Structural Requirement of Carboxyl-terminal Globular Domains of Laminin α3 Chain for Promotion of Rapid Cell Adhesion and Migration by Laminin-5*

Received for publication, February 16, 2000, and in revised form, April 18, 2000
Published, JBC Papers in Press, May 2, 2000, DOI 10.1074/jbc.M001328200

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The basement membrane protein laminin-5, a heterotrimer of laminin α3, β3, and γ2 chains, potently promotes cellular adhesion and motility. It has been supposed that the carboxy-terminal globular region of the α3 chain consisting of five distinct domains (G1 to G5) is important for its interaction with integrins. To clarify the function of each G domain, we transfected cDNAs for the full-length (wild type (WT)) and five deletion derivatives (ΔGs) of the α3 chain into human fibrosarcoma cell line HT1080, which expressed and secreted the laminin β3 and γ2 chains but not the α3 chain. The transfectants with the α3 chain cDNAs lacking G5 (ΔG5), G4–5 (ΔG4,5), G5–4 (ΔG5,4), and G2–5 (ΔG2,5) secreted laminin-5 variants at levels comparable to that with WT cDNA. However, the transfectant with the cDNA without any G domains (ΔG1–5) secreted little laminin-5, suggesting that the G domains are essential for the efficient assembly and secretion of the heterotrimer α3β3γ2. The transfectants with WT, ΔG5, and ΔG1–4,5 cDNAs survived in serum-free medium longer than those with ΔG1–4,5, ΔG2–5, and ΔG1–5 cDNAs. The transfectants with WT, ΔG5, and ΔG2–5,4,5 cDNAs secreted apparently the same size of laminin-5, which lacked G4 and G5 due to proteolytic cleavage between G3 and G4, and these laminin-5 forms potently promoted integrin α6β4-dependent cell adhesion and migration. However, the laminin-5 forms of ΔG2–5,4,5 and ΔG1–4,5 hardly promoted the cell adhesion and motility. These findings demonstrate that the G3 domain, but not the G4 and G5 domains, of the α3 chain is essential for the potent promotion of cell adhesion and motility by laminin-5.

Laminins are a family of extracellular matrix proteins that are localized mainly in basement membranes and regulate various cellular functions such as adhesion, motility, growth, differentiation, and apoptosis through interaction with specific integrins on the cell surface (1, 2). The three subunits of laminin (laminin-1 to -12) having distinct chain combinations have been identified in human thus far (3, 4). Among these laminin isoforms, laminin-5, which consists of the laminin α3, β3, and γ2 chains, is unique in the structure and biological activity. Laminin-5 was originally found as a keratinocyte-derived matrix protein named epiligrin, kaligin, or nicerin (5–7) and a laminin-like cell scattering factor, ladsin, secreted by human gastric carcinoma cells (8). Laminin-5 has a feature lacking some domains found in the amino-terminal regions (or the short arms) of the three subunits of other laminin isoforms (2). The laminin α3 chain is found in laminin-6 (α3β1γ1) and laminin-7 (α3β2γ1) besides laminin-5, but the laminin β3 and γ2 chains are found only in laminin-5. More interestingly, laminin-5 has much higher activity to promote adhesion, migration, and scattering of various types of cells than laminin-1, fibronectin, and vitronectin (8–10).

Most cultured cell lines utilize integrin α6β4 as a major receptor to adhere and migrate on the laminin-5 substrate, but integrins α6β1 and α6β2 also act as the additional receptors of laminin-5 depending on cell types (5, 9, 10). Laminin-5 is an important component of basement membranes of the skin and many other epithelial tissues (5, 6, 11). The interaction of laminin-5 with integrin α6β4 in the hemidesmosome structures is essential for the stable anchorage of basal epithelial cells to the underlying connective tissues. Defects of laminin-5 genes cause Herlitz-type junctional epidermolysis bullosa (H-JEB), which is characterized by splitting of epidermal/dermal junctions (12, 13). Similarly, targeted disruption of the laminin-5 genes or integrin α6β4 genes in mice causes severe junctional blisters and abnormal hemidesmosomes, resulting in neonatal lethality (14–16). On the other hand, the potent cell motility activity of laminin-5 has been suggested to contribute to wound healing (17) and tumor invasion (18).

