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Differential Regulation of Cellular Adhesion and Migration by Recombinant Laminin-5 Forms With Partial Deletion or Mutation Within the G3 Domain of α3 Chain

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Abstract The basement membrane protein laminin-5 promotes cell adhesion and migration. The carboxyl-terminal G3 domain in the α3 chain is essential for the unique activity of laminin-5. To investigate the function of the G3 domain, we prepared various recombinant laminin-5 forms with a partially deleted or mutated G3 domain. The deletion of the carboxyl-terminal 28 amino acids (region III) markedly decreased the cell adhesion activity with a slight loss of the cell motility activity toward BRL and EJ-1 cells. This change was attributed to the loss of Lys-Arg-Asp sequence. Further deletion of 83 amino acids (region II) led to almost complete loss of the cell motility activity. All charged amino acid residues tested in this region were not responsible for the activity loss. These results suggest that the G3 domain contains two distinct regions that differently regulate cell adhesion and migration. Analysis of laminin-5 receptors showed that integrins α3β1, α6β1, and α6β4 had different but synergistic effects on cell adhesion and migration on laminin-5. However, the structural change of the G3 domain appeared not to change integrin specificity. The present study demonstrates that the G3 domain in laminin-5 plays a central role to produce different biological effects on cells. J. Cell. Biochem. 88: 506–520, 2003.
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Key words: cell adhesion; cell migration; G3 domain; integrin; laminin-5

Extracellular matrix proteins differently regulate cellular functions such as adhesion, motility, growth, apoptosis, and differentiation by interacting with specific cell surface receptors, most importantly integrins. Recently much progress has been attained in the analysis of integrin-derived signal transduction pathway [Howe et al., 1998; Giancotti and Ruoslahti, 1999]. However, the mechanism by which different ligand–integrin interactions produce different biological effects has been poorly understood.

Laminins are a family of extracellular matrix proteins that are localized mainly in basement membranes of various tissues and play essential roles in the maintenance of tissue architecture and the control of cellular functions [Aumailley and Rousselle, 1999; Colognato and Yurchenco, 2000]. Each laminin molecule is composed of three different subunits, α, β, and γ chains, and the heterotrimer linked by disulfide bonds forms the well-known cross-shape structure. To date, primary structures of five α, three β, and three γ chains have been determined, and at least 15 laminin isoforms with different

Abbreviations used: LN5, laminin-5; CM, conditioned medium; WT, wild type; PBS, phosphate-buffered saline; FN, fibronectin.

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combinations of the three chains have been reported [Colognato and Yurchenco, 2000]. All laminin α chains contain a large globular (G) domain at their carboxyl (C)-terminal region, which consists of five homologous, tandem aligned small G subdomains (G1-5), often called laminin G-like modules (LG1-5) [Timpl et al., 2000]. Recently, the crystal structures of LG5 and LG4-5 of the laminin α2 chain were reported, showing that each module has a compact β sandwich structure formed by two antiparallel seven-stranded β-sheets [Hohenester et al., 1999; Tisi et al., 2000]. Similar module structures have been shown in neurexin [Rudenko et al., 1999] and sex hormone-binding globulin [Grishkovskaya et al., 2000]. Many extracellular or transmembrane proteins are predicted to have similar LG modules [Timpl et al., 2000]. The C-terminal globular region of laminins contains binding sites for integrins and many carbohydrate ligands including heparin, α-dystroglycan, and syndecans [Nomizu et al., 1995; Aumailley and Krieg, 1996; Talts et al., 1999a; Nielsen et al., 2000].

One of the laminin isoforms, laminin-5 (LN5), which consists of α3, β3, and γ2 chains, has a unique characteristic. It promotes adhesion, migration, and scattering of various types of cultured cells much more strongly than laminin-1, laminin-2/4, fibronectin, and vitronectin [Miyazaki et al., 1993; Kikkawa et al., 1994; Rousselle and Aumailley, 1994]. These activities of LN5 are mediated mainly by three integrin receptors: α3β1, α6β1, and α6β4 [Carter et al., 1991; Kikkawa et al., 1994; Niessen et al., 1994; Rousselle and Aumailley, 1994]. In vivo LN5 is a major substrate for the adhesion of many types of epithelial cells. In the skin, the stable binding of the epidermis to the dermis is maintained by the interaction of LN5 with integrin α6β4 in the hemidesmosome structure of basal keratinocytes [Nievers et al., 1999]. On the other hand, the cell migration-promoting activity of LN5 is thought to contribute to wound repair [Ryan et al., 1994] and tumor invasion [Pyke et al., 1995]. However, it is unknown how LN5 exerts such potent cell adhesion and motility activities. It also remains unclear whether or not the LN5 and receptor interaction for cell adhesion is identical to that for cell migration. For answering these questions, it seems important to analyze quantitatively the interactions between the LN5 variants and the receptors.

LN5 is produced initially as a 460-kDa complex, and immediately after secretion its α3 and γ2 chains in most part undergo specific proteolytic processing, producing a 400-kDa form of LN5. These proteolytic processings modulate the biological activity of LN5 [Giannelli et al., 1997; Goldfinger et al., 1998; Koshikawa et al., 2000]. Recently, we found that the α3 chain of 190 kDa is cleaved between the G3 and G4 domains, releasing the G4-5 fragment [Hirosaki et al., 2000; Tsubota et al., 2000]. The mature LN5 containing the processed 160 kDa α3 chain with the G1-3 domain strongly promotes cell adhesion and migration [Hirosaki et al., 2000], while the G4-5 fragment synergistically enhances the cell motility activity of the processed LN5 presumably by interacting with heparansulfate proteoglycans on cell surface [Tsubota et al., 2000]. However, further deletion of the G3 domain in a recombinant LN5 leads to marked loss of the high cell adhesion and motility activities, indicating an indispensable role of the G3 domain [Hirosaki et al., 2000]. A recent study has shown that a bacterially expressed recombinant G3 domain of rat laminin α3 chain supports cell adhesion and migration by binding integrin α3β1[Shang et al., 2001]. In order to clarify the role of the G3 domain in the expression of the characteristic biological activity of LN5, we have prepared various recombinant LN5 forms with partial deletion or mutation within the G3 domain and investigated the structure-function relationship in LN5.

MATERIALS AND METHODS

Antibodies

Mouse monoclonal antibodies against an amino-terminal region of human laminin α3 chain (amino acid residues 109-331) have been established in our laboratory with the support of the Eiken Chemical (Tokyo, Japan) [Hirosaki et al., 2000]. One of these antibodies, LSα3c6, was used for immunoblotting and immunoaffinity purification of recombinant LN5 proteins. The monoclonal antibody against human laminin γ2 chain (D4B5) was described previously [Mizushima et al., 1998; Koshikawa et al., 1999]. A monoclonal antibody against human laminin β3 chain, kalinin B1, was purchased from Transduction Laboratories (Lexington, KY). Function-blocking antibodies against integrins used are the anti-α2 integrin

antibody P1E6, the anti-α3 integrin antibody P1B5, and the anti-β1 integrin antibody P4C10 from Life Technologies Inc. (Gaithersburg, MD), the anti-α5 integrin antibody P1D6, and the anti-α6 integrin antibody GoH3 from PharMingen (San Diego, CA), and the anti-β4 integrin antibody ASC-3 from Chemicon (Temecula, CA).

