Journal of Cellular Biochemistry 92:701–714 (2004)

ARTICLES

Regulation of Biological Activity of Laminin-5 by Proteolytic Processing of γ2 Chain

Takashi Ogawa,[1,2] Yoshiaki Tsubota,[1] Masato Maeda,[1,2] Yoshinobu Kariya,[1,3] and Kaoru Miyazaki[1,2]*

[1]Division of Cell Biology, Kihara Institute for Biological Research, Yokohama City University, 641-12 Maioka-cho, Totsuka-ku, Yokohama 244-0813, Japan
[2]Graduate School of Integrated Sciences, Yokohama City University, 641-12 Maioka-cho, Totsuka-ku, Yokohama 244-0813, Japan
[3]Kihara Memorial Yokohama Biotechnology Foundation, 641-12 Maioka-cho, Totsuka-ku, Yokohama 244-0813, Japan

Abstract Laminin-5 (LN5), which regulates both cell adhesion and cell migration, undergoes specific extracellular proteolytic processing at an amino-terminal region of the γ2 chain as well as at a carboxyl-terminal region of the α3 chain. To clarify the biological effect of the γ2 chain processing, we prepared a human recombinant LN5 with the 150-kDa, non-processed γ2 chain (GAA-LN5) and natural LN5 with the 105-kDa, processed γ2 chain (Nat-LN5). Comparison of their biological activities demonstrated that GAA-LN5 had an about five-times higher cell adhesion activity but an about two-times lower cell migration activity than Nat-LN5. This implies that the proteolytic processing of LN5 γ2 chain converts the LN5 from the cell adhesion type to the cell migration type. It was also found that human gastric carcinoma cells expressing the LN5 with the non-processed γ2 chain is more adherent but less migratory than the carcinoma cells expressing a mixture of LN5 forms with the processed γ2 chain and with the unprocessed one. The functional change of LN5 by the proteolytic processing of the γ2 chain may contribute to elevated cell migration under some pathological conditions such as wound healing and tumor invasion. J. Cell. Biochem. 92: 701–714, 2004.

Key words: laminin-5; cell migration; cell adhesion; proteinase; processing

The major basement membrane components laminins, which consist of three different subunits, α, β and γ, linked by disulfide bonds, regulate various cellular functions, such as adhesion, motility, growth, differentiation, and apoptosis, through the interaction with specific receptors on the cell surface [Aumailley and Rousselle, 1999; Colognato and Yurchenco, 2000]. To date, at least 15 laminin isoforms have been identified [Koch et al., 1999]. Among them, laminin-5 (LN5) (α3β3γ2), a major adhesive component of the epidermal basement membrane [Carter et al., 1991; Rousselle et al., 1991], is unique in its structure and biological activity. In vitro, LN5 promotes cellular scattering, migration, and adhesion [Miyazaki et al., 1993; Kikkawa et al., 1994; Rousselle and Aumailley, 1994]. In vivo, LN5 plays an essential role in the stable anchorage of basal keratinocytes to the underlying connective tissues [Nievers et al., 1999]. The association of LN5 with integrin α6β4 is critical to form the stable hemidesmosome structures in the skin [Baker et al., 1996; Rousselle et al., 1997]. Therefore, genomic defects in any of the three LN5 subunits cause a lethal skin disease, Herlitz's junctional epidermolysis bullosa [Aberdam

Abbreviations used: BMP-1, bone morphogenic protein-1; ECM, extracellular matrix; EGF, epidermal growth factor; FCS, fetal calf serum; LN5, laminin-5; STI, soybean trypsin inhibitor; GAA-LN5, a recombinant laminin-5 with a non-processed, mutant γ2 chain; MKN-28/GAA, MKN-28 cells producing GAA-LN5; MKN-28/Mock, MKN-28 cells transfected with an empty vector; MKN-28/WT, MKN-28 cells producing WT-LN5; Nat-LN5, a natural laminin-5 with a processed γ2 chain; WT-LN5, a recombinant laminin-5 with the wild-type γ2 chain.

Grant sponsor: Ministry of Education, Culture, Sports, Science and Technology of Japan; Grant sponsor: Ministry of Welfare and Labor of Japan.

*Correspondence to: Kaoru Miyazaki, PhD, Division of Cell Biology, Kihara Institute for Biological Research, Yokohama City University; 641-12 Maioka-cho, Totsuka-ku, Yokohama 244-0813, Japan.
E-mail: miyazaki@yokohama-cu.ac.jp

Received 20 November 2003; Accepted 1 March 2004
DOI 10.1002/jcb.20112

et al., 1994; Pulkkinen et al., 1994; Kivirikko et al., 1995]. On the other hand, the potent cell-migration-promoting activity of LN5 is thought to contribute to wound healing [Ryan et al., 1994; Goldfinger et al., 1999] and tumor invasion [Pyke et al., 1994]. However, it is unclear how LN5 regulates the apparently opposite cellular functions, namely cell adhesion and cell migration.

Proteolytic processing of extracellular matrix (ECM) proteins modulates their biological activities [Egeblad and Werb, 2002; Schenk and Quaranta, 2003]. Human LN5 is synthesized and secreted as a precursor form consisting of a 190-kDa α3 chain, a 135-kDa β3 chain, and a 150-kDa γ2 chain. After secretion, the α3 and γ2 chains undergo specific extracellular proteolytic processing to convert to the mature form containing a 160-kDa α3 chain and a 105-kDa γ2 chain [Marinkovich et al., 1992]. These proteolytic cleavages of LN5 are expected to affect the biological functions. The cleavage of the 190-kDa α3 chain, which occurs between the G3 and G4 modules of the globular (G) domain [Hirosaki et al., 2000; Tsubota et al., 2000], has been suggested to increase the cell adhesion activity of LN5 but decrease its cell migration activity [Goldfinger et al., 1998]. On the other hand, the proteolytic cleavage of the 150-kDa γ2 chain of rat LN5 to the 80-kDa form by gelatinase A (MMP-2) or MT1-MMP elevates the cell migration activity of the LN5 [Giannelli et al., 1997; Koshikawa et al., 2000]. These studies present attractive models in which the cell adhesion and cell migration activities of LN5 are regulated by proteolytic processing. However, these conclusions are still controversial, because their results were not obtained by direct comparison of the biological activities between the processed and unprocessed LN5 forms. Our recent study with human recombinant laminin-6 (LN6) proteins demonstrated that the cleavage of the 190-kDa α3 chain to the 160-kDa form converts the latent LN6 to the active form, increasing both the cell adhesion and migration activities [Hirosaki et al., 2002]. It is also known that in the physiological processing of human LN5, the LN5 with the 80-kDa γ2 chain is not produced [Veitch et al., 2003]. Recent studies have shown that a metalloproteinase family of bone morphogenic protein-1 (BMP-1) are physiological processing enzymes of the γ2 and α3 chains of LN5 [Amano et al., 2000; Veitch et al., 2003]. These metalloproteinases convert the 150-kDa γ2 chain of human LN5 exclusively to the 105-kDa form. However, a human LN5 containing only the unprocessed, 150-kDa γ2 chain or only the processed, 105-kDa γ2 chain has not been isolated so far. To clarify the biological significance of the γ2 chain and its proteolytic cleavage, we prepared the LN5 forms with the 150-kDa γ2 chain and with the 105-kDa γ2 chain and compared their biological activities.

MATERIALS AND METHODS

Cells and Culture Conditions

The human gastric adenocarcinoma cell line MKN-28, which has been described in a previous study [Miyata et al., 2000], was cultured in RPMI 1640 medium supplemented with 10% fetal calf serum (FCS) at 37°C in a humidified atmosphere of 5% CO_2/95% air. The non-tumorigenic Buffalo rat liver epithelial cell line BRL and the human bladder carcinoma cell line EJ-1 have been used in previous studies [Miyazaki et al., 1993; Kariya et al., 2003]. BRL and EJ-1 cells were cultured in DME/F12 medium (Gibco BRL, Rockville, MD) supplemented with 10 % FCS. Normal human epidermal keratinocytes were maintained in a serum-free keratinocyte growth medium (KGM) supplemented with insulin, EGF, hydrocortisone and bovine pituitary extract. The medium was changed every second day. Cells after two or three passages were used for experiments. The human keratinocytes and the KGM medium were purchased from Clonetecs (San Diego, CA).