For understanding the molecular basis for the unique bifunctional properties of laminin-5, the stable adhesion and motility, it seems important to clarify its structural and functional relationships. All laminin α chains have a large carboxyl-terminal globular domain consisting of a tandem array of five small globular domains (or modules) (G1 to G5) (1, 2). These G domains are autonomous folding units (19). They contain binding sites for β1 integrins (20) and heparin (21), as well as α-dystroglycan in some laminin isoforms (22, 23). Our previous study with recombinant G domains of the laminin α3 chain has shown that the G2 domain contains an integrin α6β4-binding site, and the G4 and G5 domains weakly interact with heparan sulfate proteoglycans (24). To clarify the functions of the G domains of the laminin α3 chain, we have prepared recombinant laminin-5 proteins serially lacking G domains of the α3

*This work was supported by a Grant-in-aid from the Ministry of Education, Science, Sports and Culture of Japan. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.
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This paper is available on line at http://www.jbc.org

22495
Cloned into the XbaI site of mammalian expression plasmid vector pEF-BOS-CITE-NEO2, a modified version of pEF-BOS-CITE-NEO (27). Before cDNA transfection, HT1080 cells were cloned by the limiting dilution method. The cDNA expression plasmid vectors were transfected into a HT1080 cell clone by the calcium-phosphate precipitation method. Selection of stable transfectants and subsequent amplification of the introduced cDNAs were carried out with 500 μg/ml geneticin (Life Technologies, Inc.).

**Electrophoretic Analyses**—Northern blotting analysis of the laminin α3, β3, and γ2 chains were performed by the method described previously (26). SDS-polyacrylamide gel electrophoresis (PAGE) was performed on 6% gels under nonreducing or reducing conditions. Separated proteins were stained with Comassie Brilliant Blue R-250. For immuno-blotting, proteins separated by SDS-PAGE were transferred onto nitrocellulose membranes. The three subunits of laminin-5 were detected by the alkaline phosphatase method with chain-specific antibodies.

**Assay of Cell Adhesion**—Adhesion of BRL cells or C-41 cells to purified laminin-5 proteins and extracellular matrices deposited by various HT1080 transfectants was assayed as described previously (24). To prepare the matrices, each transfectant clone of HT1080 cells (5 × 10³ cells) was inoculated and incubated on 96-well plastic plates (Sumibec Medical, Tokyo, Japan) in DME/F12 medium containing 10% FCS at 37 °C for 4 days. Then the transfectant cells were detached from the plates with 0.05% trypsin and 0.5 mM EDTA and Mg²⁺-free phosphate-buffered saline. Complete detachment of cells was confirmed under a microscope. The plates were washed twice with phosphate-buffered saline and blocked with 200 μl of 1.2% (w/v) bovine serum albumin in phosphate-buffered saline at 37 °C for 1.5 h. These plates were used as the matrix-coated plates for the assay of cell adhesion. Cells adhered to the laminin-5 proteins or the HT1080 matrices were stained with Hoechst 33342 for 1.5 h, and the fluorescent intensity of each well was measured using a CytoFluor 2350 fluorometer (Millipore, Bedford, MA).

**Assay of Cell Migration and Cell Scattering**—Migration and scattering of BRL cells on purified laminin-5 proteins and extracellular matrices contributed by various HT1080 transfectants were assayed as reported previously (9). For the cell migration assay, the matrices of HT1080 transfectants were prepared in 25-cm² tissue culture flasks (Beckton Dickinson, Franklin Lakes, NJ) as described above. Cell migration on these substrates was monitored at 37 °C with time-lapse video equipment in which a video camera (MCK-385, Olympus, Tokyo) was mounted on an inverted microscope (IX-70; Olympus) and connected to a time-lapse control unit (LVR-3000AN, Sony, Tokyo, Japan). The length of cell migration was measured with a video micrometer (VM-30; Olympus).

Purification of Recombinant Laminin-5—Four transfectants of HT1080 cells were cultured in confluence in roller bottles (Beckton Dickinson) with DME/F12 medium containing 10% FCS (200 ml/bottle). The confluent cultures were washed twice with and incubated in serum-free DME/F12 medium. The serum-free conditioned medium was harvested every 2 days, clarified by centrifugation, and subjected to protein precipitation with 80%-saturated ammonium sulfate. The precipitated proteins were dissolved in and dialyzed against 20 mM Tris- and Mg²⁺-free buffered saline and blocked with 0.1% (v/v) Brij35 and 0.1% (v/v) CHAPS and then applied to molecular sieve chromatography on a Sepharose 4B (Amersham Pharmacia Biotech). Fractions containing laminin-5 proteins were pooled and subjected to immunofluorescence and motility assays (26). Bound proteins were eluted from the affinity column with 0.05% (v/v) trifluoroacetic acid and immediately neutralized with 1/3 volume of 1.0 M Tris-HCl (pH 7.5). The eluted laminin-5 fractions were further purified by immunofluorescence chromatography with the anti-laminin-γ2 monoclonal antibody (D4B5). The recombinant laminin-5 proteins thus purified were stored at 4 °C in the presence of 0.005% Brij35 and 0.1% CHAPS.