Cells and Culture

Human fibrosarcoma cell line HT1080 and human bladder carcinoma cell line EJ-1 were obtained from Japanese Cancer Resources Bank (JCRB), Tokyo. Buffalo rat liver-derived epithelial cell line BRL has been used in previous studies [Miyazaki et al., 1993; Kikkawa et al., 1994]. Chinese hamster ovary CHO K1 cell lines which had been transfected with human integrin cDNAs were described before [Lampe et al., 1998]. Human leukemia cell line K562, which had been transfected with human integrin cDNAs, were reported previously [Zhang et al., 1999]. Integrin expression profiles of the transfectants were verified by flow cytometry prior to the assay. These cells were maintained in DMEM/F12 medium (Life Technologies Inc.) supplemented with 10% fetal calf serum (FCS) (Biowhittaker, Walkersville, MD), penicillin, and streptomycin sulfate.

Construction of Expression Plasmids

Human laminin α3 chain cDNA has been cloned from a cDNA library of a gastric cancer cell line [Mizushima et al., 1996]. The cDNA clone LS/CX was used as a template to construct vectors encoding various α3 chain mutants by PCR with TaKaRa Ex Taq (Takara Shuzo, Tokyo, Japan). PCR primers used are listed in Table I. Each reverse primer included a stop codon followed by the *Eco*R V site. PCR products were ligated into the T-Easy vector (Promega, Madison, WI) and verified by sequencing. The *Cla* I/*Sac* I fragments from T-Easy G3 #2, #3, Cys, D/R/R, and R/E/D were ligated into the *Cla* I/*Sac* I sites of LS/CX to make LS/CX G3 #2, #3, Cys, D/R/R, and R/E/D, respectively. To prepare LS/CX G3 #1, #2-2, and #2-3, the *Xho* I/*Eco*R V fragment from each T-Easy G3 clone was inserted into the corresponding restriction sites of LS/CX G3 #3. To prepare LS/CX D/R/D, the *Hind*III/*Eco*R V fragment from T-Easy D/R/D was inserted into the corresponding restriction sitesof LS/CX G3 #2. To prepare the expression vector, the *Xba* I/*Eco*R V insert of each mutant was subsequently cloned into the *Xba* I/*Sma* I site of the mammalian expression vector pEF-BOS-CITE NEO2, a modified version of pEF-BOS-CITE NEO [Miyata et al., 1998]. The

TABLE I. Primers Used for Construction of Expression Plasmids

G2-5′	5′-CAG GAT CCG TTC TGA GCT TGT AC-3′	(nucleotides 3445-3459, sense)
G3-5′	5′-CAG GAT CCA GTG GTG TCG TTA GA-3′	(nucleotides 2851-2865, sense)
G3 #1-3′	5′-GCG ATA TCA GCC GCT ATC GCT GGT-3′	(nucleotides 3625-3639, antisense)
G3 #2-3′	5′-GCG ATA TCA GTT ACT GGT CAA ATC-3′	(nucleotides 3874-3888, antisense)
G3 #3-3′	5′-GCG ATA TCA AAA CCT GGT AGA ACC-3′	(nucleotides 3958-3972, antisense)
G3 #4-3′	5′-GCG ATA TCA CTT GGT CTT GTT AAA CC-3′	(nucleotides 3968-3984, antisense)
G3 #2-2-3′	5′-GCG ATA TCA GTT GTC GCT TAT TAC AGA TAC-3′	(nucleotides 3691-3711, antisense)
G3 #2-3-3′	5′-GCG ATA TCA ACT TGA AAT GTG TTT TAG CC-3′	(nucleotides 3763-3780, antisense)
G3 Cys-2-5′	5′-GCC AGT TTA AAC AAA CCA CCT TTT C-3′	(nucleotides 3919-3943, sense)
G3 Cys-2-3′	5′-GCC TCC CAG GGA CAC ATC TCT CTT-3′	(nucleotides 3897-3918, antisense)
G3 Cys-1-5′	5′-GCT ATT AGC AAT GTT TTT GTC CAG-3′	(nucleotides 3823-3846, sense)
G3 Cys-1-3′	5′-ACC CTC AAA ATT GCT CCC GCC C-3′	(nucleotides 3801-3822, antisense)
KRD-5′	5′-GCA GCT GTG TCC CTG GGA GGC TGC-3′	(nucleotides 3898-3921, sense)
KRD-3′	5′-GGC GAG AGA GTT ACT GGT CAA ATC TAG G-3′	(nucleotides 3870-3897, antisense)
LNK-5′	5′-GCC GCA CCA CCT TTT CTA ATG TTG C-3′	(nucleotides 3928-3952, sense)
LNK-3′	5′-TGC ACT GCA GCC TCC CAG GGA CAC ATC-3′	(nucleotides 3901-3927, antisense)
KRLKH-5′	5′-GCA GCC ATT TCA AGT TCC CGG CAG TCT-3′	(nucleotides 3766-3792, sense)
KRLKH-3′	5′-TAG GGC TGC GCT ATT TCT CAG AAG CTG GTC-3′	(nucleotides 3736-3765, antisense)
D/R/D-5′	5′-TCT GGA CTA GCC CTT CTC ATC GCT GAC-3′	(nucleotides 3712-3738, sense)
D/R/D-3′	5′-GTT GGC GCT TAT TAC AGA TAC ATA ATG C-3′	(nucleotides 3684-3711, antisense)
D/R/R-5′	5′-AGG CTA AAA CAC ATT TCA AGT TTC GCC CAG-3′	(nucleotides 3760-3789, sense)
D/R/R-3′	5′-TTT GCT ATT TGC CAG AAG CTG GGC ATC-3′	(nucleotides 3733-3759, antisense)
R/E/D-5′	5′-GCA GTC CTA GCT TTG ACC AGT AAC TC-3′	(nucleotides 3865-3891, sense)
R/E/D-3′	5′-AGG ACT CAG TGA TAA GGC CTG GAC-3′	(nucleotides 3838-3864, antisense)

The underlines indicate the restriction site of *Eco*R V in primers 1–8 and the double underlines do the substituted nucleotides in primers 9–24. Primers 1 and 3 were used for G3 #1, primers 2 and 4 for G3 #2, primers 2 and 5 for G3 #3, primers 2 and 6 for G3 #4, primers 1 and 7 for G3 #2-2, and primers 1 and 8 for G3 #2-3. G3 #3 in the T-Easy vector was used as a template for the construction of a vector encoding G3 Cys, in which alanine was substituted stepwise for two cysteine residues. G3 #4 in the T-Easy vector was used as a template for the construction of vectors encoding various alanine substitutes.

expression vectors for the wild type and ΔG3-5 were described previously [Hirosaki et al., 2000].

Expression and Purification of Recombinant LN5 Mutant With Deletion Within G3 Domain

The three subunits of laminins are intracellularly assembled into the heterotrimer linked with disulfide bonds and then secreted from cells [Matsui et al., 1995]. In the present study, human fibrosarcoma cell line HT1080, which expresses the laminin β3 and γ2 chains but not the α3 chain [Mizushima et al., 1996], were stably transfected with expression vectors containing various mutants of human laminin α3 chain cDNA by the calcium-phosphate method and selected with 500 μg/ml G418 (Gibco). Secretion of recombinant LN5 proteins with mutations in the G3 domain of the α3 chain was analyzed by immunoblotting of serum-free conditioned medium (CM) with the monoclonal antibody against human laminin α3 chain. LN5 mutants secreted from HT1080 transfectants were purified by essentially the same method as reported previously [Hirosaki et al., 2000]. Serum-free CMs were collected in roller bottles, concentrated by ammonium sulfate, and then applied to molecular-sieve chromatography on a Sepharose 4B column (Amersham Pharmacia Biotech, Uppsala, Sweden). Fractions containing LN5 were pooled and applied to a gelatin-Sepharose 4B column to remove fibronectin. LN5 in the unbound fractions from the gelatin column was purified by immunoaffinity chromatography with the anti-laminin α3 monoclonal antibody. Bound proteins were eluted from the affinity column with 0.05% (v/v) trifluoroacetic acid and immediately neutralized with a small volume of 2 M Tris-HCl, pH 7.5. The recombinant LN5 proteins thus purified were stored at 4°C in the presence of 0.005% Brij35 and 0.1% CHAPS. Protein concentrations were determined using a Bio-Rad protein assay kit (Hercules, CA) with bovine serum albumin as a standard.