Construction of Non-Processed γ2 cDNA Vector and Transfection

A human laminin γ2 chain cDNA [Kallunki et al., 1992] has been cloned by the RT-PCR method from the human gastric cancer cell line STKM-1 in our laboratory. In order to prepare a non-processed form of laminin γ2 chain, two amino acid residues at the processing site of the γ2 chain, Asp^{435} and Glu^{436}, were replaced with alanine (Ala) by the site-directed mutagenesis using the inverted PCR method [Imai et al., 1991]. The *Bam*HI/*Eco*RI fragment of laminin γ2 cDNA (number 85–1,942) was cloned into the *Eco*RI/*Bam*HI sites of pBluescript II KS(+) and used as the template for the mutagenesis PCR. Whole sequence of the template was amplified with a mutagenesis primer pair designed in the inverted tail-to-tail directions. The sequences of

primers used are as follows: primer γ2 GAA-3′ (sense, number 1,422-1,447), 5′-cGccAATCCT-GACATTGAGTGTGCTG-3′; and primer γ2 GAA-5′ (antisense, number 1,396–1,421), 5′-gCgCCTGAATAACAATCTCCTGTGTC-3′, where the underlines indicate additional base substitutions to introduce a unique *Kas*I site for rapid screening of mutated clones, and small letters indicate a mismatch sequence for the base substitution to convert both Asp^{435} and Glu^{436} to Ala. The amplified linear DNA was self-ligated to transform *E. coli*. The mutated clones thus created, were screened by *Kas*I digestion and these sequences were verified by DNA sequence analysis. The *Apa*I/*Sca*I fragment (559–1,183) and the *Apa*I/*Kpn*I fragment (vector region, 1–559) of the wild-type laminin γ2 cDNA in pBluescript II KS(+) were replaced with the corresponding region of the mutated fragments to construct the full-length mutant γ2 cDNA pBluescript II KS(+) GAA. pBluescript KS (+) GAA was digested with *Eco*RV/*Xba*I at the multi-cloning site. The full-length mutant cDNA thus prepared was inserted into the *Xba*I/*Sma*I-sites of the mammalian expression vector pEF-BOS-CITE-NEO [Miyata et al., 1998]. The expression vector was transfected into MKN-28 cells by the calcium phosphate method [Chen and Okayama, 1987].

Preparation of Conditioned Medium and ECM of MKN-28 Transfectants

MKN28 transfectants were grown to confluence in 90-mm culture dishes with RPMI 1640 medium containing 10% FCS. The confluent cultures were washed twice with and incubated in serum-free RPMI 1640 medium containing 1 mM soybean trypsin inhibitor (STI) (Sigma, St. Louis, MO). The serum-free culture medium was collected 2-days later, concentrated 250-fold by ammonium sulfate precipitation at 80% saturation, and used as a conditioned medium. The cells in the culture dishes were removed by incubating with 10 mM EDTA in phosphate-buffered saline (PBS), and the ECM proteins remaining on the plastic surface were dissolved in 1 ml of the SDS sample buffer. These samples were analyzed by immunoblotting.

Purification of Recombinant LN5 and Natural LN5

To prepare recombinant LN5 forms, conditioned media were prepared from serum-free roller bottle cultures of MKN-28 cells transfected with γ2 chain cDNAs as described above. LN5 was purified by molecular-sieve chromatography on a Sepharose 4B column and the following affinity chromatography with the anti-γ2-chain monoclonal antibody (D4B5) as reported elsewhere [Hirosaki et al., 2000; Kariya et al., 2003]. Similarly, a natural LN5 was purified from the conditioned medium of the human squamous cell carcinoma line HSC-4.

Assay of Cell Attachment Activity

The cell attachment assay was performed as describe previously [Mizushima et al., 1997]. Ninety-six-well microtiter plates (Coster, Acton, MA) were coated with various concentrations of substrate proteins in PBS at 4°C overnight and then blocked with the saline containing 1.2% (w/v) bovine serum albumin at 37°C for 1.5 h. Cells were suspended in a serum-free medium at a density of $2 \sim 4 \times 10^5$ cells/ml, and 0.1 ml aliquot was inoculated per well of the plates. After incubation at 37°C, non-adherent cells were removed by gentle agitation and then by adding 0.1 ml of 15% (v/v) Percol (Amersham Bioscience, Piscataway, NJ) to the wells, and adherent cells were fixed with 2.5% (v/v) glutaraldehyde and stained with 0.1 ml of a mixture of 0.0005% (w/v) Hoechst 33342 and 0.001% (w/v) Triton X-100 for 1.5 h. The fluorescent intensity of each well of the plates was measured using a CytoFluor 2350 fluorometer (Millipore, Bedford, MA).

Assays of Cell Scattering and Cell Migration

The cell-scattering activity of two forms of LN5 toward BRL cells was assayed as reported previously [Hirosaki et al., 2000]. Cells were plated at a density of 7,000 cells/well onto 24-well culture plates (Sumibe Medical, Tokyo, Japan) containing 0.5 ml/well of DME/F12 plus 1% FCS. In the standard assay, test samples were directly added to the culture medium and incubated at 37°C. After 2 days in incubation, the total cells and single cells in each field were separately counted under a microscope. The degree of cell scattering was expressed as the percentage of single cells in each field. In the assay of cell migration, test samples were coated on 24-well plates as described above, and the cell migration on these substrates was monitored at 37°C with a time-lapse video equipment. The length of cell migration was measured using a video micrometer (VM-30; Olympus, Tokyo, Japan).

SDS–PAGE and Immunoblotting Analyses

SDS–PAGE was performed on 5 or 6% gels under reducing or non-reducing conditions. In the analyses of purified proteins, separated proteins were stained with a Wako silver staining kit II (Wako, Osaka, Japan). In immunoblotting analysis, proteins resolved by SDS–PAGE were transferred to nitrocellulose membranes and detected by the alkaline phosphatase method with specific antibodies. To detect the three subunits of LN5, mouse monoclonal antibodies against the human laminin α3 chain (LSα3c4) and γ2 chain (D4B5) [Mizushima et al., 1998; Koshikawa et al., 1999] and a mouse monoclonal antibody against the human laminin β3 chain from Transduction Laboratories (Lexington, KY) were used.

Determination of Protein Concentrations

Protein concentrations were determined using a Bio-Rad protein assay kit (Hercules, CA) with bovine serum albumin as a standard. Relative concentrations of three forms of LN5 were normalized for the concentration of laminin β3 chain, which was determined by an enzyme-linked immunosorbant assay with the anti-laminin-β3 monoclonal antibody 29E [Koshikawa et al., 1999].

RESULTS

Expression of LN5 With Non-Processed, 150-kDa Laminin γ2 Chain in MKN-28 Cells

The proteolytically processed 105-kDa γ2 chain of LN5 has an amino-terminal sequence starting at Asp^{435} [Miyazaki et al., 1993], suggesting that the 150-kDa γ2 chain is cleaved between Gly^{434} and Asp^{435} in the second epidermal growth factor (EGF)-like repeat of domain III (Fig. 1A). The cleavage sequence Gly-Asp is commonly found in the LN5 proteins of human, mouse, and rat. Mammalian tolloid (mTLD) or other members of the BMP-1 family participate in this processing event [Amano et al., 2000; Veitch et al., 2003]. The amino acid positions P1′ (Asp^{435}) and P2′ (Glu^{436}) in the human laminin γ2 chain appear to be important amino acid residues for the proteolytic processing [Prockop et al., 1998]. In order to obtain the LN5 with the non-processed, 150-kDa γ2 chain, we substituted these two amino acid residues with Ala by the site-directed mutagenesis of the γ2 cDNA (Fig. 1B). Mammalian expression vectors containing the mutant γ2 cDNA (γ2/GAA) or the wild-type γ2 cDNA (γ2/WT) were constructed, and these vectors as well as the empty vector (Mock) were transfected into the human gastric adenocarcinoma cell line MKN-28, which is known to constitutively express the laminin α3 and β3 chains but scarcely the γ2 chain [Mizushima et al., 1996].

The secretion and deposition of the LN5 subunits by the MKN28 transfectants were analyzed by immunoblotting with LN5 antibodies. Because MKN28 cells secrete a high activity of trypsin [Miyata et al., 1999], the transfected cell lines were cultured in a serum-free medium supplemented with STI. The serum-free conditioned media obtained from the cultures of both MKN-28 transfectants with the γ2/GAA cDNA (MKN-28/GAA) and with the γ2/WT cDNA (MKN-28/WT) contained the 160-kDa α3 chain, the 135-kDa β3 chain and the 150-kDa, and/or 105-kDa γ2 chains at high levels, but the conditioned medium from the control transfectant (MKN-28/Mock) contained little LN5 subunits (Fig. 2A). As expected, MKN-28/GAA secreted almost a single band of the 150-kDa γ2 chain, while MKN-28/WT secreted the 105-kDa γ2 chain as a major component and the 150-kDa γ2 chain as a minor component. Similar immunoblotting patterns were obtained when the LN5 subunits deposited on the ECM were analyzed (Fig. 2B). The mixture of LN5 with the 150-kDa γ2 chain and with the 105-kDa γ2 chain was found in the ECM from MKN-28/WT cells, while the LN5 with only the 150-kDa γ2 chain was found in the ECM from MKN-28/GAA cells (right panel in Fig. 2B). Unlike the conditioned media, the ECM fractions from both cell types contained the 190-kDa, unprocessed form and the 145-kDa, N-terminally cleaved form of the α3 chain as minor components in addition to the major 160-kDa α3 chain (left panel in Fig. 2B). Only a single band of the 135-kDa β3 chain was found in both the conditioned media and the ECMs from the three cell types. These electrophoretic patterns of the α3 and β3 chains confirmed that the overexpression of the mutated γ2 chain (GAA-γ2) affected neither the secretion or deposition of the α3 and β3 chains nor their proteolytic processing.