**RESULTS**

**Expression of Full-length and Carboxyl-terminally Deleted Derivatives of Laminin α3 Chain in HT1080 Cells**—To assess the contribution of the five G domains of the laminin α3 chain to cell adhesion and cell motility activities of laminin-5, we tried to prepare recombinant laminin-5 variants serially lacking G domains. Human fibrosarcoma cell line HT1080, which expresses the laminin β3 and γ2 chains but not the α3 chain (26), was chosen as the recipient cells to transfect the α3 chain cDNAs.
Expression vectors containing the cDNAs for wild type (WT) and the following five deletion derivatives of human laminin α3 chain were constructed: the α3 chains without G5 (ΔG5); without G4 and G5 (ΔG4–5); without G3, G4, and G5 (ΔG3–5); without G2, G3, G4, and G5 (ΔG2–5); and without any G domains (ΔG1–5) (Fig. 1). These expression vectors and the control vector without any α3 chain cDNA were transfected into HT1080 cells, and stable transfectant clones were isolated.

When cell morphology and growth rate were compared among the HT1080 transfectants, the difference is not evident between the control and cDNA transfectants under usual serum-containing culture conditions. However, when incubated in serum-free culture medium, HT1080 transfectants of WT, ΔG5, and ΔG3–5 survived much longer than the other cDNA transfectants and the control transfectant (data not shown). This suggested that the laminin-5 forms with G1–3 might prevent HT1080 cells from apoptosis in serum-free medium.

Next, we examined gene expression of the three laminin-5 subunits in the control and cDNA transfectants of HT1080 cells by Northern blotting. As expected, all of the five transfectants of the α3 chain cDNAs expressed the α3 chain mRNA with different sizes at comparable levels, whereas the control transfectant expressed no positive signal (Fig. 2A). In contrast, the laminin β3 and γ2 chain mRNAs were expressed in the control transfectant as well as the cDNA transfectants (Fig. 2, B and C). The transfection of the laminin α3 chain cDNAs did not affect the transcriptional levels of the β3 and γ2 chain genes in any clones.

**Fig. 1. Schematic diagram of cDNAs for full-length laminin α3EpA (α3) chain and its carboxyl-terminally deleted derivatives.** The full-length cDNA (WT) and partially deleted cDNAs (ΔG0, ΔG1–3, ΔG2–5, ΔG3–5, and ΔG4–5) are shown by closed bars. The scale in base pairs (bp) is shown on the top. The domain structures of laminin α3 chain (open column) are indicated by IIIa, I/II, and G1 to G5. c, conserved cysteine residues in I/II domain. The shaded portion indicates the signal peptide. Numbers in parentheses indicate the length of the cDNAs in base pairs.

**Fig. 2. Expression of three laminin-5 subunits in control and laminin α3 chain cDNA transfectants of HT1080 cells.** Total cellular RNAs were isolated from the indicated transfectants of HT1080 cells and subjected to Northern blotting for laminin α3 chain (LNα3A), β3 chain (LNβ3), γ2 chain (LNγ2), and glyceraldehyde-3-phosphate dehydrogenase (G3PDH). The RNA from the gastric adenocarcinoma cell line STKM-1, which secretes a high level of laminin-5, was included as a positive control. Different sizes of the α3 chain mRNAs are detectable in the cDNA transfectants but not in the control transfectant (Control), which was transfected with the control vector without laminin α3 cDNA. The laminin α3 cDNA transfectants of WT and ΔGs are shown in Fig. 1. Experimental conditions are described in the text.

Considered that the G domains of the α3 chain are required for the assembly and subsequent secretion of the heterotrimer α3β3γ2.

As described above, the HT1080 transfectants of WT, ΔG5, and ΔG3–5 secreted apparently the same size (160 kDa) of the α3 chain. It is known that the mature α3 chain of 190 kDa is proteolytically processed to the 160-kDa form (28). When the conditioned media of the control HT1080, HT1080/WT, and HT1080/ΔG5 were analyzed by Western blotting with a rabbit polyclonal antibody against a recombinant G4 protein, the 190-
the mature laminin bodies against laminin onto nitrocellulose membranes, and then probed with monoclonal anti-secreted from control and cDNA transfectants of HT1080 cells.

The secretion of the laminin 22498 kDa). Other experimental conditions are described in the text.

Western blotting analysis with the anti-laminin 2 chain antibody detected almost a single band of the 135-kDa mature 3 chain (105 kDa). Therefore, we judged the 160-kDa band as the 3 chain.

Other experimental conditions are described in the text.

kDa band in HT1080/WT and the 175-kDa band in HT1080/ΔG were clearly detected, whereas the 160-kDa band in both transfectants was scarcely detected by the antibody (Fig. 4). Therefore, we judged the 160-kDa band as the 3 chain that had been proteolytically cleaved between the G3 and G4 domains.

