contained the laminin α5 chain (350 kDa), β1 chain (220 kDa), and γ1 chain (210 kDa), but the control HEK293 ECM did not (Fig. 5A). However, when the conditioned media of both cell lines were analyzed, the α5 chain was detected at a high level in HEK293 cells but at a very low level in β3SA-HEK cells. Under non-reducing conditions, immunoblotting with the anti-laminin-α5-chain antibody detected an immunoreactive band at about 800 kDa in both conditioned media, indicating that both cell lines secreted the laminin α5 chain as Lm511 (data not shown). These results indicate that Lm511 is efficiently deposited on β3SA-ECM but poorly on the HEK293 ECM.

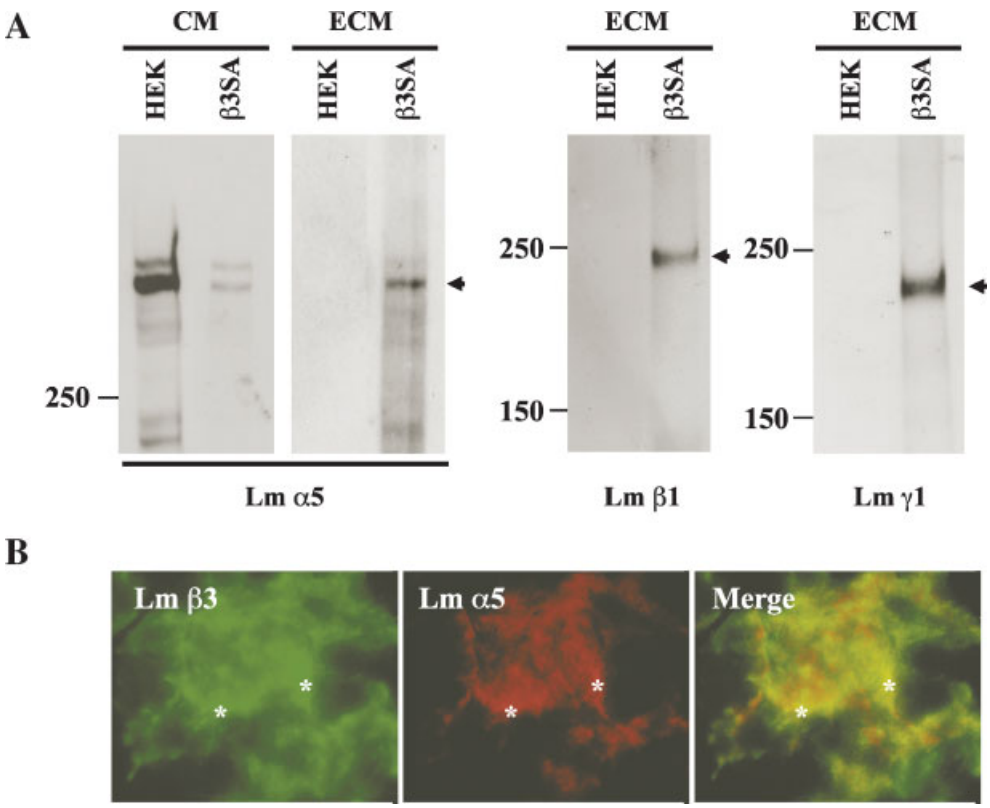

Fig. 5. Secretion and deposition of Lm511 in cultures of HEK293 and β3SA-HEK cell lines. **A**: HEK293 (HEK) and β3SA-HEK (β3SA) cell lines were inoculated in duplicate 90-mm dishes at a density of 2×10^6 cells/dish. One dish was incubated in the growth medium (DMEM/F12 plus 10% FCS) for 6 days, and the resultant ECM was collected as described in Materials and Methods. Another dish was incubated in the growth medium for 3 days and then in serum-free medium for 3 days. The serum-free conditioned medium (CM) for the last 2 days was collected and concentrated 50-folds. The ECMs and CMs from the two cell lines, the amounts of which were equivalent to 20% and 5% of whole dish, respectively, were run on a 4%–7.5% SDS–PAGE gel under reducing conditions and immunoblotted with antibodies against the laminin α5 (Lmα5), laminin β1 (Lmβ1), and laminin γ1 (Lmγ1) chains. Arrowheads indicate the respective laminin chains. **B**: β3SA-HEK cells were cultured in the growth medium for 3 days, and then the cells were removed. The resultant ECM was immuno-stained with the anti-laminin-β3-chain mAb LE-12C (Lmβ3, green) and the anti-laminin-α5-chain mAb 4C7 (Lmα5, red). Asterisks indicate co-localized areas of β3SA and laminin α5 chain.