We also confirmed by immunoblotting under non-reducing conditions that the laminin α3, β3, and γ2 chains were secreted as the LN5

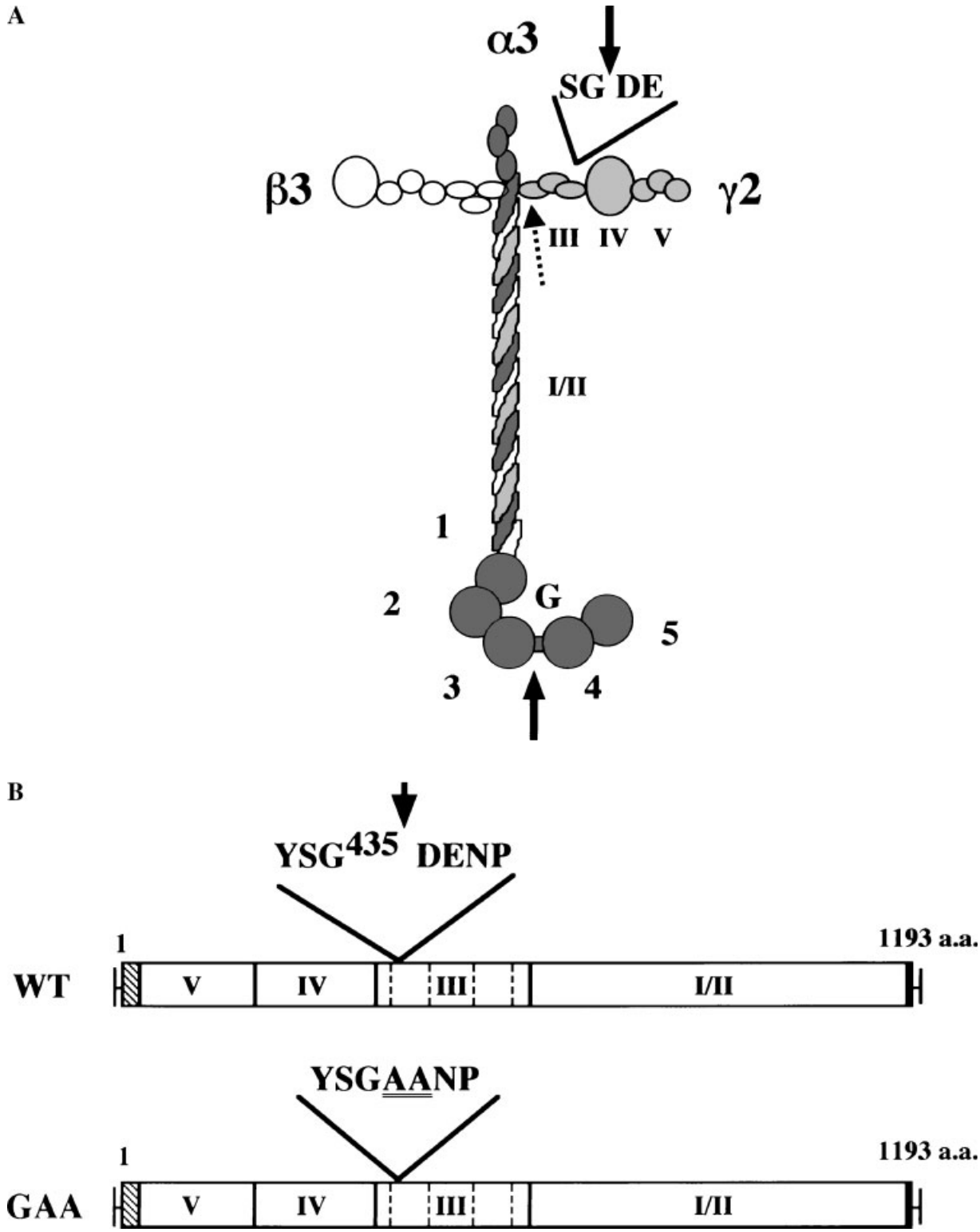

Fig. 1. Domain structures of LN5 and its proteolytic processing sites. **A**: Schematic structure of LN5 molecule (α3β3γ2). Arrows, major cleavage sites in the α3 and γ2 chains in human LN5; dashed arrow, cleavage site in rat LN5 to produce the 80-kDa γ2 chain; G, a carboxyl-terminal globular domain of the α3 chain. Roman numerals indicate domains of the γ2 chain. **B**: Domain structures and processing sequences of wild-type (WT) and mutated (GAA) human laminin γ2 chains. The arrow indicates the proteolytic processing site which produces the 105-kDa γ2 chain. Both D(Asp)^{435} and E(Glu)^{436} in the wild-type γ2 chain (WT) were substituted with A(Ala) by the site-directed mutagenesis of the γ2 cDNA to prepare the processing-resistant mutant γ2 chain (GAA). These cDNA constructs were inserted into the mammalian expression vector pEF-BOS-CITE-NEO and transfected into MKN-28 cells. Scale bars indicate the number of amino acid residues. Shadow boxes indicate the signal sequence. Roman numerals indicate domains.

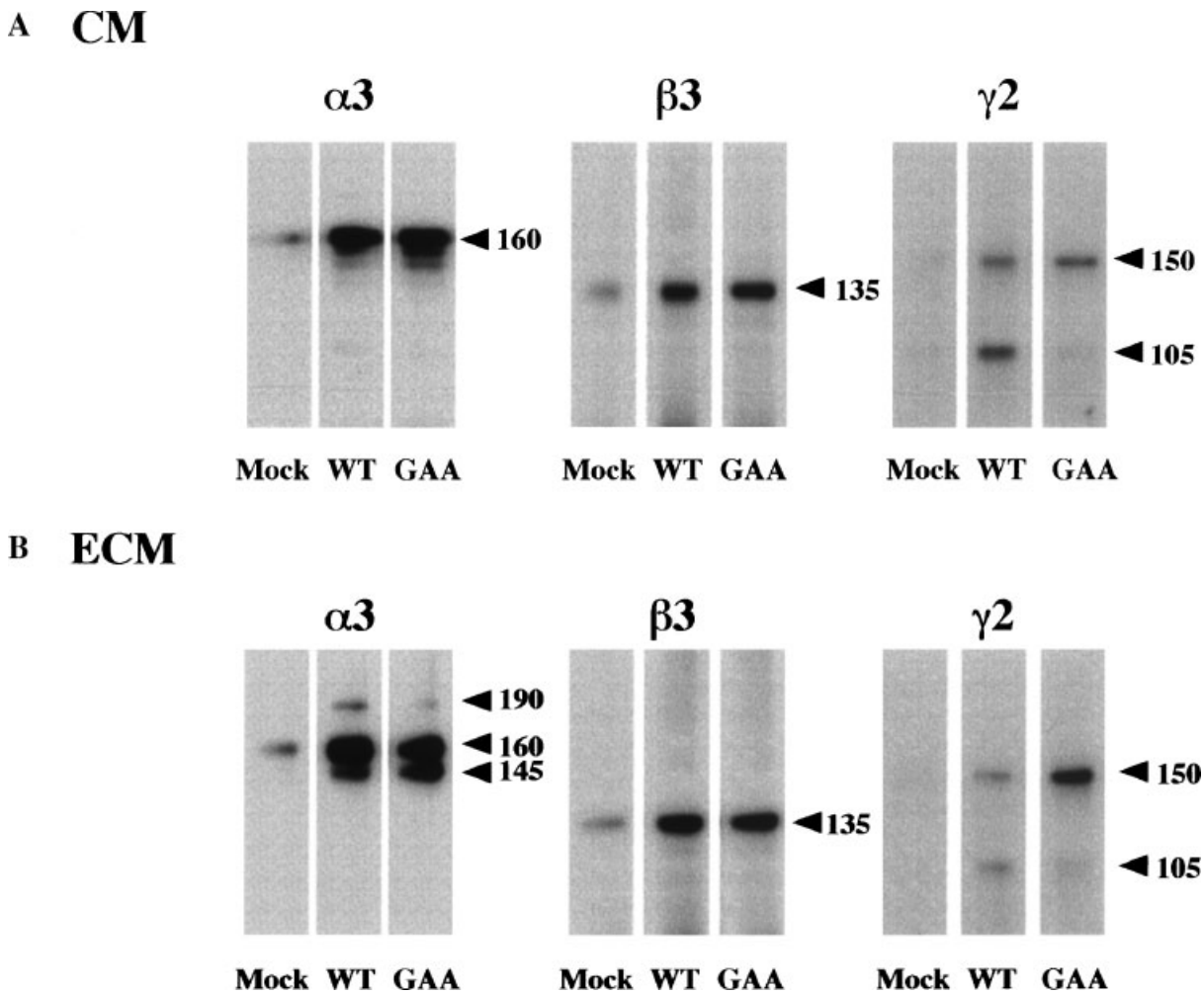

Fig. 2. Analysis of LN5 subunits secreted in soluble form and deposited on plastic surface by three kinds of MKN-28 transfectants. MKN-28 transfectants were grown to confluence in 90-mm dish, and the conditioned medium (CM) (**A**) and ECM fractions (**B**) were prepared and analyzed by immunoblotting with the antibodies to the laminin α3 chain, β3 chain, and γ2 chain as described in ''Materials and Methods.'' Mock, MKN-28/Mock cells; WT, MKN-28/WT cells; GAA, MKN-28/GAA cells. Arrowheads, LN5 subunits and their molecular sizes in kDa. The two minor α3 chains of 190 and 145 kDa in ECM (B) correspond to the unprocessed α3 chain and the processed one lacking the amino-terminal region, respectively.

heterotrimers in the cultures of the three transfectants (data not shown).

Like the parent MKN-28 cells, the three kinds of MKN-28 transfectant secreted active trypsin to the culture media at high levels. Therefore, when they were cultured in a serum-free medium without STI, any of the LN5 subunits were scarcely detected in the immunoblotting analysis of the conditioned media, suggesting that the secreted trypsin had degraded the LN5 subunits (data not shown).