Western blotting analysis with the anti-β3 chain antibody detected almost a single band of the 135-kDa mature β3 chain in the conditioned media of all transfectants (Fig. 3B). On the other hand, analysis with the anti-γ2 chain antibody detected the mature γ2 chain of 150 kDa and its proteolytically processed form of 105 kDa at relatively irregular intensity (Fig. 3C). It is noteworthy that the control transfectant secreted significant amounts of the β3 and γ2 chains. This indicates that the secretion of the laminin β3 and γ2 chains does not depend on the co-expression of the α3 chain.

To confirm the secretion of laminin-5, immunoprecipitation with the anti-laminin γ2 chain monoclonal antibody was carried out with the conditioned media of some HT1080 transfectants. The anti-γ2 chain antibody precipitated both the laminin 2 chain (105 kDa) and the processed laminin 2 chain (105 kDa). Other experimental conditions are described in the text.

The conditioned media of HT1080/WT and HT1080/ΔG5, as compared with their processed forms (data not shown).

Cell Adhesion Activity of Matrices Deposited by HT1080 Transfectants—To examine the cell adhesion activity of the laminin-5 deletion forms, the matrices deposited by HT1080 transfectants were tested with Buffalo rat liver cell line BRL, which has been used for the assays of cell scattering activity and cell adhesion activity of laminin-5 (8, 9). This cell line adheres not only to laminin-5 but also to fibronectin. When the relative amounts of the laminin α3 chain proteins deposited by HT1080 transfectants were determined using the anti-laminin-α3 antibody, all of the matrices except for the control and ΔG1–5 contained the laminin α3 chains at similar levels (data not shown). When the cell adhesion activity was assayed at 20 min after seeding, BRL cells attached and spread on the matrices of the other transfectants including WT, ΔG5, and HT1080/ΔG4–5 but not on the matrices of the other transfectants (Fig. 5, closed columns). When assayed at 1 h after seeding, all of the matrices supported the adhesion of BRL cells at almost the same activity (Fig. 5, open columns). However, there was a morphological difference of BRL cells between the matrices from the transfectants of laminin α3 cDNAs with and without the G3 domain. BRL cells spread more on the matrices of WT, ΔG5, and ΔG4–5 than on those of the others (data not shown). It has previously been reported that the RGD peptide inhibits adhesion of BRL cells to fibronectin by about half but does not inhibit that to laminin-5 at all (9). When BRL cells were treated with a RGD-containing peptide (GRGDSP) or a control peptide (GRGESP), the cell adhesion to the ΔG4–5 matrix but not that to the ΔG4–5 matrix, was effectively inhibited by the GRGDSP peptide (Fig. 6). This suggests that BRL cells preferentially attach to and spread on laminin-5 in the matrices of ΔG4–5, ΔG5, and WT, whereas they slowly attach to fibronectin or other cell adhesion proteins in the matrices of the other transfectants including ΔG5. This possibility was confirmed using the adhesion assay with human cervix epidermoid carcinoma cell line C-4I, which is able to adhere to laminin-5 but not fibronectin, vitronectin,
or laminin-1. When C-4I cells were seeded on the matrices of HT1080 transfectants, they effectively attached to and spread only on the matrices of WT, ΔG5, and ΔG4–5, even at 1.5 h after seeding (Fig. 7). When effect of function-blocking anti-integrin antibodies was examined, antibodies to integrins α3 and β1 strongly inhibited the cell adhesion to the ΔG4–5 matrix and to purified laminin-5, indicating that C-4I cells had adhered to the laminin-5 deposited on the matrix through integrin α3β1 (Table I). The anti-α3-integrin antibody weakly inhibited both cell adhesion to the ΔG4–5 matrix and to the purified laminin-5, but antibodies to integrins α2 and α5 rather stimulated the cell adhesion. All these results strongly suggested that the G3 domain of the laminin α3 chain is essential for the potent cell adhesion activity of laminin-5.

Cell Migration Activity of Matrices Deposited by HT1080 Transfectants—Laminin-5 has potent cell scattering- and cell migration-stimulating activities toward BRL cells (8, 9). The cell scattering activity of the matrices deposited by each HT1080 transfectant was analyzed using BRL cells. BRL cells showed marked cell scattering on the matrices of WT, ΔG5, and ΔG4–5 but not on the others (data not shown).