SDS–PAGE and Immunoblotting

SDS–PAGE was performed on 6% gels under reducing or non-reducing conditions. Separated proteins were stained with a Wako silver staining kit II (Wako, Osaka, Japan). For immunoblotting analysis, proteins resolved by SDS–PAGE were transferred onto nitrocellulose membranes and detected by the ECL detection reagents (Amersham Pharmacia Biotech).

Cell Adhesion Assay

Cell adhesion assay was performed as described previously [Mizushima et al., 1997]. Briefly, each well of 96-well ELISA plates (Costar, Cambridge, MA) was coated with a substrate protein and then blocked with bovine serum albumin. Cells (2×10^5) were placed into each well with serum-free DMEM/F12 medium, and incubated at 37°C for 1 h. After non-adherent cells had been removed, adherent cells were fixed and stained with Hoechst 33432. The fluorescent intensity of each well of the plates was measured with a CytoFluor 2350 fluorometer (Millipore, Bedford, MA). For inhibition assay, the cell suspension was incubated with function-blocking anti-integrin antibodies for 20 min at room temperature before inoculation.

Assays of Cell Scattering and Migration

Cell-scattering activity of recombinant LN5 proteins were assayed as reported previously [Tsubota et al., 2000]. BRL cells (7×10^3) were suspended in DMEM/F12 plus 1% FCS and inoculated into each well of 24-well plates. Each sample was directly added into the cultures and incubated at 37°C for about 40 h. After cell staining with Giemsa, total cells and scattered, single cells were counted in three randomly selected microscope fields. Typically, the number of total cells ranged from 500 to 700. The degree of cell scattering was expressed by the percentage of single cells to the total cells. In one experiment, test proteins were coated on culture plates before inoculation of cells.

Cell migration was assayed as reported before [Hirosaki et al., 2000]. EJ-1 cells (1.0×10^4 cells in DMEM/F12 plus 1% FCS) were inoculated into each well of 24-well plates pre-coated with test proteins. After pre-incubation for 1 h at 37°C, cell movement was monitored for 10 h with a time-lapse video camera, and migration distance was determined by measuring the total length of the random path that each cell covers.

RESULTS

Expression and Purification of Recombinant LN5 Mutants

To determine the functional sites for the cell adhesion and motility activities within the G3 domain of laminin α3 chain, we first prepared

recombinant LN5 variants which were deleted inside the G3 domain. To design the G3 deletion mutants, we tentatively divided the G3 domain into four regions (I to IV), taking note of the presence of an *N*-linked carbohydrate motif (Asn-Xaa-(Ser/Thr)) and two cysteine residues which were predicted to form a disulfide bridge (Fig. 1A). Region I (amino acid residues 1149-1213) covers the N-terminal, about one-third sequence of the G3 domain. Regions II (amino acid residues 1214-1296) and III (amino acid residues 1297-1324) contain the first and second cysteine residues, respectively, and region IV (amino acid residues 1325-1337) covers the sequence between the carbohydrate motif and the proteolytic cleavage site. We also noticed the possible β-strand structures of the G3 domain, which were predicted from those of the G5 domain of laminin α2 chain reported by Hohenester et al. [1999]. The C-terminal end of each deletion mutant was placed at a linker sequence between two possible β-strands (Fig. 1B). Expression vectors containing the cDNAs for the full-length (LS/CX WT: nucleotides -105139) and the following three deletion mutants of human laminin α3 chain were constructed: LS/CX #1 (nucleotides -103639; amino acid residues 1-1213) containing region I; LS/CX #2 (nucleotides -103888; amino acid residues 1-1296) containing regions I and II; and LS/CX #3 (nucleotides -103972; amino acid residues 1-1324) containing regions I, II, and III (Fig. 1, Table II).

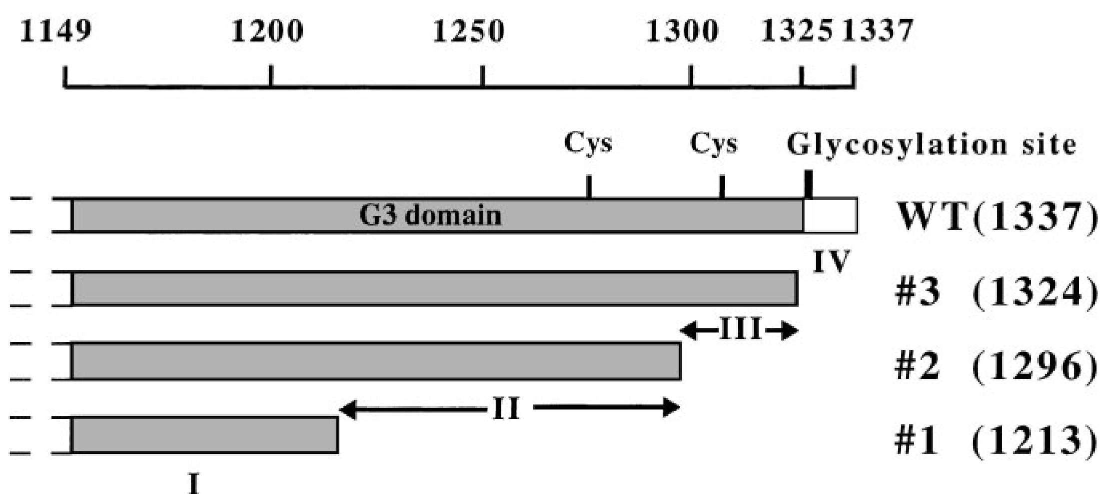
Human fibrosarcoma cell line HT1080, which expresses the laminin β3 and γ2 chains but not the α3 chain [Mizushima et al., 1996], was transfected with each of full-length and mutated cDNAs of the α3 chain. The transfected HT1080 cells secreted the wild-type (WT) and mutated recombinant LN5 proteins #1, #2, and #3 into the respective culture media. Each recombinant protein was purified by a combination of molecular-sieve chromatography, gelatin column chromatography, and immunoaffinity chromatography with an anti-laminin α3 chain antibody. The purified recombinant proteins of WT, #1, #2, and #3 were analyzed by SDS–PAGE and immunoblotting with anti-laminin α3, β3, and γ2 chain antibodies (Fig. 2). SDS–PAGE under non-reducing conditions separated each protein sample into two major bands of approximately 450 and 400 kDa, which correspond to the LN5 forms with the 150-kDa γ2 chain and with the 105-kDa γ2 chain, respectively (Fig. 2A) [Hirosaki et al., 2000]. SDS–PAGE under reducing conditions revealed that the four recombinant LN5 proteins contained proteins of 160–140, 140, and 105 kDa (Fig. 2B). Immunoblotting with the anti-laminin α3 antibody identified the laminin α3 chain of 160 kDa in WT, 158 kDa in #3, 156 kDa in #2, and 148 kDa in #1 (Fig. 2C). Immunoblotting with the anti-laminin β3 and γ2 chain antibodies identified a single band of the laminin β3 chain (140 kDa) (Fig. 2D) and two bands (150 and 105 kDa) of the laminin γ2 chain in each LN5 sample, respectively (Fig. 2E). These results indicated that the partially deleted α3 chains were associated with the endogenous β3 and γ2 chains to form the LN5 heterotrimers.