Phenotypic Changes of MKN-28 Cells by Overexpression of Processed or Non-Processed LN5

In order to investigate autocrine effects of the secretion of processed and non-processed LN5 proteins, the three MKN28 transfectants were compared with respect to cell adhesion and migration properties. When each transfected cell line was seeded to plastic plates containing a serum-free medium, all kinds of cell lines poorly adhered to the plates. When incubated in the serum-free medium with STI for 4 h, MKN-28/WT and MKN-28/GAA appeared more spread than the control transfectant MKN-28/Mock (Fig. 3A). When adherent cells were quantified in the presence or absence of STI, the cell attachment efficiency was slightly higher for MKN-28/GAA than MKN-28/WT, and for MKN-28/WT than MKN-28/Mock (Fig. 3B). Although the presence of STI in the medium did not change the relative cell attachment efficiencies of the three cell lines, it shortened

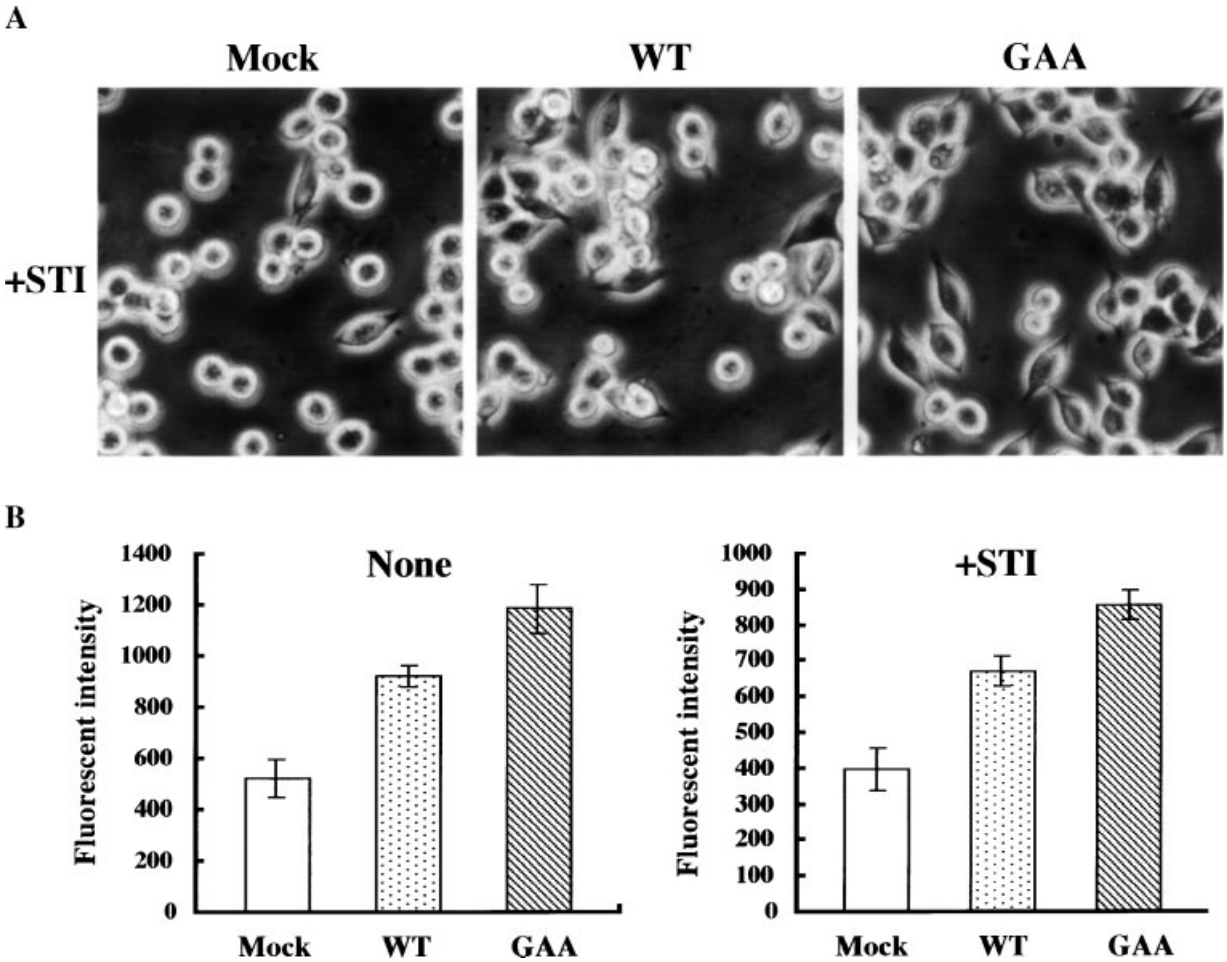

Fig. 3. Adhesion of three kinds of MKN-28 transfectants. **A**: MKN-28/Mock (Mock), MKN-28/WT (WT), and MKN-28/GAA (GAA) cells were plated on 24-well plates in a serum-free medium supplemented with 1 mM STI, and phase-contrast photomicrographs were taken after 4-h incubation. **B**: The three MKN-28 transfectants were plated and incubated in a serum-free medium without STI for 8 h (**left panel**), or with 1 mM STI for 4 h (**right panel**). After the incubation, the relative numbers of adherent cells were determined as described in ''Materials and Methods.'' Each value represents the mean of the fluorescent intensities of adherent cells in triplicate wells.

the time required for sufficient cell attachment from 8 h to 4 h, suggesting that STI prevented or suppressed the degradation of secreted LN5 by trypsin.

Next, we compared the cell migration activities of the three transfectants. As shown in Figure 4, MKN-28/WT cells were more migratory than MKN-28/GAA and MKN-28/Mock cells. This is in contrast to the cell adhesion activity of the cell lines. These results suggest that the LN5 with the non-processed γ2 chain has a higher cell adhesion activity but a lower cell migration activity than one with the processed γ2 chain toward the producing cells.

Purification of LN5 Variants

In order to confirm the differential biological activities of the LN5 variants, we purified LN5 proteins from the serum-free conditioned media of MKN-28/GAA cells and MKN-28/WT cells. Because MKN-28/WT cells secreted both the 105-kDa and 150-kDa γ2 chains, we also purified LN5 from the conditioned medium of the human squamous cell carcinoma line HSC-4, which preferentially secreted the LN5 with the 105-kDa γ2 chain. For the purification, LN5 was separated by molecular-sieve chromatography on a Sepharose 4B column, followed by affinity chromatography on an anti-laminin-γ2 monoclonal antibody column. The purified materials were analyzed by SDS–PAGE under reducing conditions (Fig. 5A) and immunoblotting (Fig. 5B). The LN5 from MKN-28/GAA cells, designated GAA-LN5, was composed of the 160-kDa α3 chain, the 150-kDa γ2 chain, and the 135-kDa β3 chain (Fig. 5B). As expected, the

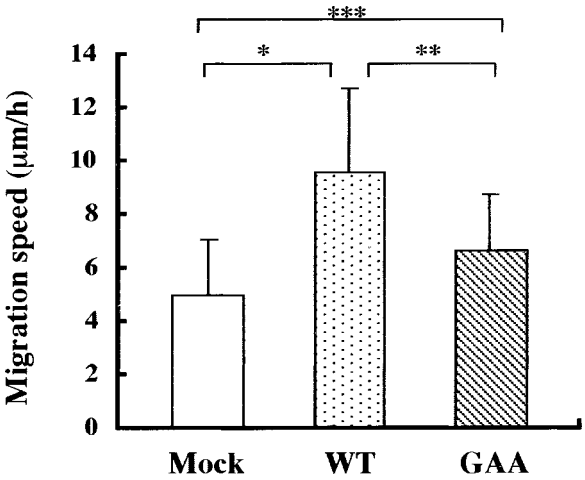

Fig. 4. Migration activity of three kinds of MKN-28 transfectants. MKN-28/Mock (Mock), MKN-28/WT (WT), and MKN-28/GAA (GAA) cells were plated on 24-well plates in RPMI1640 medium plus 1% FCS. After incubation for 1 h, the migration of the MKN-28 cells were monitored by video for 10 h. Each bar represents the mean ± S.D. of the cell migration speeds of at least 25 cells. The asterisks *, **, and *** indicate statistical differences ($P < 0.001$), ($P < 0.01$), and ($P < 0.05$), respectively.

processed, 105-kDa γ2 chain was scarcely detected. The LN5 purified from MKN-28/WT cells, named WT-LN5, contained both the 150-kDa and 105-kDa γ2 chains, in addition to the 160-kDa α3 chain and the 135-kDa β3 chain (Fig. 5A,B). On the other hand, the natural LN5 purified from HSC-4 cells, designated Nat-LN5, was mainly composed of the 160-kDa α3 chain, the 135-kDa β3 chain, and the 105-kDa γ2 chain (Fig. 5B). This LN5 scarcely contained the unprocessed, 150-kDa γ2 chain. Therefore, in the following experiments we mainly used Nat-LN5 and GAA-LN5 to examine the effect of the proteolytic processing of the 150-kDa γ2 chain to the 105-kDa form. The relative concentrations of the three forms of LN5 were normalized based on the concentration of the β3 chain determined by the immunoblotting (Fig. 5B) and ELISA assay.