We further examined localization of β3SA and Lm511 in β3SA-ECM by immunohistochemistry (Fig. 5B). Immunoreactivity for β3SA was intensely detected where cells were located. Although immunoreactive signals for Lm511 were very weak, they were detected only at the places where strong β3SA signals were detected.

Cooperative Effect of Lm511/521 and β3SA on Cell Attachment and Spreading

Lm511 (α5β1γ1) efficiently promotes cell adhesion and motility via integrins, α3β1, α6β1, and α6β4 [Kikkawa et al., 1998]. Therefore, Lm511 was considered to be responsible for the strong cell attachment and spreading activities of β3SA-ECM. However, the amount of Lm511 in β3SA-ECM was estimated to be less than 0.5 μg per 90-mm culture dish as analyzed by immunoblotting with the anti-α5-chain antibody. This concentration of Lm511 was equivalent to that obtained when the dish was coated with 0.1 μg/ml of purified Lm511. However, Lm511/521 supported efficient cell adhesion only at concentrations over 1 μg/ml in our assay (data not shown). These results suggested that β3SA might cooperatively function with Lm511. To ascertain this possibility, cells were inoculated into wells pre-coated with a mixture of a constant concentration (0.1 μg/ml) of purified Lm511/521 and various concentrations of β3SA (0–5.0 μg/ml). Although Lm511/521 alone did not promote cell attachment or cell spreading, the mixture of Lm511/521 and β3SA promoted both cell attachment and cell spreading in dependence on the concentration of β3SA (Fig. 6A,B).

We also examined the effects of the function-blocking antibodies against integrins on the cell attachment to the mixture of Lm511/521 and β3SA. The anti-β1-integrin antibody alone or the combination of the anti-α3- and anti-α6-integrin antibodies almost completely blocked the cell attachment to the mixed substrate. This suggested that the cell adhesion to Lm511/521 plus β3SA was mediated by integrins α3β1 and α6β1. The inhibitory effects of the antibodies were more prominent than their effects toward the cell attachment to β3SA-ECM (Figs. 6C and 4A).

Mechanism of β3SA-Induced Lm511 Deposition

As shown above, Lm511 was efficiently deposited in the culture of β3SA-HEK cells but not HEK293 cells. To investigate its mechanism, deposition of Lm511 was examined under various conditions. HEK293 cells were incubated for 3 days on non-treated plates, plates coated with purified β3SA, ones coated with the conditioned medium of β3SA-HEK cells, or the ECM obtained from 1-day culture of β3SA-HEK cells, and then Lm511 deposited on the plates were analyzed by immunoblotting (Fig. 7A). Lm511 was significantly deposited on the plates only when HEK293 cells were incubated with β3SA-ECM. However, when the conditioned medium of HEK293 cells was incubated with the ECM, Lm511 was not deposited. Furthermore, HEK293 cells scarcely deposited Lm511 on the plates coated with purified β3SA or the conditioned medium of β3SA-HEK cells. These results indicate that the deposition of Lm511 is a cell-mediated process. It is also clear that β3SA-ECM has a different activity from purified β3SA or the conditioned medium of β3SA-HEK cells although the great majority of the ECM was β3SA.

To show difference between soluble forms and deposited form of β3SA, purified β3SA, the conditioned medium of β3SA-HEK cells, and β3SA-ECM were analyzed by SDS–PAGE under non-reducing and reducing conditions. As shown in Figure 7B, non-reduced β3SA in the ECM could not migrate from the top of the gel, whereas β3SA in a purified sample and the conditioned medium mostly migrated to a position of approximately 65 kDa. Under reducing conditions, β3SA in all samples migrated to the same position. These results suggested that β3SA protein might be deposited as a polymerized form or a large complex form.