To analyze whether or not each recombinant LN5 was folded properly, they were subjected to trypsin digestion followed by reducing SDS–PAGE (Fig. 2F). When the recombinant proteins WT, #3, #2, and #1 were incubated with 2 ng/μl trypsin for one h, the heat-denatured WT-LN5 was rapidly and unlimitedly degraded whereas the others were fairly resistant to the digestion and produced limitedly cleaved fragments. This suggested that the LN5 mutants might maintain conformations similar to that of the natural LN5.

We also constructed expression vectors containing cDNAs which encoded the following

Fig. 1. Structures of G3 domains in various recombinant LN5 mutants. **A**: Schematic structures of wild-type (WT) and three deletion forms of G3 domain (#3, #2, and #1) in laminin α3 chain. G3 domains are shown by shaded bars, and the spacer region between G3 and G4 domains is shown by an open bar. The amino acid position is shown on the top. Numbers in parentheses indicate the C-terminal amino acid position of the α3 chain in the four LN5 forms. I, II, III, and IV indicate region I (amino acid residues 1149-1213), region II (amino acid residues 1214-1296), region III (amino acid residues 1297-1324), and region IV (amino acid residues 1325-1337), respectively. Cys indicates cysteine residues. **B**: Amino acid sequence of G3 domain. Possible β-strand structures were deduced from the crystal structure of the G5 domain of laminin α2 chain [Hohenester et al., 1999] and are indicated by arrows and alphabetical letters (A to N) above the sequence. The C-terminal end of each deletion mutant is indicated by heavy arrows with # numbers under the sequence. The amino acid residues indicated by bold letters with symbols (open and closed circles, asterisks) or within boxes were substituted to alanine in the respective LN5 mutants. A double line shows a potential N-glycosylation site. An arrowhead indicates the proteolytic processing site of the laminin α3 chain in the spacer sequence.

A



B

A B C

(1149) SASFSRGGQLSFTDLGLPPTDHLQASFGFQ (1178)

D E F

(1179) TFQPSGILLDHQTWTRNLQVTLEDGYIELS (1108)

G H

(1209) TSDSGGPIFKSPQTYMDGLLHYVSVIS**D**NS (1238)

#1 #2-2

I J K

(1239) GL**R**LLI**DD**QLL**R**NS**KR**L**KH**ISSS**R**QSLRLG (1268)

#2-3

L

(1269) GSNFEGCISNVFVQ**R**LSLSP**E**VL**D**LTSNSL (1298)

#2

M N

(1299) **KRD**VSLGGCS**LNK**PPFLMLLKGSTRFNKTK (1328)

#3

(1329) TFRINQLLQDTPVASPRSVKVWQDACSPLP (1358)

Fig. 1.

TABLE II. Mutants of Laminin α3 Chain cDNAs Transfected Into HT1080 Cells and Secretion of Recombinant Laminin-5

Clone name	Amino acid position	Secretion of laminin-5
Control		
WT		+
Deletion		
#3	132 5-	+
#2	129 7-	+
#2-2	123 8-	−
#2-3	126 1-	−
#1	121 4-	+
Alanine substitution		
Cys	127 5, 130 7	+
KRD	129 9-130 1	+
LNK	130 9-131 1	+
KRLKH	125 3, 125 4, 125 6, 125 7	+
D/R/D	123 6, 124 1, 124 5	−
D/R/R	124 6, 125 0, 126 2	+
R/E/D	128 3, 128 9, 129 2	+

Expression vector of each laminin α3 chain cDNA was constructed and transfected into HT1080 cells, and the secretion of the recombinant laminin-5 was verified by non-reducing immunoblotting with the anti-α3 chain antibody.

laminin α3 chains partially lacking region II of the G3 domain: #2-2 (amino acid residue 1-1237) and #2-3 (amino acid residue 1-1260) (Fig. 1B). When these cDNAs were transfected into HT1080 cells, no laminin α3 chains were secreted into the culture medium, suggesting that these mutant α3 chains could not form the stable heterotrimers with the β3 and γ2 chains, or were mis-folded and degraded before forming the heterotrimers (Table II).

Cell Adhesion Activity of LN5 Variants

To compare the biological activity of the LN5 variants, Buffalo rat liver-derived epithelial cell line BRL and human bladder carcinoma cel1 line EJ-1, both of which do not secrete but respond to LN5 [Miyazaki et al., 1993], were used. First, four kinds of recombinant LN5 variants, #1, #2, #3, and WT, were assayed at various concentrations for cell adhesion activity toward BRL cells (Fig. 3A). The purified LN5 proteins of WT and #3 showed a nearly identical dose−response curve for cell adhesion, indicating that the N-linked carbohydrate chain of G3 domain is not necessary for cell adhesion activity. In contrast, the effective concentration for the half maximal activity (ED_{50}) of #2 was about 5.6-times higher than that of WT or #3, indicating that region III of the G3 domain contains an important site for the cell adhesion activity of LN5. The cell adhesion activities of #2 and #1 were almost identical to that of LN5 ΔG3-5, which lacked the whole sequence of the G3, G4, and G5 domains [Hirosaki et al., 2000].

We next substituted the two cysteine residues, which are predicted to form a disulfide bond from the crystal structure of laminin α2 G4 and G5 domains [Timpl et al., 2000], with alanine by the point mutation of the α3 cDNA and prepared the LN5 mutant (Table II). When the LN5 mutant Cys was assayed for cell adhesion activity, it was slightly less active than WT or #3 but much more active than #2 and #1 (Fig. 3A). These results show that the disulfide bond between the two cysteine residues is not essential for the cell adhesion activity of LN5 and probably for stabilizing the tertiary structure of the G3 domain.

Charged, especially basic, amino acid residues are thought to be important for interaction between the G domains of some laminin α chains and integrins or heparin-like cell surface receptors [Hohenester et al., 1999; Talts et al., 1999a; Nielsen et al., 2000]. To localize cell-binding sites in the LN5 G3 domain, we employed the alanine-scanning mutagenesis. Expression vectors for six kinds of alanine-substituted α3 chains, D/R/D, D/R/R, KRLKH, R/E/D, KRD, and LNK, were constructed (Fig. 1B) (Table II). These expression vectors were individually transfected into HT1080 cells, and the secreted LN5 mutants, which had alanine substitution in some charged amino acids of the G3 domain, were purified from the conditioned media of the respective cDNA transfectants. Only the mutated α3 chain D/R/D was not secreted into the culture medium, suggesting that the amino acid sequence Asp1236-Asp1245 in region III is