Cell Adhesion Activity

To elucidate the effect of the γ2 chain cleavage on the biological activity of LN5, we first compared the cell adhesion activities of the three LN5 forms with the non-processed, 150-kDa γ2 chain (GAA-LN5), with a mixed composition of the 150-kDa, and 105-kDa γ2 chains (WT-LN5), and with the processed, 105-kDa γ2 chain (Nat-LN5). When BRL cells were plated on the three forms of LN5, they could attach to the LN5 dose-dependently (Fig. 6A). The effective concentration for the half-maximal cell adhesion (ED_{50}) was approximately 130 ng/ml for GAA-LN5, 500 ng/ml for WT-LN5, and 700 ng/ml for Nat-LN5 (the processed form). When BRL cells were incubated for 1 h on the plates coated at 250 ng/ml of LN5, most of cells were spread on GAA-LN5, but they only weakly attached to Nat-LN5 without spreading (Fig. 6B). On the plates coated at 500 ng/ml of LN5, the cells became spread even on Nat-LN5 (data not shown).

Normal human epidermal keratinocytes (Fig. 6C) and the human bladder carcinoma cell line EJ-1 (data not shown) showed almost the same response to these LN5 forms. To compare the coating efficiencies of Nat-LN5 and GAA-LN5, we analyzed the amount of the LN5 proteins bound to the plates by the ELISA assay. As expected, there was no significant difference between Nat-LN5 and GAA-LN5 (data not shown). These results demonstrate that the non-processed LN5 (GAA-LN5) has an about five-times higher cell adhesion activity than the processed LN5 (Nat-LN5).

When the effects of function-blocking anti-integrin antibodies on the cell adhesion were examined, the anti-integrin α3 and anti-integrin β1 antibodies almost completely blocked the attachment of EJ-1 cells to Nat-LN5 and GAA-LN5, indicating that both LN5 variants recognized integrin α3β1 as a primary receptor (data not shown).

Cell Motility Activity

LN5 promotes not only cell adhesion but also cell scattering and migration [Miyazaki et al., 1993; Kikkawa et al., 1994]. Both cell scattering and migration on LN5 are thought to reflect the enhanced cellular motility. The cell-scattering activity toward BRL cells was assayed with the two forms of LN5 as reported before [Kariya et al., 2003]. When GAA-LN5 and Nat-LN5 were directly added to the culture medium of BRL cells at a concentration of 12.5 ng/ml, Nat-LN5 more efficiently scattered BRL cells than GAA-LN5 (Fig. 7A). The cell-scattering activity of GAA-LN5 appeared to be about 30% lower than that of Nat-LN5 as compared at their half-maximal concentrations (Fig. 7B).

We next monitored the migration of BRL cells on the LN5 substrata by video microscopy. This analysis showed that Nat-LN5 supported an

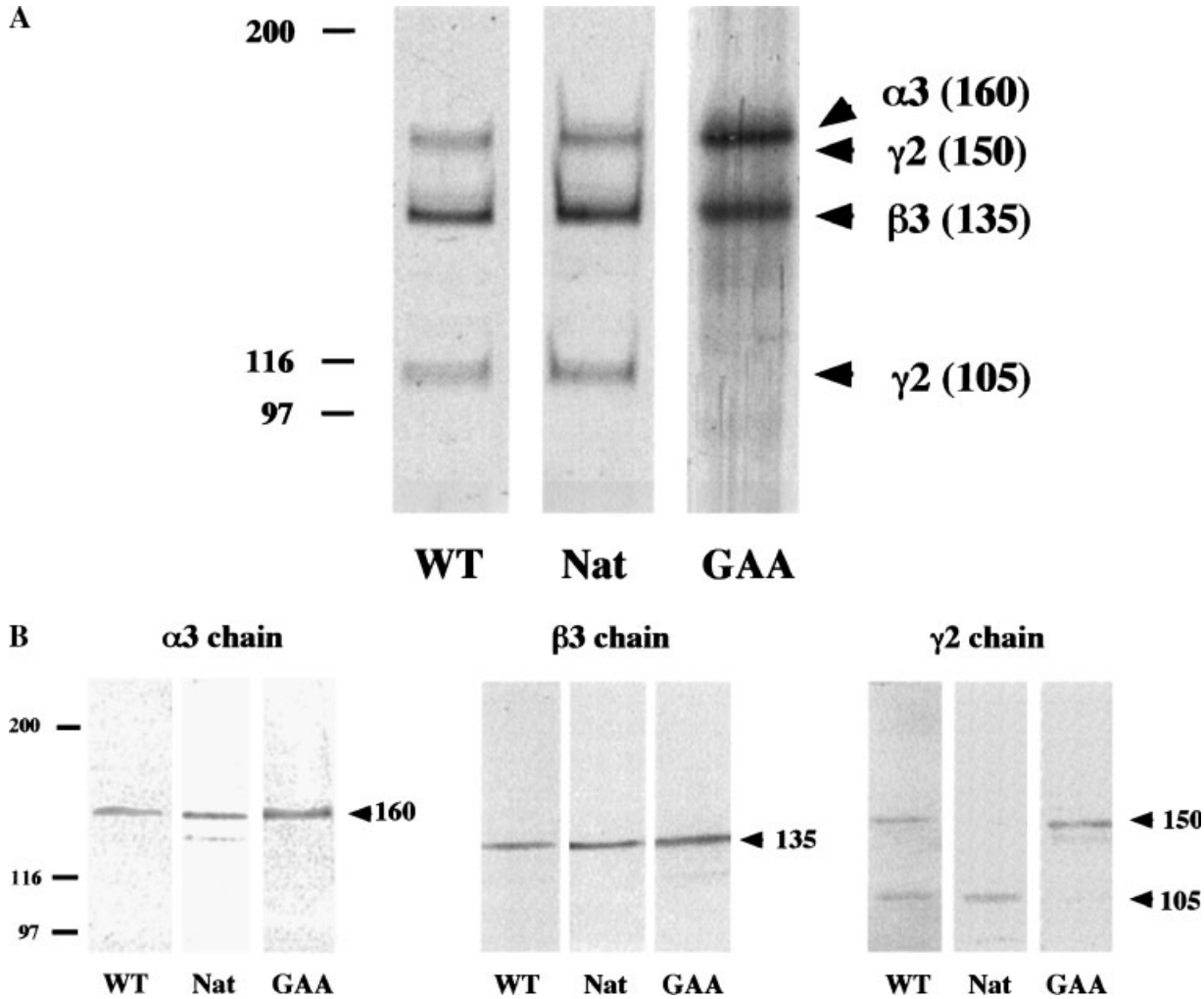

Fig. 5. Electrophoretic profiles of purified recombinant and natural LN5 proteins. **A**: SDS–PAGE under reducing conditions. Two recombinant LN5 proteins, WT-LN5 (WT) and GAA-LN5 (GAA), were purified from the serum-free conditioned media of the respective MKN-28 transfectants. Similarly, a natural LN5 (Nat-LN5) (Nat) was purified from HSC-4 cells. These proteins were separated by SDS–PAGE on a 6% gel under reducing conditions and stained with silver. The 105-kDa, processed γ2 chain is found in Nat-LN5 and WT-LN5, but not in GAA-LN5. The 160-kDa α3 chain and the 150-kDa γ2 chains were not clearly separated under the electrophoretic conditions. Arrowheads, LN5 subunits with molecular sizes in kDa. **B**: Immunoblotting of Nat-LN5 (Nat), GAA-LN5 (GAA), and WT-LN5 (WT). Twenty ng of three purified LN5 proteins were separated by SDS–PAGE under reducing conditions and then analyzed by immunoblotting with the antibodies against the α3, β3, and γ2 chains. Arrowheads indicate the major bands of the three subunits with apparent molecular sizes in kDa.

about two-times faster cell migration than GAA-LN5 as compared at the same weight concentration, and this difference became greater at their minimal effective concentrations for cell adhesion (Fig. 8A). EJ-1 carcinoma cells also migrated significantly faster on Nat-LN5 than on GAA-LN5 (Fig. 8B). The normal human mammary gland epithelial cell line MCF10A and the human epidermoid carcinoma cell line A431, both of which produced endogenous LN5, also showed the same response to these LN5 forms (data not shown). These results demonstrate that the processed LN5 (Nat-LN5) has significantly higher cell migration activity than the non-processed LN5 (GAA-LN5). This means that the proteolytic processing of γ2 chain enhances the cell migration activity of LN5.