DISCUSSION

To clarify the biological function of the short arm of laminin β3 chain, we investigated the properties of ECMs deposited by HEK293 cells and their transfectants overexpressing the wild-type or truncated Lm332, or the short arm of laminin β3 chain. Although the β3 short arm produced by β3SA-HEK cells was effectively deposited on culture plates, its partial deletion from Lm332 did not have significant effect on the deposition of Lm332. This suggests that the α3 and γ2 chains also support the matrix deposition of Lm332. It has been reported that the C-terminal, globular domain LG4-5 of the α3 chain has heparin-binding activity and

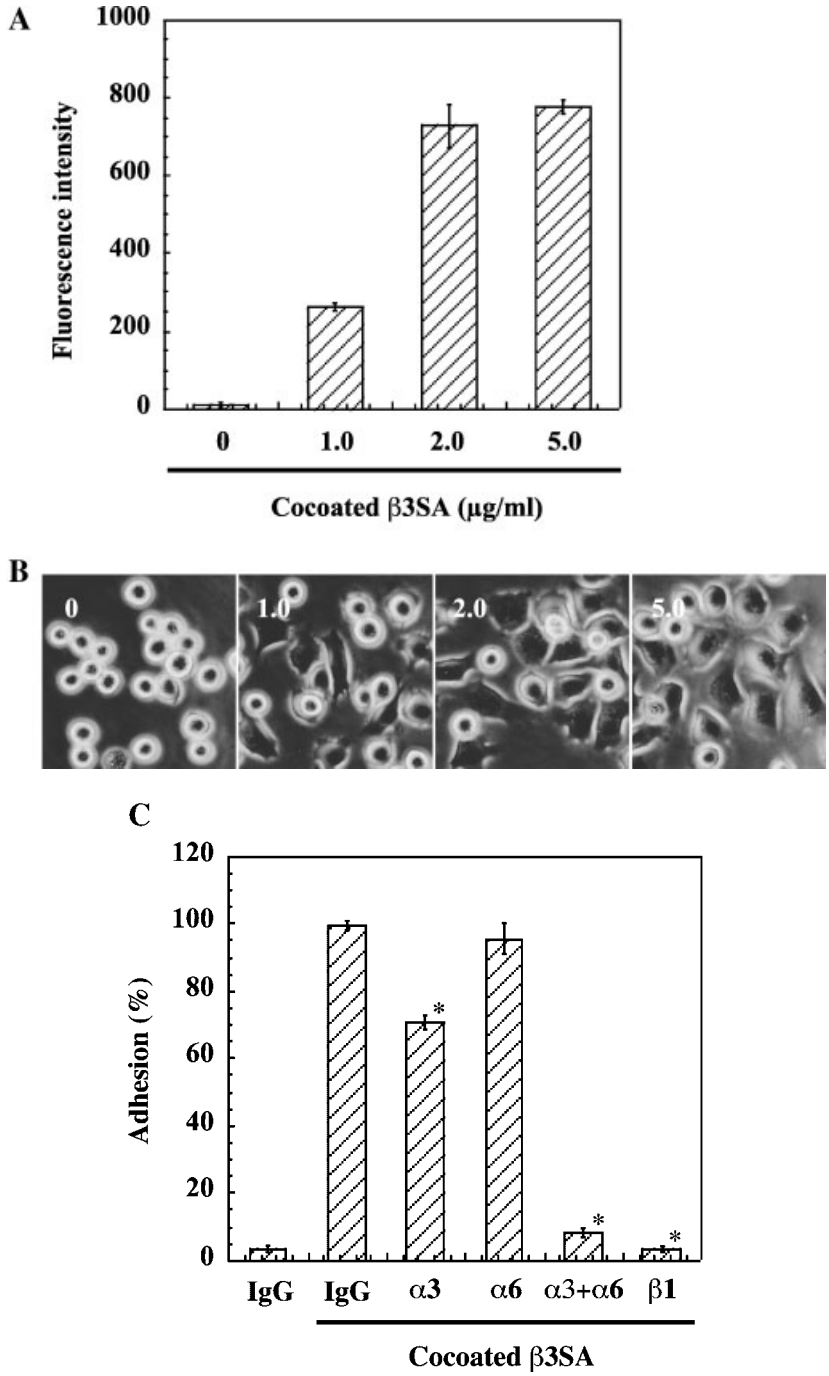

Fig. 6. Effect of β3SA on cell adhesion to Lm511/521. **A**: The indicated concentrations of β3SA were coated with 0.1 μg/ml of human Lm511/521. EJ-1 cells were inoculated into each well and incubated for 20 min. The relative numbers of adherent cells were determined as described in Figure 3A. Each point represents the mean ± SD (bar) for triplicate assays. **B**: The cell morphology in the above cultures was examined under a phase-contrast microscope. The values on the pictures indicate the concentration of β3SA (μg/ml). **C**: Effects of function-blocking, anti-integrin antibodies on the cell attachment to the mixed substrate (0.1 μg/ml Lm511/521 plus 5 μg/ml β3SA) were examined as described in Figure 4A. Cell attachment in the presence of a control mouse IgG to Lm511/521 plus β3SA was taken 100%. *, $P < 0.001$.