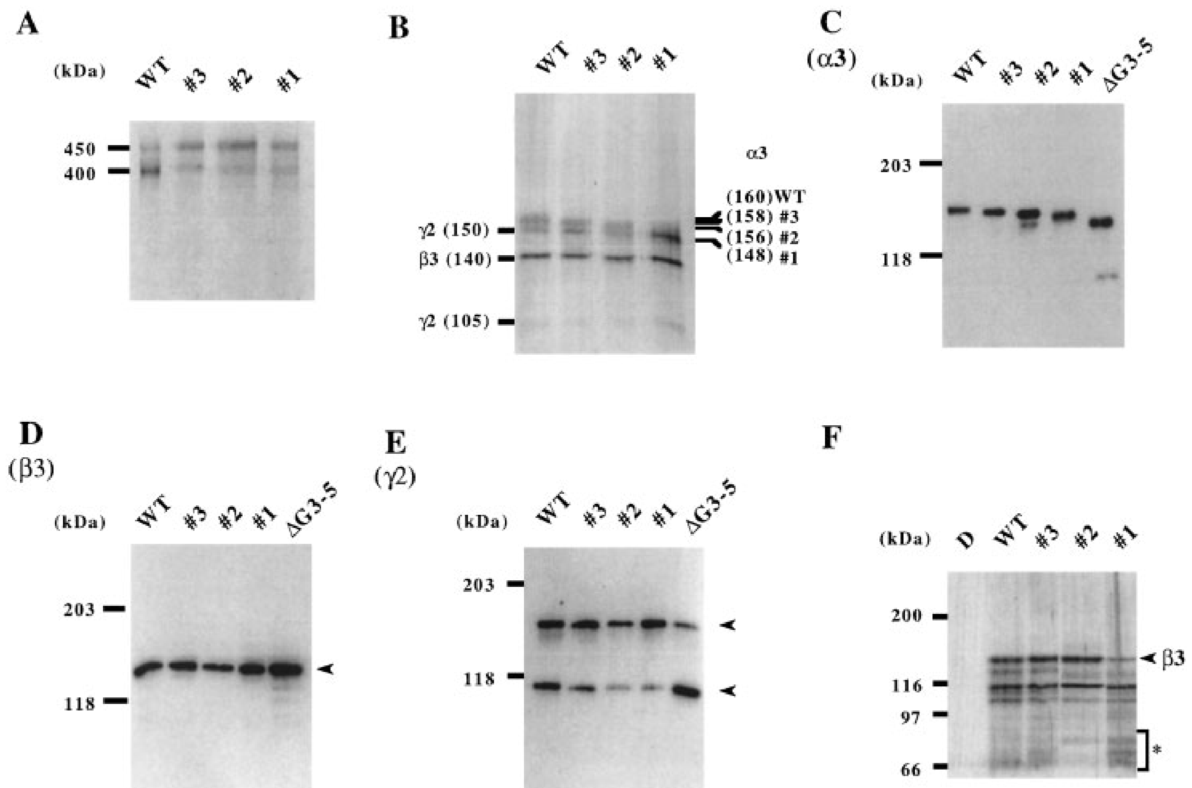


Fig. 2. Immunoblotting and silver staining of purified recombinant LN5 forms. The purified proteins of the control (WT) and three deletion forms (#3, #2, and #1) of LN5 were run on 6% gels under non-reducing (**A**) or reducing (**B–F**) conditions and stained with silver. For immunoblotting analysis, the proteins were transferred onto nitrocellulose membranes, and then probed with monoclonal antibodies against the laminin α3 chain (C), β3 chain (D), and γ2 chain (E). Ordinate, molecular mass in kDa. In A, the two major bands of ~450 and 400 kDa correspond to the LN5 forms with the 150-kDa γ2 chain and with the processed 105-kDa γ2 chain (the lower band in E), respectively. The 400-kDa LN5 is the dominant form in WT-LN5. An additional minor band under the 400-kDa band may be LN5 with the 80-kDa γ2 chain. In F, susceptibility of the four recombinant LN5 proteins to trypsin was examined. WT-LN5 and the three deletion LN5 forms (200 ng protein each) were incubated with 2 ng/μl trypsin-TPCK in 20 μl of a reaction mixture containing 30 mM Tris-HCl, 0.005% Brij35, and 0.1% CHAPS at room temperature for 1 h and then analyzed by SDS–PAGE under reducing conditions. *, proteolytic fragments. See B for the electrophoretic patterns before the trypsin treatment.

important for the conformation of G3 domain (Table II).

The purified recombinant LN5 mutants were assayed for cell adhesion activity (Fig. 3B). Except for KRD, these LN5 mutants showed essentially the same cell adhesion activity as WT. The cell adhesion activity of KRD was about one-third that of WT. These results indicate that among the 5 mutated sequences only the KRD sequence in region III is important for the cell adhesion activity of LN5. It is very likely that the decrease of cell adhesion activity in the deletion mutant #2 is in large part due to the loss of the KRD sequence in region III. To examine the adhesion activity of the KRD sequence, we synthesized a peptide containing KRD (amino acid residues 1289-1306) and tested its cell attachment activity. Culture plates coated with the synthetic peptide did not support the attachment of BRL cells, although the peptide of the integrin-binding sequence α3G2A present in the G2 domain dose-dependently supported the cell attachment [Mizushima et al., 1997] (data not shown). This suggests that the LN5 receptor may recognize the specific conformation of the KRD-containing region rather than the short KRD sequence.

We also assayed the cell adhesion activity of the deletion and substitution mutants of LN5 using EJ-1 cells. The results with EJ-1 cells were almost the same as those with BRL cells (data not shown).

Cell Motility Activity of LN5 Variants

To examine the effect of the G3 deletion on cell motility, cell scattering activity of LN5 mutants

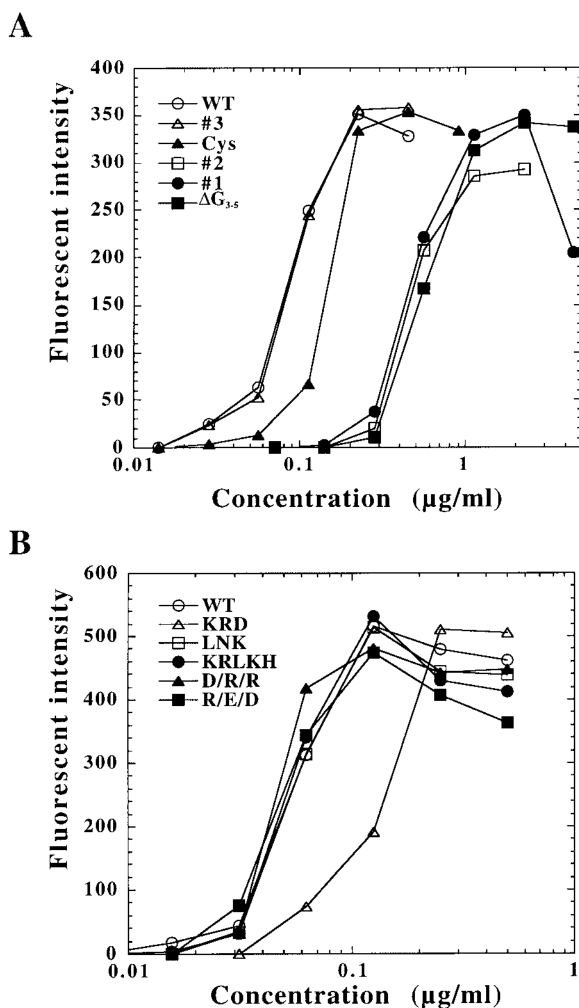


Fig. 3. Cell adhesion activity of LN5 mutants toward BRL cells. Ninety-six-well plates were coated with the indicated concentrations of each LN5 form. **A**: WT (open circle), #3 (open triangle), Cys (closed triangle), #2 (open square), #1 (closed circle), and ΔG3-5 (closed square); (**B**) WT (open circle), KRD (open triangle), LNK (open square), KRLKH (closed circle), D/R/R (closed triangle), and R/E/D (closed square). BRL cells were plated on each substrate in serum-free medium and incubated at 37°C for 1 h. After the incubation, relative numbers of adherent cells were determined by measuring fluorescent intensity. Each point represents the mean of triplicate assays. Three independent experiments gave similar results. Other detailed experimental conditions are described in the text.