DISCUSSION

In the present study, we, for the first time, prepared a recombinant human LN5 with only the non-processed, 150-kDa γ2 chain (GAA-LN5) and a natural LN5 with only the

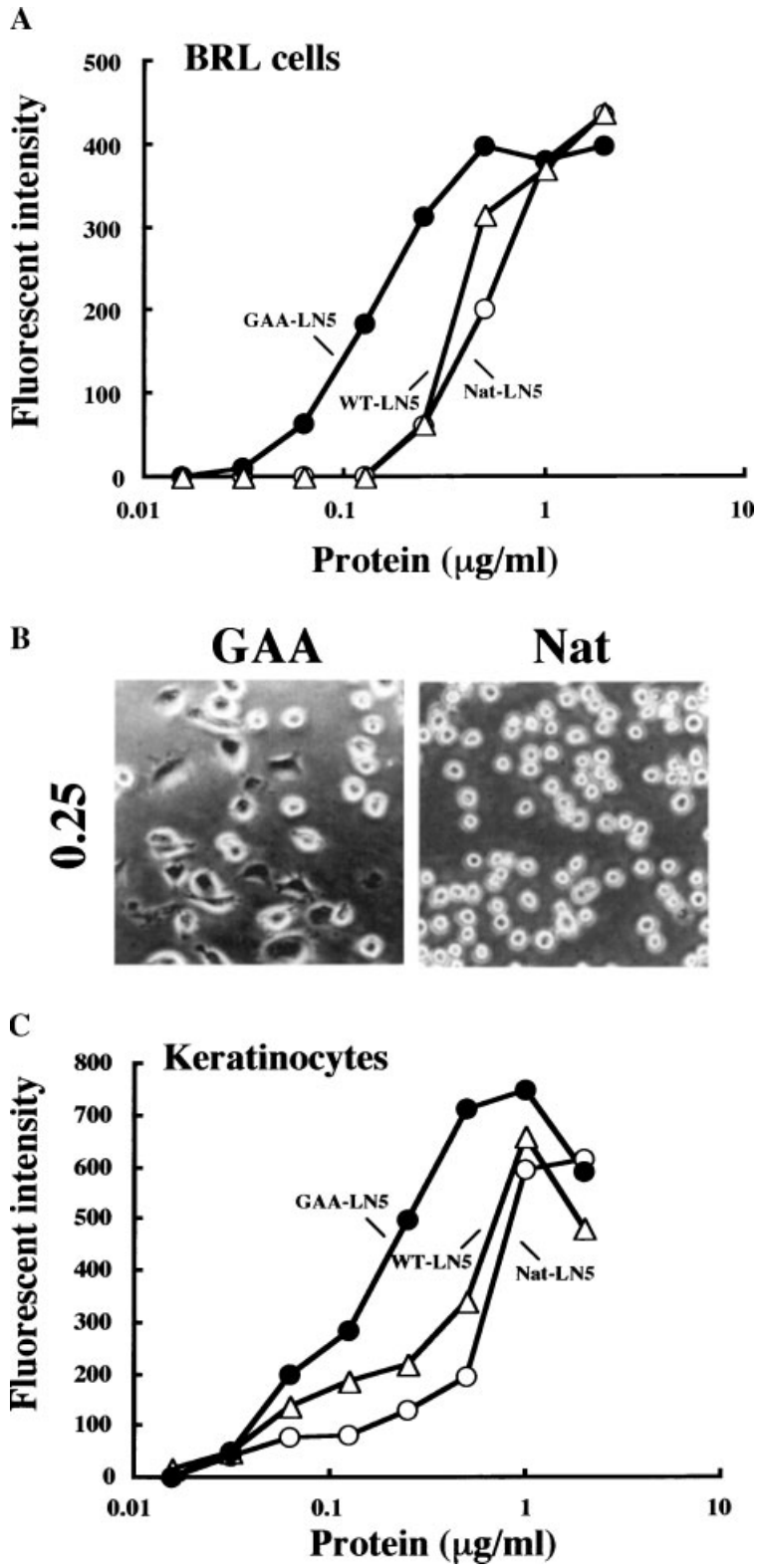

Fig. 6. Cell adhesion activity of three forms of purified LN5. **A**: Effects of various concentrations of Nat-LN5 (○), WT-LN5 (△), and GAA-LN5 (●) on attachment of BRL cells to plastic plates. A total of 96-well plates were coated with the indicated concentrations of each LN5 protein. BRL cells were plated on each substrate in serum-free medium and incubated for 1 h. After the incubation, the relative numbers of adherent cells were determined as described in ''Materials and Methods.'' Each value represents the mean of the fluorescent intensities of adherent cells in triplicate wells. **B**: Morphology of BRL cells incubated in serum-free medium for 1 h on plastic plates pre-coated with 0.25 mg/ml GAA-LN5 (GAA) or Nat-LN5 (Nat). **C**: Effects of various concentrations of Nat-LN5 (○), WT-LN5 (△), and GAA-LN5 (●) on attachment of normal human keratinocytes onto plastic plates. Experiments were done as described above.

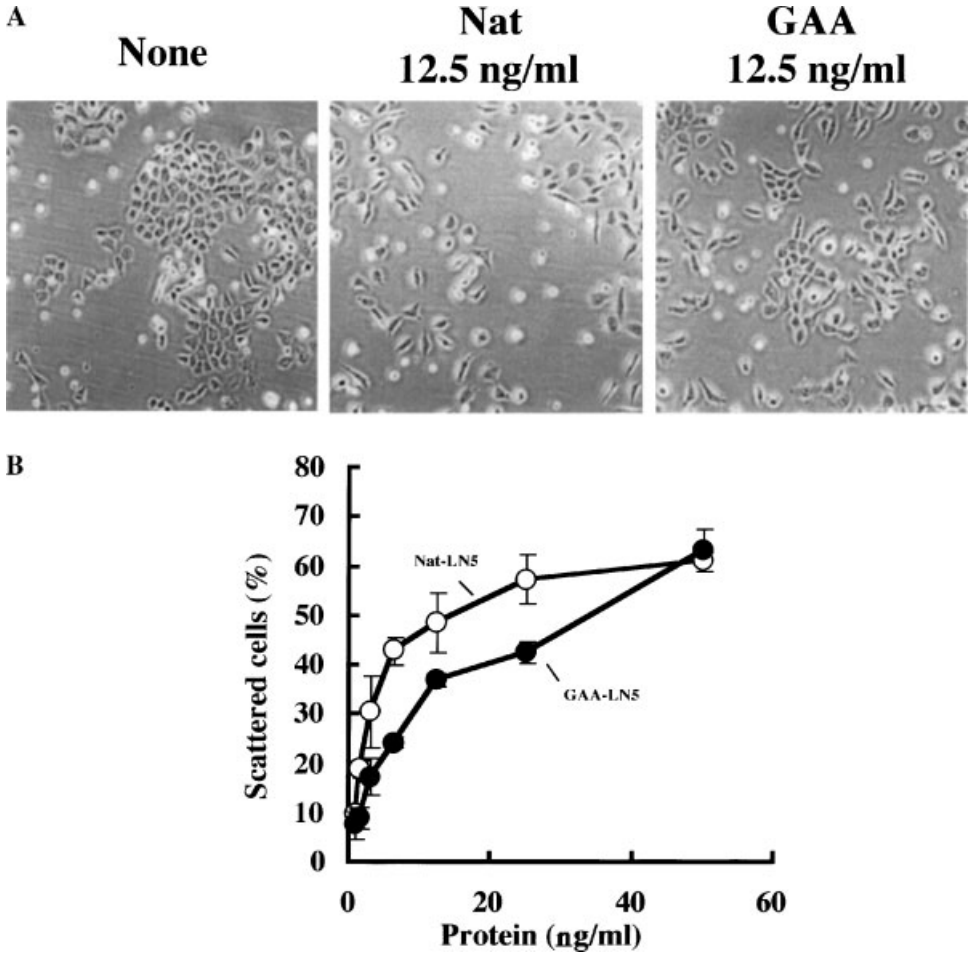

Fig. 7. Cell-scattering activity of Nat-LN5 and GAA-LN5. **A**: Morphology of BRL cells incubated with Nat-LN5 or GAA-LN5. BRL cells were cultured for 2 days in DME/F12 medium supplemented with 1% FCS without (None) or with 12.5 ng/ml Nat-LN5 (Nat) or GAA-LN5 (GAA), which were directly added into the culture medium. **B**: Effects of various concentrations of Nat-LN5 (○) and GAA-LN5 (●) on scattering of BRL cells. Cell scattering is expressed as the percentage of single cells in each field. Each value represents the mean ± SD for four different fields. At least 100 cells were counted in each field.

processed, 105-kDa γ2 chain (Nat-LN5) and compared their biological activities. The results demonstrate that GAA-LN5 has a higher cell adhesion activity and a lower cell motility activity than Nat-LN5. This implies that the proteolytic processing of the LN5 γ2 chain converts the LN5 from the cell adhesion type to the cell migration type.

Much attention has been focused on the proteolytic processing of LN5. Originally, Giannelli et al. [1997] reported that when rat LN5 with the unprocessed 150-kDa γ2 chain was treated with gelatinase A, the LN5 digest stimulated cell migration more strongly than the unprocessed LN5 in the Boyden chamber assay. Gelatinase A cleaves the 150-kDa γ2 chain at a carboxyl-terminal site of domain III, producing the 80-kDa γ2 chain (see Fig. 1A). The gelatinase-activating enzyme MT1-MMP also cleaves the γ2 chain at two different sites, converting it to the 100-kDa form as an intermediate form and finally the 80-kDa form [Koshikawa et al., 2000]. Because the LN5 digest in their experiments contained both the LN5 with the processed 80-kDa γ2 chain and the short arm fragments of the γ2 chain, it was unknown which product was responsible for the enhanced cell migration activity. Recently, the same group reported that a 30-kDa recombinant protein corresponding to the laminin-type EGF-like domain III in the γ2 chain short arm, which can be released by the MT1-MMP cleavage of the γ2 chain at two different sites (see Fig. 1), bound and activated the EGF receptor to stimulate cell migration [Schenk et al., 2003]. Their results suggest that the