To compare the cell migration activity of laminin-5 deletion forms, BRL cells were incubated on the matrix deposited by each HT1080 transfectant. The cell migration on the matrix was monitored for 12 h using a time-lapse video recorder (Fig. 8). The migration speed was about 5–10 times higher on the matrix of WT than on the control matrix, suggesting that laminin-5 was responsible for the high motility of BRL cells. Similar elevated cell motility was observed on the matrices of ΔG5 and ΔG4–5, whereas this activity was remarkably decreased by losing the G3 domain. The matrix of ΔG4–5 slightly stimulated the migration of BRL cells as compared with those of the control transfectants, ΔG5–6 and ΔG1–5. These results indicate that the G3 domain of the laminin α3 chain is indispensable for the strong cell motility activity of laminin-5 and that the G2 domain also has a low cell motility activity.

Purification of Recombinant Laminin-5 Deletion Forms and Their Subunit Composition—HT1080 cells are expected to secrete some cell adhesion proteins such as fibronectin and collagens. To rule out the effects of these intrinsic matrix proteins, we purified recombinant laminin-5 variants and investigated their biological activities. Four types of laminin-5, WT, ΔG5, ΔG3–5, and ΔG2–5, were prepared from the conditioned media of

\[ \text{Relative number of adherent cells (%) } \]

| Antibodies | Relative number of adherent cells (%) |
|------------|---------------------------------------|
| PBS        | 100                                   |
| Control    | 99 ± 11                               |
| IgG        | 131 ± 17                              |
| Anti-α3    | 25 ± 3.4                              |
| Anti-α5    | 130 ± 16                              |
| Anti-α6    | 86 ± 11                               |
| Anti-β1    | 36 ± 4.6                               |

\[ \text{Effect of function-blocking antibodies specific to various integrin subunits on adhesion of C-4I cells to matrix of HT1080/ΔG4–5 and to laminin-5} \]

\[ \text{Adhesion of BRL cells to extracellular matrices deposited by control and cDNA transfectants of HT1080 cells. BRL cells were plated and incubated on the matrices of HT1080 transfectants in serum-free medium for 90 min, and the relative numbers of adherent cells were determined by measuring fluorescent intensity. Each point represents the mean ± S.D. for triplicate cultures. Other experimental conditions are described in the text.} \]

\[ \text{TABLE I} \]

\[ \text{Carboxyl-terminal G Domains of Laminin α3 Chain} \]

or laminin-1.² When C-4I cells were seeded on the matrices of HT1080 transfectants, they effectively attached to and spread on only the matrices of WT, ΔG5, and ΔG4–5, even at 1.5 h after seeding (Fig. 7). When effect of function-blocking anti-integrin antibodies was examined, antibodies to integrins α3 and β1 strongly inhibited the cell adhesion to the ΔG4–5 matrix and to purified laminin-5, indicating that C-4I cells had adhered to the laminin-5 deposited on the matrix through integrin α3β1 (Table I). The anti-α3-integrin antibody weakly inhibited both cell adhesion to the ΔG4–5 matrix and to the purified laminin-5, but antibodies to integrins α2 and α5 rather stimulated the cell adhesion. All these results strongly suggested that the G3 domain of the laminin α3 chain is essential for the potent cell adhesion activity of laminin-5.

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² Y. Kikkawa and K. Miyazaki, unpublished data.
the respective cDNA transfectants. Each recombinant laminin-5 was separated by molecular sieve chromatography followed by immunoadfinity chromatography using the anti-laminin-α3 monoclonal antibody LSA3c4. The laminin-5 preparations slightly contained the laminin β1 chain (220 kDa) and the laminin γ1 chain (210 kDa), besides the laminin-5 subunits, suggesting that they contained laminin-6. Therefore, these preparations were finally applied to an anti-laminin-γ2 antibody (DA85) column to remove laminin-6. The total amount of laminin-5 and laminin-6 in conditioned medium was estimated to be about 100 μg/liter in WT, 60–80 μg/liter in ΔG5 and ΔG3–5, and less than 50 μg/liter in ΔG2–5. The recovery of laminin-5 ranged between 25 and 50% in the final laminin-5 preparations. The purified materials contained the laminin α3 chains (160–120 kDa), the β3 chain (135 kDa), the γ2 chain (150 kDa), and the proteolytically processed laminin γ2 chain (105 kDa) (Fig. 9, left). The approximate size of the laminin α3 chain was 160 kDa in WT and ΔG5, 140 kDa in ΔG3–5, and 120 kDa in ΔG2–5 (Fig. 9, right). The laminin-5 forms of WT and ΔG5 were considered to be essentially identical to the laminin-5 form of ΔG2–5 (see Figs. 3A and 4). When the laminin-5 of WT was analyzed by Western blotting with the anti-G4 antibody, no immunoreactive band was detected at a low molecular weight region, indicating that the cleaved fragment of G4–5 was not associated with the purified laminin-5 (data not shown).

The fractions eluted from the anti-laminin-α3 antibody column slightly contained the non-processed α3 chains (190 kDa in WT and 175 kDa in ΔG5) and the laminin β1 and γ1 chains, but these proteins passed through the anti-laminin-γ2 antibody column. This suggested that the non-processed α3 chains belonged to laminin-6.