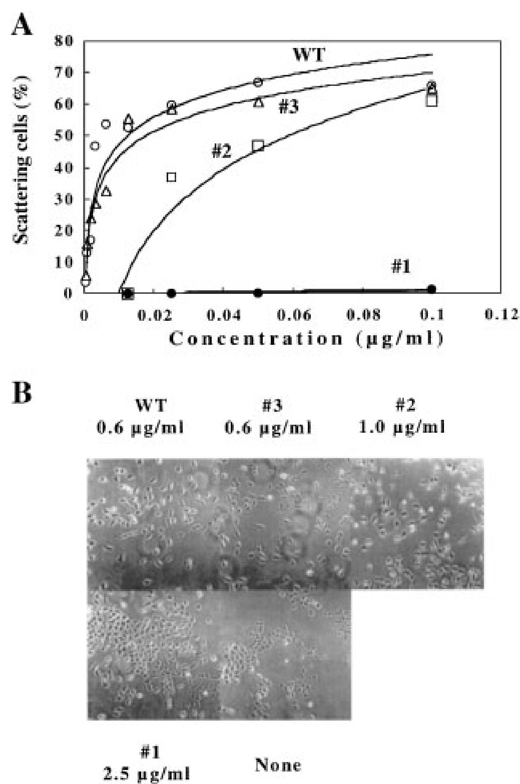


Fig. 4. Cell scattering activity of LN5 mutants in soluble and insoluble conditions toward BRL cells. **A**: Assay with soluble LN5 proteins. Five hundred microliters of BRL cell suspension (1.4×10^4 cells/ml in DMEM/F12 medium supplemented with 1% FCS) was inoculated into each well of 24-well plates. The four LN forms were individually added into the culture of BRL cells at the indicated concentrations and incubated for 40 h. At least 500 total cells and scattered cells were counted in each of three randomly selected microscopic fields. Other experimental conditions are described in the text. WT (open circle), #3 (open triangle), #2 (open square), and #1 (closed circle). **B**: Assay with insoluble LN5 proteins. Each well of 24-well plates was coated without (*None*) or with 0.6 μg/ml WT, 0.6 μg/ml #3, 1.0 μg/ml #2, or 2.5 μg/ml #1. BRL cells were inoculated into the wells, and cell morphology was examined under a phase-contrast microscope after incubation for 40 h.

was assayed by directly adding each sample into the culture medium of BRL cells in the presence of 1% serum (Fig. 4). WT and #3 showed essentially the same scattering activity at an ED_{50} of 6 ng/ml (Fig. 4A). #2 also showed cell scattering activity at an ED_{50} of about 20 ng/ml, whereas #1 did not show any activity at 100 ng/ml (Fig. 4A). The scattering activity of #1 was not observed even when the final concentration was increased to 0.4 μg/ml (data not shown). The cell scattering activity toward BRL cells was also assayed using culture plates pre-coated with the LN5 mutants at concentrations capable of supporting cell adhesion. In this assay, #2 showed significant cell scattering activity at a slightly higher concentration (1.0 μg/ml) than #3 and WT (0.6 μg/ml), but #1 showed little activity even at 2.5 μg/ml (Fig. 4B). When the cell scattering activity of the LN5 mutants with the alanine substitution was assayed by directly adding the test samples into the BRL culture, there was no significant difference between WT and the LN5 mutants including Cys and KRD

(data not shown). All these results suggest that the scattering activity of LN5 require mainly region II rather than the cell adhesion site of region III. Most of charged amino acids in region II are unlikely to be responsible for the cell motility activity. Furthermore, these results imply that the cell adhesion activity of LN5 mutants is not correlated with the cell scattering activity.

To confirm the different cell motility activity of LN5 mutants, migration speed of EJ-1 cells was measured on plastic plates pre-coated with WT and the three deletion mutants of LN5 as well as fibronectin by video-microscopy (Fig. 5). Although fibronectin and #1-LN5 hardly supported cell migration, the other three LN5 forms strongly promoted cell migration in the order of WT > #3 > #2. This confirmed that #2-LN5 maintained the high cell motility activity though it had mostly lost the high cell adhesion activity.

As shown above, #2-LN5 has much higher cell motility activity than #1-LN5 though they have similar cell adhesion activity. When BRL cells and EJ-1 cells were seeded onto plates pre-coated with 0.56 μg/ml of #2-LN5 or #1-LN5 in serum-free medium, both types of cells could spread well on #2-LN5 but very poorly on #1-LN5 (data not shown). This suggested that the cell motility activity might be correlated with the cell spreading activity rather than cell adhesion activity.

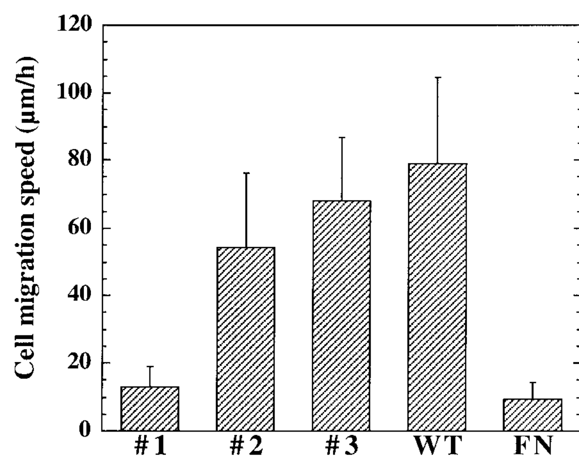


Fig. 5. Effects of LN5 mutants on migration of EJ-1 cells. Five hundred microliters of EJ-1 cell suspension (2×10^4 cells/ml in DMEM/F12 medium supplemented with 1% FCS) was inoculated into 24-well plates pre-coated with 0.35 μg/ml of each recombinant LN5 protein or 10 μg/ml fibronectin (FN). Each bar represents the mean ± SD for 15 cells.

Interaction of LN5 Variants With Integrins

LN5 is known to interact with integrins α3β1, α6β1, and α6β4 as the receptors for cell adhesion. It seems likely that these integrins play different roles in cell adhesion and migration, and the LN5 deletion mutants may have different integrin-binding specificities. To examine these specificities, we performed some additional experiments.

To test integrin requirement for cell adhesion and motility on LN5, EJ-1 cell line (clone 8) was used. This cell line expressed high levels of α3 and β1 integrins, a moderate level of α6 integrin and a low level of β4 integrin, suggesting that they produce integrins α3β1and α6β1, and some integrin α6β4 (data not shown). When EJ-1 cells were treated with function-blocking anti-integrin antibodies before seeding, the anti-α3 (P1B5) and -β1 (P4C10) integrin antibodies inhibited the adhesion of EJ-1 cells to WT-LN5 (Fig. 6). The anti-α6 antibody did not inhibit cell adhesion by itself, but treatment with a combination of the anti-α3 and anti-α6 antibodies completely inhibited the cell adhesion. When cell morphology was checked, the anti-α6 antibody as well as the anti-α3 and -β1 antibodies, but not the anti-β4 antibody, inhibited cell spreading (data not shown). These results suggest that in EJ-1 cells integrin α3β1 plays a primary role in cell attachment to LN5, while integrin α6β1 plays a supplementary role. Both integrins α3β1 and α6β1 seem important for the cell spreading on LN5.