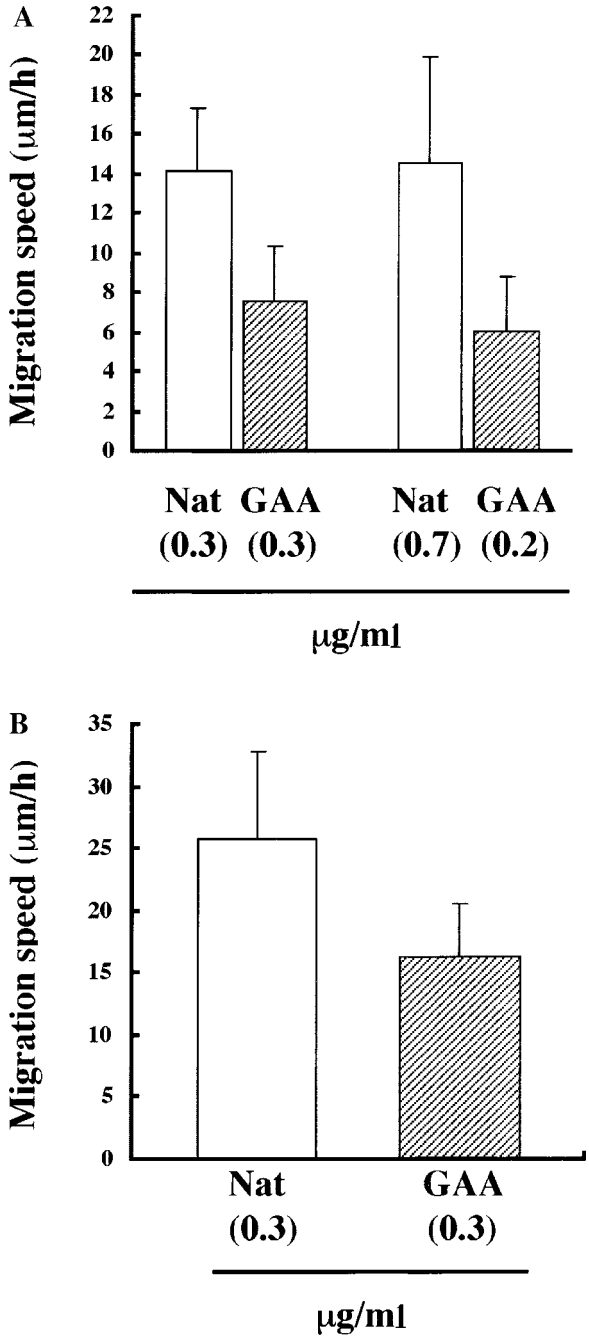

Fig. 8. Cell migration activity of Nat-LN5 and GAA-LN5. BRL cells (**A**) and EJ-1 cells (**B**) were suspended in DMEM/F12 medium supplemented with 1% FCS, plated on 24-well plates pre-coated with the indicated concentrations of Nat-LN5 (Nat) or GAA-LN5 (GAA), and incubated for 1 h to allow the cell spreading. After the incubation, the migration of the cells was monitored by video for 10 h. Each bar represents the mean ± S.D. of the cell migration speeds of at least 15 cells. In BRL cells (A), the cell migration speeds on Nat-LN5 and GAA-LN5 were compared at the same weight/ml concentration (**left two columns**) and at the concentrations capable of supporting the same level of cell adhesion (**right two columns**).

domain III fragment of γ2 chain, rather than the LN5 with the processed 80-kDa γ2 chain, is responsible for the enhanced cell migration activity. Another group has reported that a blocking antibody against the domain III of the γ2 chain suppressed the LN5-dependent cell migration [Salo et al., 1999]. These reports suggest that the domain III of the γ2 chain is a cell migration-stimulatory domain in LN5 [Schenk et al., 2003]. Unlike rat LN5, however, the 80-kDa γ2 chain is not produced in human LN5, because the processing site of rat γ2 chain is not conserved in the human γ2 chain. Neither MT1-MMP nor MMP-2 cleaves the γ2 chain of human LN5, although they cleave that of rat LN5 [Veitch et al., 2003]. In the present study, we found that the LN5 with the 150-kDa γ2 chain (GAA-LN5) is more adhesive but less motile than one with the 105-kDa γ2 chain (Nat-LN5). As the γ2 chain is cleaved at the amino-terminal region of domain III, the 105-kDa γ2 chain of Nat-LN5 looses domains IV and V but still retains most part of domain III (Fig. 1B). It seems likely that the proteolytic processing of the γ2 chain to the 105-kDa form may expose domain III, allowing its efficient binding to EGF receptor and resultant induction of intracellular migratory signal. However, further studies are needed to show that the LN5 with the processed γ2 chain binds to EGF receptor or other receptors besides integrins.

Gagnoux-Palacios et al. [2001] overexpressed a cDNA encoding the laminin γ2 chain without the cleavage sequence in γ2-deficient human keratinocytes to produce LN5 with an non-processed, 150-kDa γ2 chain. These keratinocytes were more resistant to trypsin treatment for the detachment from the substrate and more efficiently deposited the LN5 on the matrix than the cells expressing the LN5 with the wild-type γ2 chain. They also suggested that the globular domain IV in the γ2 short arm is required for the matrix assembly and the cell attachment activity of LN5. In consistence with their findings, MKN-28 cells expressing GAA-LN5 were more adherent than those expressing WT-LN5. However, we could detect no significant difference with respect to the deposition of LN5 on the matrix between the two LN5 forms with the processed γ2 chain and with the non-processed one (Fig. 2B). MKN-28 cells may not produce a matrix component capable of binding to domain IV of the γ2 chain. In addition, we found that the GAA-LN5-expressing cells were less motile

than the WT-LN5-expressing cells. It seems possible that the loss of domain IV contributes to both the elevated cell migration activity and the lowered cell adhesion activity of the LN5 with the processed γ2 chain.

The present study indicates that the proteolytic processing of the γ2 chain from the 150-kDa form to the 105-kDa form converts the LN5 from the adhesion type to the motility type. LN5 plays a critical role in stable adhesion of basal epithelial cells to the basement membrane, while LN5 is believed to contribute to cell migration under some pathological conditions. Indeed, LN5 has been reported to be expressed in keratinocytes at edges of tissue injuries [Ryan et al., 1994] and in carcinoma cells at invasion fronts [Pyke et al., 1995]. The functional change of LN5 by the proteolytic processing of the γ2 chain is likely to contribute to the elevated cell migration under these pathological conditions.

LN5 undergoes proteolytic cleavage not only at the amino-terminal region of the γ2 chain but also at the G domain of the α3 chain. We have analyzed LN5 secreted by many human cancer cell lines and found that the LN5 trimers in these cell lines almost exclusively contain the processed α3 chain [Mizushima et al., 1996], whereas they contain a mixture of the processed and unprocessed γ2 chains (Fig. 2) [unpublished data]. This suggests that the processing of the γ2 chain, but not the α3 chain, is regulated by unknown conditions. Recent studies have suggested that the processing of the LN5 subunits are catalyzed by proteinases of the BMP-1 metalloproteinase family [Amano et al., 2000; Veitch et al., 2003]. However, the regulatory mechanism of these enzymes, including the regulation by endogenous proteinase inhibitors, remains unknown. It seems important to investigate the regulation of the γ2 chain processing, especially in pathological conditions such as wound healing and tumor invasion, where the enhanced cell migration is critical.

ACKNOWLEDGMENTS

We thank Dr. H. Yasumitsu, Dr. S. Higashi, and Dr. T. Hirosaki for helpful suggestions and discussions.

REFERENCES

Aberdam D, Galliano MF, Vailly J, Pulkkinen L, Bonifas J, Christiano AM, Tryggvason K, Uitto J, Epstein E, Jr., Ortonne JP, et al. 1994. Herlitz's junctional epidermolysis bullosa is linked to mutations in the gene (*LAMC2*) for the gamma 2 subunit of nicein/kalinin (LAMININ-5). Nat Genet 6:299–304.

Amano S, Scott IC, Takahara K, Koch M, Champliaud MF, Gerecke DR, Keene DR, Hudson DL, Nishiyama T, Lee S, Greenspan DS, Burgeson RE. 2000. Bone morphogenetic protein 1 is an extracellular processing enzyme of the laminin 5 gamma 2 chain. J Biol Chem 275:22728–22735.

Aumailley M, Rousselle P. 1999. Laminins of the dermoepidermal junction. Matrix Biol 18:19–28.

Baker SE, Hopkinson SB, Fitchmun M, Andreason GL, Frasier F, Plopper G, Quaranta V, Jones JC. 1996. Laminin-5 and hemidesmosomes: Role of the alpha 3 chain subunit in hemidesmosome stability and assembly. J Cell Sci 109:2509–2520.

Carter WG, Ryan MC, Gahr PJ. 1991. Epiligrin, a new cell adhesion ligand for integrin alpha 3 beta 1 in epithelial basement membranes. Cell 65:599–610.

Chen C, Okayama H. 1987. High-efficiency transformation of mammalian cells by plasmid DNA. Mol Cell Biol 7: 2745–2752.

Colognato H, Yurchenco PD. 2000. Form and function: The laminin family of heterotrimers. Dev Dyn 218:213–234.

Egeblad M, Werb Z. 2002. New functions for the matrix metalloproteinases in cancer progression. Nat Rev Cancer 2:161–174.

Gagnoux-Palacios L, Allegra M, Spirito F, Pommeret O, Romero C, Ortonne JP, Meneguzzi G. 2001. The short arm of the laminin gamma2 chain plays a pivotal role in the incorporation of laminin 5 into the extracellular matrix and in cell adhesion. J Cell Biol 153:835–850.