**Biological Activity of Purified Recombinant Laminin-5 Deletion Forms**—The purified recombinant laminin-5 deletion forms were assayed for cell adhesion activity using BRL cells as the indicator. When each recombinant laminin-5 preparation was precoated on plastic plates at different concentrations, WT and ΔG5 promoted the cell adhesion in a concentration-dependent manner, but neither ΔG3–5 nor ΔG2–5 supported the cell adhesion even at the maximum concentration tested (Fig. 10). This confirmed that the G3 domain is essential for the potent cell adhesion activity of laminin-5. As expected from the above SDS-PAGE analysis, WT and ΔG5 showed essentially the same activity.

The purified recombinant laminin-5 forms were also assayed for cell-scattering activity using BRL cells (Fig. 11). When BRL cells were incubated with each recombinant protein in 1% FCS-containing medium for 2 days, typical cell scattering was observed with WT and ΔG5. ΔG2–5 induced weak cell scattering of BRL cells as compared with the negative control, but ΔG2–5 did not at all. These results were very consistent with the previous experiments with matrices deposited by HT1080 transfectants (Fig. 8). It is clear that the G3 domain of the laminin α3 chain is indispensable for the potent cell motility activity of laminin-5, as well as its cell adhesion activity.

**DISCUSSION**

The present study demonstrated that the laminin-5 lacking both the G4 and G5 domains in the α3 chain had potent activity to promote adhesion and migration of BRL cells, but deletion of the G3 domain caused marked decrease of the biological activity of laminin-5. This implies that the G3 domain plays an indispensable role in the expression of biological activity of laminin-5. HT1080 cells expressing laminin-5 forms with the G3 domain were more resistant to apoptosis in serum-free culture medium than those without the G3 domain. This is...
consistent with the previous report that laminin-5-deficient keratinocytes exhibit reduced survival as compared with normal cells (14).

Laminin-5 has been reported to have several integrin-binding sites in the G domains of the laminin α1 chain. Two or more integrin recognition sequences, which are adjacent or located in distinct G domains, are likely to cooperate in ligand binding (20, 29). However, our previous study with four recombinant G domains of the laminin α1 chain showed that only the G2 domain contains an integrin αβ1β2 binding activity, although the activity of the G3 domain was not examined (24). The recombinant G2 protein and the integrin αβ1β2-binding peptide (αG2A) have a very low cell adhesion activity compared with intact laminin-5 (24). In addition, the G2 domain and intact laminin-5 lose their cell adhesion activity by heating (8, 24). These imply that a specific conformation of the G2 domain produced by the interaction with the G1, G3, and some other parts of the α3, β3, and γ2 chains might be important for the high affinity binding of laminin-5 to integrins. Correspondingly, Talts et al. (19, 23) have recently shown that recombinant G1–3 protein of laminin α2 chain has cell adhesion activity similar to that of native laminin-2/4, although each of G1, G2, and G3 recombinant proteins does not. However, any natural or recombinant laminin-2/4 forms without G4 and G5 domains has not been reported.

Laminin-5 is synthesized initially as a high molecular weight precursor protein that undergoes specific processing to smaller forms after being secreted (28, 30, 31). The size reduction is a result of proteolytic processing of the α3 and γ2 subunits from 190 to 160 and from 150 to 105 kDa, respectively (8, 28, 31). In this study, we first demonstrated that the laminin α3 chain was proteolytically cleaved between the G3 and G4 domains in HT1080/WT cell line, producing the 160-kDa α3 chain. Both the naturally processed laminin-5 with the 160-kDa α3 chain and the recombinant laminin-5 lacking G4 and G5 (ΔG4,5) showed high cell adhesion and cell motility activities. This clearly indicates that the G4 and G5 domains are not essential for the stimulation of cell adhesion and motility. We have recently found that the G4-G5 fragment of laminin α3 chain is secreted from some human carcinoma cell lines. The G4-G5 fragment appeared not to be associated with laminin-5 after the proteolytic cleavage. It has been reported that recombinant G4 and G5 domains bind to heparan sulfate proteoglycans as major cell surface receptors, and the G5 recombinant protein has some activity to stimulate cell migration (24). Therefore, it is conceivable that the secreted G4-G5 fragment acts on cells in cooperation with or independently of the laminin-5 with the 160-kDa α3 chain.