regulates the deposition of Lm332 [Tsubota et al., 2005]. The short arm of laminin γ2 chain, especially the laminin 4 domain, also contributes to the deposition of Lm332 into ECM [Gagnoux-Palacios et al., 2001]. β3SA-HEK cells, which do not secrete Lm332, deposited a recombinant β3 short arm (β3SA) protein as a major ECM component. β3SA was likely to form

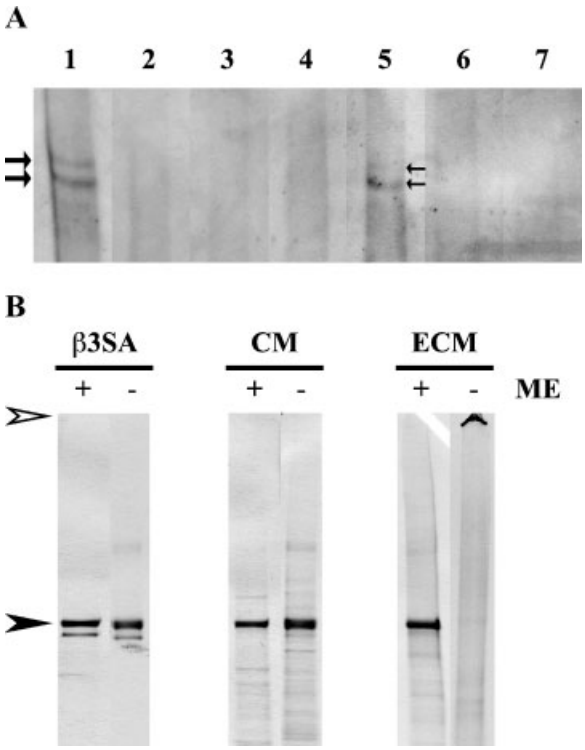

Fig. 7. Deposition of Lm511 secreted by HEK293 cells, and characterization of β3SA deposited by β3SA-HEK cells. **A**: Deposition of Lm511 by HEK293 cells under various conditions. The following culture substrates were prepared: non-treated wells (**lane 7**); wells coated with 5 μg/ml of purified β3SA (**lane 2**); wells coated with the serum-free conditioned medium (CM) of β3SA-HEK cells (**lane 3**); wells with the ECM obtained from 1-day culture of β3SA-HEK cells (β3SA-ECM) (**lanes 4–6**), in which Lm511 was scarcely detected (lane 4). HEK293 cells (lanes 2, 3, 5, and 7) or the serum-free conditioned medium of HEK293 cells (lane 6) was incubated for 3 days in the wells shown above, and Lm511 deposited on the plates was analyzed by immunoblotting with the anti-laminin-α5-chain mAb. **Lanes 1** and 4 indicate control β3SA-ECMs prepared from 4-days culture and 1-day culture of β3SA-HEK, respectively. The CMs of β3SA-HEK cells and HEK293 cells were prepared as described in Figure 5. Other experimental conditions were described in Materials and Methods. **B**: Comparison of electrophoretic patterns of β3SA present in conditioned medium (CM) and ECM. The purified β3SA, concentrated CM and ECM were obtained from the culture of β3SA-HEK cells and analyzed by SDS–PAGE under reducing (+ME) and non-reducing (−ME) conditions. Separated proteins were stained with Coomassie Brilliant Blue R-250 (CBB). The open arrowhead indicates the β3SA band immobilized on the gel top, and the closed arrowhead indicates a band corresponding to the β3SA monomer.

a large molecular complex or network structure in the ECM of β3SA-HEK cells (β3SA-ECM), because it could not migrate on SDS–PAGE under non-reducing conditions (Fig. 7B). Immunohistochemical staining also suggested that the deposition of β3SA was a cell-mediated process (Fig. 5B).