Integrins that mediate migration of EJ-1 cells on WT-LN5 were analyzed by the use of the anti-integrin antibodies (Fig. 6B). Both anti-α3 and anti-β1 integrin antibodies strongly inhibited the cell migration. Unlike the case of cell adhesion (Fig. 6A), cell migration was inhibited by the anti-α6 integrin antibody alone. This inhibition may be related with the suppression of cell spreading by the anti-α6 integrin.

To examine whether the LN5 variants interact with each integrin, we used two additional cell lines, CHO and K562, which had been transfected with some integrin cDNAs. Parent CHO cells express endogenous integrins α5β1 and α3β1 [Symington et al., 1993]. CHO-α6 and CHO-α6β4 cells have been transfected with an α6 integrin cDNA and with both α6 and β4 integrin cDNAs, respectively [Lampe et al., 1998].

When the parent CHO, CHO-α6, and CHO-α6β4 cells were seeded on the plates pre-coated

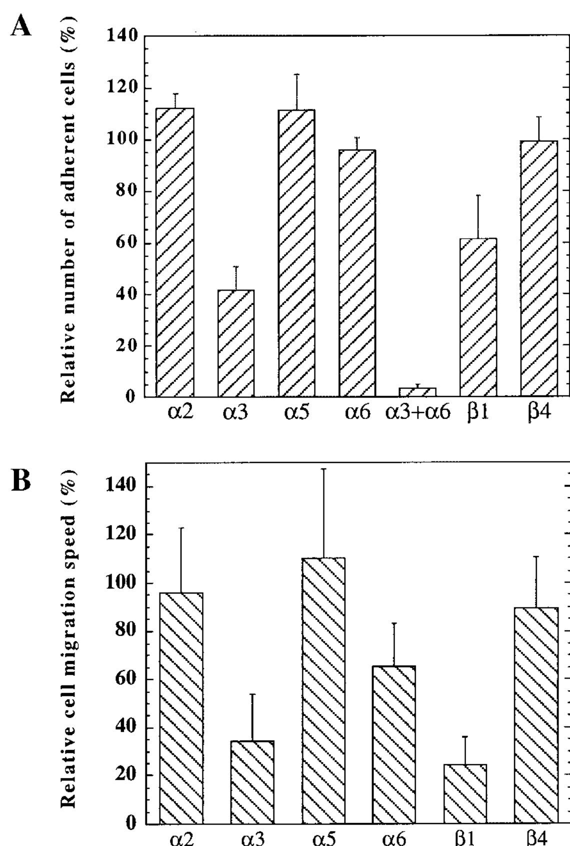


Fig. 6. Effects of anti-integrin antibodies on adhesion or migration of EJ-1 cells on WT-LN5. EJ-1 cells were preincubated with the indicated anti-integrin antibodies (1:100 dilution) or PBS (−) at room temperature for 20 min and then seeded on the plates pre-coated with 0.35 μg/ml WT-LN5. **A**: Cell attachment to LN5 after incubation for 1 h. The relative number of adherent cells in the PBS (−) control was taken as 100%. Each point represents the mean ± SD for triplicate assays. Other experimental conditions are described in the text. **B**: Cell migration on LN5. After pre-incubation for 1 h to allow cell attachment, the migration of EJ-1 cells on substrate was monitored by video microscopy for 10 h. The relative cell migration speed of PBS (−)-treated cells was taken as 100%. Each bar represents the mean ± SD for 15 cells.

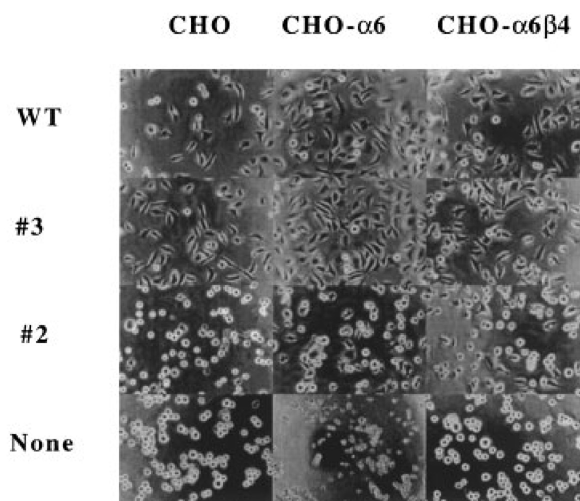


Fig. 7. Morphology of integrin-transfected CHO cells on LN5 mutants. Three CHO cell lines, CHO, CHO-α6, and CHO-α6β4, were incubated for 1 h on the plates which had previously coated without (*None*) or with 1 μg/ml WT, 1 μg/ml #3, or 4.5 μg/ml #2 at 37°C for 1 h. The CHO cells attached to but never spread on #2, whereas part of the CHO-α6 cells and the CHO-α6β4 cells spread on the same substrate.

with various concentrations of WT, #3, #2, or #1, all cell lines attached to WT and #3 more efficiently than to #2, but did not attach to #1 (data not shown). The effective concentration for cell attachment on each LN5 form was significantly lower in CHO-α6β4 than in the parent CHO and CHO-α6, suggesting that the expression of integrin α6β4 increases the efficiency of cell attachment to these LN5 forms. The cell morphology of the three CHO cell lines on these LN5 variants is shown in Figure 7. The parent CHO cells spread on WT and #3, showing spindle-shaped morphology, but did not spread on #2. CHO-α6 cells showed more flat morphology on each LN5 form than the parent cells, indicating that integrin α6β1 stimulates cell spreading on any of WT, #3, and #2 LN5 forms. The spreading of CHO-α6 cells on #2-LN5 was significantly inhibited by the pre-treatment with the anti-α6 integrin antibody (data not shown). CHO-α6β4 cells appeared to spread slightly more than the parent cells on #2-LN5. These results demonstrate that all of the integrins α3β1, α6β1, and α6β4 can interact with not only WT and #3 but also #2 and they synergistically enhance cell attachment or cell spreading.

We also used CHO cells transfected with exogenous α3 integrin cDNA (CHO-α3). There was no significant difference in the efficiency of cell attachment and spreading between the parent CHO and CHO-α3 cells, showing that the increase in the amount of integrin α3β1 on cell surface does not lead to more efficient cell spreading (data not shown). Furthermore, we used two human leukemia cell lines, K562-α3 and K562-α6, which produce integrins α3β1 and α6β1, respectively, in addition to endogenous integrin α5β1 on cell surface [Faull et al., 1993; Zhang et al., 1999]. They attached to WT- and #3-LN5 forms but weakly to #2-LN5 (data not shown). This also suggests that the active sites in regions III and II can recognize both integrins α3β1 and α6β1.

DISCUSSION

In the present study, we prepared many recombinant LN5 variants with amino acid substitution or C-terminal deletion within the G3 domain of the α3 chain. The deletion of region III (amino acid residues 1298-1324) in the G3 domain lead to a marked loss of the cell adhesion activity, while the additional deletion of region II (amino acid residues 1214-1297) lead to almost complete loss of the cell motility activity. This suggests that the G3 domain may contain at least two important sites that differently regulate cell adhesion and motility. The characteristic cell adhesion and motility activities of LN5 seem to be largely dependent on these two functional sites in the G3 domain. The lack of the potent cell adhesion activity in #2-LN5 is likely to be attributed to the loss of the KRD sequence present in region III, because the mutation of this sequence decreased the cell adhesion activity of LN5. In contrast to the KRD sequence in region III, the cell motility-responsible site in region II did not depend on any of the charged amino acid residues changed within the region.