Goldfinger et al. (32) have compared biological activities of extracellular matrices containing different forms of laminin-5 and found that the laminin-5 with the unprocessed, 190-kDa α3 chain has high cell motility activity, whereas one with the processed, 160-kDa α3 chain supports stable cell adhesion. However, we have observed the high motility activity of the laminin-5 with the 160-kDa α3 chain toward BRL cells and many human carcinoma cell lines (8). It is evident that the laminin-5 with the 160-kDa α3 chain exhibits potent cell motility activity toward some cell types. It has also been reported that the processing of the laminin γ2 chain by the matrix metalloproteinases gelatinase A and MT1-MMP regulates the cell motility activity of laminin-5 (33, 34). For clarifying the structure-function relationship of laminin-5, it seems essential to isolate the laminin-5 forms with processed and unprocessed α3 and γ2 chains. In this respect, it should be noted that HT1080/WT cells secreted both the 160- and 190-kDa α3 chains, but only the 160-kDa α3 chain was purified as a laminin-5 complex. Our recent attempt to isolate the 190-kDa α3 chain has shown that this α3 chain exists as a laminin-6 but not laminin-5 form. This suggests that the proteolytic processing of the laminin α3 chain occurs specifically in laminin-5.

The present study showed that the control HT1080 cells, which do not express the α3 chain, secreted the laminin β3 and γ2 chains into culture medium. Various laminin subunits are assembled in the rough endoplasmic reticulum. Several groups have proposed the mechanism for the assembly of laminin subunits. In laminin-1 and laminin-5, a disulfide-linked βγ heterodimer is formed as a presumed intermediate, and α chain is added at a subsequent stage (35–37). In laminin-1, the α1 chain can be secreted as a single subunit, whereas the β1 and γ1 chains cannot (37). When the β1 and γ1 chains are overexpressed separately or together, they remain intracellular as the disulfide-linked dimer of β1γ1 or β1β1. We recently found that the laminin γ2 chain is solely overexpressed at the invasion front of gastric carcinomas, and the γ2 chain monomer is se-

3 Y. Tsubota, H. Mizushima, T. Hirosaki, S. Higashi, H. Yasumitsu, and K. Miyazaki, unpublished data.

4 T. Hirosaki, H. Mizushima, K. Moriyama, and K. Miyazaki, unpublished data.
creted from gastric carcinoma cells in vitro (25). These results clearly indicate that the laminin-5 subunits are secreted differently from the laminin-1 subunits. Furthermore, the present study indicated that HT1080/G3, which lacked all G domains, secreted only a trace amount of the laminin α3 chain into culture medium and contained little α3 protein in the cytoplasm despite the high expression of its mRNA. Therefore, the G domains appear to be essential for the formation of stable heterotrimer of laminin-5. The failure of subunit assembly may cause the prompt degradation of the α3 chain inside the cells.

In conclusion, we first demonstrated that the G3 domain, but not G4 and G5 domains, of the laminin α3 chain is required for the potent activity of laminin-5 to promote cell adhesion and migration. The physiological meaning of the proteolytic cleavage and the biological activity of the G4-G5 fragment are currently under investigation.

Acknowledgments—We thank to Drs. H. Yasumitsu and S. Higashi for helpful discussion.