Giannelli G, Falk-Marzillier J, Schiraldi O, Stetler-Stevenson WG, Quaranta V. 1997. Induction of cell migration by matrix metalloprotease-2 cleavage of laminin-5. Science 277:225–228.

Goldfinger LE, Stack MS, Jones JC. 1998. Processing of laminin-5 and its functional consequences: Role of plasmin and tissue-type plasminogen activator. J Cell Biol 141:255–265.

Goldfinger LE, Hopkinson SB, deHart GW, Collawn S, Couchman JR, Jones JC. 1999. The alpha3 laminin subunit, alpha6beta4 and alpha3beta1 integrin coordinately regulate wound healing in cultured epithelial cells and in the skin.1. J Cell Sci 112:2615–2629.

Hirosaki T, Mizushima H, Tsubota Y, Moriyama K, Miyazaki K. 2000. Structural requirement of carboxyl-terminal globular domains of laminin alpha 3 chain for promotion of rapid cell adhesion and migration by laminin-5. J Biol Chem 275:22495–22502.

Hirosaki T, Tsubota Y, Kariya Y, Moriyama K, Mizushima H, Miyazaki K. 2002. Laminin-6 is activated by proteolytic processing and regulates cellular adhesion and migration differently from laminin-5. J Biol Chem 277: 49287–49295.

Imai Y, Matsushima Y, Sugimura T, Terada M. 1991. A simple and rapid method for generating a deletion by PCR. Nucleic Acids Res 19:2785.

Kallunki P, Sainio K, Eddy R, Byers M, Kallunki T, Sariola H, Beck K, Hirvonen H, Shows TB, Tryggvason K. 1992. A truncated laminin chain homologous to the B2 chain: Structure, spatial expression, and chromosomal assignment. J Cell Biol 119:679–693.

Kariya Y, Tsubota Y, Hirosaki T, Mizushima H, Puzon-McLaughlin W, Takada Y, Miyazaki K. 2003. Differential regulation of cellular adhesion and migration by recombinant laminin-5 forms with partial deletion or mutation within the G3 domain of alpha3 chain. J Cell Biochem 88:506–520.

Kikkawa Y, Umeda M, Miyazaki K. 1994. Marked stimulation of cell adhesion and motility by ladsin, a laminin-like scatter factor. J Biochem 116:862–869.

Kivirikko S, McGrath JA, Baudoin C, Aberdam D, Ciatti S, Dunnill MG, McMillan JR, Eady RA, Ortonne JP, Meneguzzi G, et al. 1995. A homozygous nonsense mutation in the alpha 3 chain gene of laminin 5 (*LAMA3*) in lethal (Herlitz) junctional epidermolysis bullosa. Hum Mol Genet 4:959–962.

Koch M, Olson PF, Albus A, Jin W, Hunter DD, Brunken WJ, Burgeson RE, Champliaud MF. 1999. Characterization and expression of the laminin gamma3 chain: A novel, non-basement membrane-associated, laminin chain. J Cell Biol 145:605–618.

Koshikawa N, Moriyama K, Takamura H, Mizushima H, Nagashima Y, Yanoma S, Miyazaki K. 1999. Overexpression of laminin gamma2 chain monomer in invading gastric carcinoma cells. Cancer Res 59:5596–5601.

Koshikawa N, Giannelli G, Cirulli V, Miyazaki K, Quaranta V. 2000. Role of cell surface metalloprotease MT1-MMP in epithelial cell migration over laminin-5. J Cell Biol 148:615–624.

Marinkovich MP, Lunstrum GP, Burgeson RE. 1992. The anchoring filament protein kalinin is synthesized and secreted as a high molecular weight precursor. J Biol Chem 267:17900–17906.

Miyata S, Miyagi Y, Koshikawa N, Nagashima Y, Kato Y, Yasumitsu H, Hirahara F, Misugi K, Miyazaki K. 1998. Stimulation of cellular growth and adhesion to fibronectin and vitronectin in culture and tumorigenicity in nude mice by overexpression of trypsinogen in human gastric cancer cells. Clin Exp Metastasis 16:613–622.

Miyata S, Koshikawa N, Higashi S, Miyagi Y, Nagashima Y, Yanoma S, Kato Y, Yasumitsu H, Miyazaki K. 1999. Expression of trypsin in human cancer cell lines and cancer tissues and its tight binding to soluble form of Alzheimer amyloid precursor protein in culture. J Biochem 125:1067–1076.

Miyata S, Koshikawa N, Yasumitsu H, Miyazaki K. 2000. Trypsin stimulates integrin alpha(5)beta(1)-dependent adhesion to fibronectin and proliferation of human gastric carcinoma cells through activation of proteinase-activated receptor-2. J. Biol. Chem. 275:4592–4598.

Miyazaki K, Kikkawa Y, Nakamura A, Yasumitsu H, Umeda M. 1993. A large cell-adhesive scatter factor secreted by human gastric carcinoma cells. Proc Natl Acad Sci USA 90:11767–11771.

Mizushima H, Miyagi Y, Kikkawa Y, Yamanaka N, Yasumitsu H, Misugi K, Miyazaki K. 1996. Differential expression of laminin-5/ladsin subunits in human tissues and cancer cell lines and their induction by tumor promoter and growth factors. J Biochem 120:1196–1202.

Mizushima H, Takamura H, Miyagi Y, Kikkawa Y, Yamanaka N, Yasumitsu H, Misugi K, Miyazaki K. 1997. Identification of integrin-dependent and -independent cell adhesion domains in COOH-terminal globular region of laminin-5 alpha 3 chain. Cell Growth Differ 8:979–987.

Mizushima H, Koshikawa N, Moriyama K, Takamura H, Nagashima Y, Hirahara F, Miyazaki K. 1998. Wide distribution of laminin-5 gamma 2 chain in basement membranes of various human tissues. Horm Res 2:7–14.

Nievers MG, Schaapveld RQ, Sonnenberg A. 1999. Biology and function of hemidesmosomes. Matrix Biol 18:5–17.

Prockop DJ, Sieron AL, Li SW. 1998. Procollagen N-proteinase and procollagen C-proteinase. Two unusual metalloproteinases that are essential for procollagen processing probably have important roles in development and cell signaling. Matrix Biol 16:399–408.

Pulkkinen L, Christiano AM, Gerecke D, Wagman DW, Burgeson RE, Pittelkow MR, Uitto J. 1994. A homozygous nonsense mutation in the beta 3 chain gene of laminin 5 (*LAMB3*) in Herlitz junctional epidermolysis bullosa. Genomics 24:357–360.

Pyke C, Romer J, Kallunki P, Lund LR, Ralfkiaer E, Dano K, Tryggvason K. 1994. The gamma 2 chain of kalinin/laminin 5 is preferentially expressed in invading malignant cells in human cancers. Am J Pathol 145:782–791.

Pyke C, Salo S, Ralfkiaer E, Romer J, Dano K, Tryggvason K. 1995. Laminin-5 is a marker of invading cancer cells in some human carcinomas and is coexpressed with the receptor for urokinase plasminogen activator in budding cancer cells in colon adenocarcinomas. Cancer Res 55:4132–4139.

Rousselle P, Aumailley M. 1994. Kalinin is more efficient than laminin in promoting adhesion of primary keratinocytes and some other epithelial cells and has a different requirement for integrin receptors. J Cell Biol 125:205–214.

Rousselle P, Lunstrum GP, Keene DR, Burgeson RE. 1991. Kalinin: An epithelium-specific basement membrane adhesion molecule that is a component of anchoring filaments. J Cell Biol 114:567–576.

Rousselle P, Keene DR, Ruggiero F, Champliaud MF, Rest M, Burgeson RE. 1997. Laminin 5 binds the NC-1 domain of type VII collagen. J Cell Biol 138:719–728.

Ryan MC, Tizard R, VanDevanter DR, Carter WG. 1994. Cloning of the LamA3 gene encoding the alpha 3 chain of the adhesive ligand epiligrin. Expression in wound repair. J Biol Chem 269:22779–22787.

Salo S, Haakana H, Kontusaari S, Hujanen E, Kallunki T, Tryggvason K. 1999. Laminin-5 promotes adhesion and migration of epithelial cells: Identification of a migration-related element in the gamma2 chain gene (*LAMC2*) with activity in transgenic mice. Matrix Biol 18:197–210.

Schenk S, Quaranta V. 2003. Tales from the cryptic sites of the extracellular matrix. Trends Cell Biol 13:366–375.

Schenk S, Hintermann E, Bilban M, Koshikawa N, Hojilla C, Khokha R, Quaranta V. 2003. Binding to EGF receptor of a laminin-5 EGF-like fragment liberated during MMP-dependent mammary gland involution. J Cell Biol 161: 197–209.

Tsubota Y, Mizushima H, Hirosaki T, Higashi S, Yasumitsu H, Miyazaki K. 2000. Isolation and activity of proteolytic fragment of laminin-5 alpha3 chain. Biochem Biophys Res Commun 278:614–620.

Veitch DP, Nokelainen P, McGowan KA, Nguyen TT, Nguyen NE, Stephenson R, Pappano WN, Keene DR, Spong SM, Greenspan DS, Findell PR, Marinkovich MP. 2003. Mammalian tolloid metalloproteinase, and not matrix metalloprotease 2 or membrane type 1 metalloprotease, processes laminin-5 in keratinocytes and skin. J Biol Chem 278:15661–15668.