In addition, the present study revealed that β3SA-ECM strongly promoted cell attachment and spreading although purified β3SA did not support cell adhesion by itself. The strong cell adhesion activity of β3SA-ECM is most likely derived from Lm511 deposited on the ECM. Although both control HEK293 cells and β3SA-HEK cells secreted Lm511 at similar levels, Lm511 was efficiently deposited only in β3SA-HEK cells. It has been reported that Lm511, like Lm332, has high cell adhesion and motility activities [Kikkawa et al., 1998]. However, the level of Lm511 in β3SA-ECM was too low to show such a high cell adhesion activity. It is most likely that Lm511 and β3SA protein deposited on β3SA-ECM synergistically promote cellular adhesion. The synergistic cell adhesion activity of Lm511 and β3SA was confirmed by the assay with purified Lm511 and β3SA protein. However, the mechanism of the β3SA activity is not clear.

It has been reported that some cell adhesion proteins, such as laminins and fibronectin, are assembled into ECM structures via cell receptors [Lohikangas et al., 2001; Li et al., 2002; Wierzbicka-Patynowski and Schwarzbauer, 2003; Hamelers et al., 2005]. How does β3SA make Lm511 deposit on ECM? There are two possibilities. First, Lm511 may directly bind to β3SA. Odenthal et al. [2004] have reported that a recombinant N-terminal fragment (amino acid no. 1–610) of laminin β3 chain has some affinity for N-terminal fragments of laminin α1, α2, α5, β1, β2, β3, γ1, and γ3 chains as analyzed by the surface plasmon resonance. To show the direct interaction between β3SA and Lm511, we tried to precipitate Lm511 with β3SA-bound beads from the conditioned medium of A549 human lung carcinoma cells, which contained native Lm511 [Kikkawa et al., 1998]. However, Lm511 was not co-precipitated with β3SA (data not shown). In addition, when HEK293 cells or their conditioned medium was incubated on β3SA-coated plates, Lm511 was not deposited on the plates (Fig. 7A). These results exclude the possibility that Lm511 directly binds to β3SA. Second, β3SA may stimulate cells to deposit Lm511. When HEK293 cells were incubated on β3SA-ECM or on β3SA-coated plates, Lm511 was deposited on the ECM but not the β3SA-coated plates (Fig. 7A). In addition, Lm511 and β3SA were co-localized in β3SA-ECM (Fig. 5B). Therefore, it is more likely that β3SA in the ECM focally stimulates cells to deposit Lm511.

The differential effect of β3SA-ECM and β3SA coating may be attributed to a difference in the molecular state of β3SA between the two conditions. In the ECM, β3SA appeared to form a large complex or a polymerized structure (Figs. 5B and 7B). This form of β3SA is likely to have a stronger effect on target cells than the monomer protein.

Our previous study indicated that β3SA binds to an unknown cell receptor [Nakashima et al., 2005]. The present study also suggests that both the induction of the Lm511 deposition and the enhancement of Lm511-mediated cell adhesion are induced by the binding of β3SA to a cell receptor. However, we could not identify the receptor of β3SA in this study. It is known that some membrane proteins are associated with integrins and regulate integrin functions. These include syndecans [Beauvais and Rapraeger, 2004; Yokoyama et al., 2005], tetraspanins [Berditchevski and Odintsova, 1999; Nishiuchi et al., 2005], and galectins [Woo et al., 1990; Hughes, 2001]. We found that β3SA has weak heparin-binding activity (data not shown). However, heparin did not affect the biological activities of β3SA or β3SA-ECM, indicating that heparansulfate proteoglycans including syndecans are not receptors for β3SA (data not shown). In addition, we preliminarily examined whether β3SA binds to each of tetraspanins, CD151, or galectin-3. However, we failed to show these interactions (data not shown). Further studies are required to identify the β3SA receptor.

The present study strongly suggests that the short arm of laminin β3 chain is involved in both the matrix assembly of laminins and the regulation of integrin functions. It is assumed that the β3 short arm of Lm332 activates integrin activity to interact with the integrin-binding sites of Lm332, Lm511, and possibly other laminins, leading to the enhancement of the cell adhesion activity and matrix assembly of these laminins. In this regard, it is noted that Lm332 and Lm511 are colocalized in the epidermal BM [Aumailley and Rousselle, 1999; McMillan et al., 2006]. It was previously reported that the β3 short arm is proteolytically released from Lm332 in human keratinocytes [Nakashima et al., 2005]. Therefore, it seems possible that the β3 short arm, as either a released form or a functional domain of Lm332, plays an important role in the cooperation of Lm332 with Lm511 to maintain the structure and function of the epidermal BM.

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