REFERENCES

1. Timpl, R. (1996) Curr. Opin. Cell Biol. 8, 618–624
2. Aumailley, M., and Rousselle, P. (1999) Matrix Biol. 18, 19–28
3. Miner, J. H., Patton, B. L., Lenta, S. I., Gilbert, D. J., Snider, W. D., Jenkins, N. A., Copeland, N. G., and Sanes, J. R. (1997) J. Cell Biol. 137, 685–701
4. Koch, M., Olson, P. F., Albus, A., Jin, W., Hunter, D. D., Brunken, W. J., Burgeson, R. E., and Champiaud, M. F. (1999) J. Cell Biol. 145, 605–618
5. Carter, W. G., Ryan, M. C., and Gahr, P. J. (1991) Cell 65, 599–610
6. Rousselle, P., Lunstrum, G. P., Keene, D. R., and Burgeson, R. E. (1991) J. Cell Biol. 114, 567–576
7. Verrando, J., Verrando, P., Pitas, A., and Ortonne, J.-P. (1988) Biochim. Biophys. Acta 942, 45–56
8. Miyazaki, K., Kikkawa, Y., Nakamura, A., Yasumitsu, H., and Umeda, M. (1993) Proc. Natl. Acad. Sci. U. S. A. 90, 11767–11771
9. Kikkawa, Y., Umeda, M., and Miyazaki, K. (1994) J. Biochem. (Tokyo) 116, 862–869
10. Rousselle, P., and Aumailley, M. (1994) J. Cell Biol. 125, 205–214
11. Mizushima, H., Koshikawa, N., Motiyama, K., Takamura, H., Nagashima, Y., Hirahara, F., and Miyazaki, K. (1998) Hormone Res. 50, Suppl. 2, 7–14
12. Aberdam, D., Galliano, M.-F., Vailly, J., Pulkkinen, L., Bonfias, J., Christiano, A. M., Tryggvason, K., Uitto, J., Epstein Jr., E. H., Ortonne, J.-P., and Meneguzzi, G. (1994) Nat. Genet. 6, 299–304
13. Pulkkinen, L., Christiano, A. M., Gerecke, D., Burgeson, R. E., Pittelkow, M. R., and Uitto, J. (1994) Genomics 24, 357–360
14. Ryan, M. C., Lee, K., Miyashita, Y., and Carter, W. G. (1999) J. Cell Biol. 145, 1309–1323
15. Dowling, J., Yu, Q. C., and Fuchs, E. (1996) J. Cell Biol. 134, 559–572
16. Georges-Labouesse, E., Messaddeq, N., Yehia, G., Cadalbert, L., Dierich, A., and Le Meur, M. (1996) Nat. Genet. 13, 370–373
17. Ryan, M. C., Tizard, R., VanDevanter, D. R., and Carter, W. G. (1994) J. Biol. Chem. 269, 22779–22787
18. Pyke, C., Salo, S., Rainkki, E., Remer, J., Dan, K., and Tryggvason, K. (1995) Cancer Res. 55, 4132–4139
19. Talta, J. F., and Timpl, R. (1998) FEBS Lett. 426, 71–76
20. Nomizu, M., Kim, W. H., Yamamura, K., Utani, A., Song, S. Y., Otaka, A., Roller, P. P., Kleinman, H. K., Yamada, Y. (1995) J. Cell Biol. 270, 20583–20590
21. Sung, U., O’Rear, J. J., and Yurchenco, P. D. (1993) J. Cell Biol. 123, 1255–1268
22. Gee, S. H., Blacher, R. W., Deeville, P. J., Provost, P. R., Yurchenco, P. D., and Carbonetto, S. (1993) J. Biol. Chem. 268, 14972–14980
23. Talta, J. F., and Timpl, R. (1999) FEBS Lett. 458, 319–323
24. Mizushima, H., Takamura, H., Miyagi, Y., Kikkawa, Y., Yamamura, N. Y., Yasumitsu, H., Misugi, K., and Miyazaki, K. (1997) Cell Growth & Differ. 8, 979–987
25. Koshikawa, N., Motiyama, K., Takamura, H., Mizushima, H., Nagashima, Y., Yanoma, S., and Miyazaki, K. (1999) Cancer Res. 59, 5596–5601
26. Mizushima, H., Miyagi, Y., Kikkawa, Y., Yamamura, N., Yasumitsu, H., Misugi, K., and Miyazaki, K. (1996) J. Biochem. (Tokyo) 120, 1196–1202
27. Miyata, S., Miyagi, Y., Koshikawa, N., Nagashima, Y., Kato, Y., Yasumitsu, H., Hirahara, F., Misugi, K., and Miyazaki, K. (1998) Clin. Exp. Metastasis 16, 613–622
28. Marinkovich, M. P., Lonstrum, G. P., Keene, D. R., and Burgeson, R. E. (1992) J. Cell Biol. 119, 695–703
29. Pattaramalai, S., Skubitz, K. M., and Skubitz, A. P. (1996) Exp. Cell Res. 232, 281–290
30. Matsui, C., Wang, C. K., Nelson, C. F., Bauer, E. A., and Hoeffer, W. K. (1995) J. Biol. Chem. 270, 23496–23503
31. Vailly, J., Verrando, P., Champiaud, M. F., Gerecke, D., Wagman, D. W., Baudoin, C., Aberdam, D., Burgeson, R., Bauer, E., and Ortonne, J. P. (1994) Eur. J. Biochem. 219, 209–218
32. Goldfinger, L. E., Stack, M. S., and Jones, J. C. (1998) J. Cell Biol. 141, 255–265
33. Giannelli, G., Falk-Marzillier, J., Schiraldi, O., Stetler-Stevenson, W. G., and Quarranta, V. (1997) Science 277, 225–228
34. Koshikawa, N., Giannelli, G., Cirulli, V., Miyazi, K., and Quarranta, V. (2000) J. Cell Biol. 148, 615–624
35. Peters, B. P., Hartle, R. J., Krzesicki, R. F., Kroll, T. G., Perini, F., Balun, J. E., Yanoma, S., and Miyazaki, K. (1999) Arch. Biochem. Biophys. 367, 4132–4139
36. Tokida, Y., Aratani, Y., Morita, A., and Kitagawa, Y. (1990) J. Biol. Chem. 265, 18125–18129
37. Yurchenco, P. D., Quan, Y., Colonnato, H., Mathus, T., Harrison, D., Yamada, Y., and O’Rear, J. J. (1997) Proc. Natl. Acad. Sci. U. S. A. 94, 10189–10194