Recent analysis of the crystal structure of the G5 and G4-5 domains (or modules) of laminin α2 chain predicts that all G domains of laminin α chains have a compact β-sandwich structure of two anti-parallel seven-stranded β-sheets [Hohenester et al., 1999; Tisi et al., 2000]. It is likely that partial deletion or mutation of the G3 domain affects the β-sheet structure of the remaining part. We can not exclude the possibility that the loss of biological activity in some LN5 variants, e.g., #2-LN5, is due to the mis-folding of the G3 domain resulting from the C-terminal deletion. Indeed, three kinds of LN5 mutants were not secreted, presumably because of abnormal peptide folding. However, the LN5 with the alanine substitution of two cysteine residues in the G3 domain has nearly normal cell adhesion and cell scattering activities. #2-LN5 was secreted at a similar level to WT-LN5 and maintained high cell motility activity toward BRL and EJ-1 cell lines. Furthermore, the three LN5 deletion mutants of #1, #2, and #3 were resistant to trypsin digestion similarly to WT-LN5. These results suggest that the β-sheet structure of the G3 domain may be fairly stable, and #2-LN5 and other secreted LN5 mutants may have conformations close to that of the natural LN5. The result with KRD-LN5 also supports that the lowered cell adhesion activity of #2-LN5 is due to the loss of an active site rather than the mis-folding of the remaining part of G3 domain. It is also noted that laminin-2 which has been cleaved at the N-terminal region of the α2 chain by a furin-like enzyme is associated with the C-terminal fragment and maintain high biological activity [Smirnov et al., 2002].

#2-LN5 showed significantly lowered cell attachment activity as compared to WT-LN5 and #3-LN5. Although #2-LN5 and #1-LN5 had similar cell attachment activity, only the former stimulated cell motility activity at coating concentrations capable of supporting cell adhesion (Fig. 4B and Fig. 5). These results suggest that although cell adhesion is essential for cell migration, the cell adhesion activity of LN5 mutants does not necessarily correlate with the cell motility activity. LN5 has a unique cell motility activity. As shown in Figure 4A, LN5 stimulates cell motility even when it is added as a soluble ligand at a very low concentration into serum-containing culture medium. In this case, the cell motility activity of LN5 does not require LN5-dependent cell adhesion. We have recently found that LN5 stimulates cell migration on a fibronectin substrate (Kariya et al., unpublished data). It is very likely that #2-LN5 can induce an intracellular signal to promote cell motility, even if it can not support effective cell attachment. When morphological effects of #2-LN5 and #1-LN5 on BRL and EJ-1 cells were compared, the former, but not the latter, supported cell spreading (data not shown). In CHO α6 cells, #2-LN5 promoted cell spreading but less effectively than WT-LN5, whereas #1-LN5 did not support cell spreading at all (Fig. 7). Thus, the cell motility activity appears to correlate with the cell spreading activity.

LN5 recognizes at least three different integrins, $\alpha3\beta1$, $\alpha6\beta1$, and $\alpha6\beta4$ [Carter et al., 1991; Kikkawa et al., 1994; Nielsen et al., 2000]. Integrins $\alpha3\beta1$ and $\alpha6\beta4$ are thought to be major receptors for LN5, but little is known about the role of integrin $\alpha6\beta1$ [Rousselle and Aumailley, 1994]. Consistently with the past findings, our experiments with EJ-1 cells and CHO cells suggest that integrin $\alpha3\beta1$ plays a primary role in both cell adhesion and cell migration. Integrins $\alpha6\beta1$ and $\alpha6\beta4$ appear to play rather compensatory roles in cell adhesion to LN5. However, integrin $\alpha6\beta1$ seemed to contribute to cell spreading and cell migration in EJ-1 cells.

The experiments with CHO cells also showed that in the presence of integrin α3β1, integrin α6β1 promoted cell spreading (Fig. 7), while integrin α6β4 promoted cell attachment but not spreading (data not shown). These results suggest that the three integrins differently regulate cell adhesion and migration on LN5, and that interaction of LN5 with two or three different integrins more efficiently supports cell adhesion and/or migration than that with a single integrin. These facts raised a possible mechanism that integrin specificities might be altered by deletion or mutation in the G3 domain. However, the experiments with CHO and K562 cell lines suggested that the LN5 mutants of #3 and #2 interacted with each of integrins α3β1, α6β1, and α6β4. This makes the hypothesis unlikely that the LN5 variants have different integrin specificity. The integrin signals induced by the three LN5 forms #1, #2, and #3 are likely to differ quantitatively and/or qualitatively even when they interact with a common integrin. These different signals will differently promote cell adhesion and migration. Further studies are needed to show the quantitative and qualitative differences in the internal signals induced by the LN5 variants.

LN5 looses the G4-5 domain in the α3 chain immediately after secretion, and the remaining G3 domain plays an essential role in the potent promotion of cell adhesion and cell motility [Hirosaki et al., 2000; Tsubota et al., 2000]. Shang et al. [2001] showed that a recombinant glutathione-S-transferase fusion protein of rat α3-G3 domain is able to bind integrin α3β1 and to support both cell adhesion and migration, though these activities are far lower than those of LN5. Very recently, Smirnov et al. [2002] prepared recombinant laminin-2 proteins with and without G4-5 and showed that G1-3 in laminin α2 chain is required for efficient cell adhesion through integrins, while G4-5 is required for basement membrane assembly. The present study demonstrates using recombinant LN5 proteins that the G3 domain of human LN5 regulates cell adhesion and migration. However, it is unlikely that integrins interact with only the G3 domain of LN5. The C-terminal globular region G1-5 is thought to contain multiple integrin binding sites. Recent studies on the crystal structures of α2-G4 and α2-G4-5 have proposed a hypothetical model of the entire C-terminal structure of laminin α chains in which the G1-3 domain has a cloverleaf-like structure that makes each G domain contact with the rod domain [Timpl et al., 2000]. In laminin-1, the proteolytic fragment E8, which consists of a part of the rod domain and G1-3, can bind integrins α3β1, α6β1, α6β4, α7β1, and α9β1 [Aumailley and Krieg, 1996]. In laminin α2 chain, a recombinant G1-3 tandem without the rod structure appears to bind integrins α3β1 and α6β1 [Talts and Timpl, 1999b], sulfated carbohydrates, and matrix proteins such as fibulins and nidogen-2 [Talts et al., 1999a]. The strong binding of α-dystroglycan to α2-G1-3 and α2-G4-5 is not found in the individual G domains (modules), indicating that the tandem G module structure is important for the binding to receptors or matrix ligands [Talts and Timpl, 1999b]. It is unknown how many integrin molecules bind to one mature LN5 molecule, which contains G1-G3 but not G4-5. In the present study, the similar response of CHO and K562 cell lines expressing a different set of integrins to the LN5 deletion mutants suggests that the G3 domain interacts with the three integrins α3β1, α6β1, and α6β4 in a similar manner, but inducing an intracellular signal specific for each integrin. The recombinant G2 domain shows an integrin α3β1-mediated cell attachment activity [Mizushima et al., 1997], but its activity is far lower than that of the recombinant G3 protein [Shang et al., 2001]. Based on these past findings and the possible compact structure of each G domain, it is reasonable to speculate that the G1 and G2 domains support the interaction of the G3 domain with one integrin molecule. It has been reported that a recombinant G4 protein of the laminin α3 chain binds syndecans-2 and -4 [Utani et al., 2001]. The interaction of LN5 with non-integrin receptors may play some roles in regulation of cell adhesion and migration. At present, however, there is no evidence that WT-LN5 or LN5 mutants without the G4-5 domain interact with syndecans or other non-integrin receptors.

Many laminin isoforms have a similar C-terminal structure of the α chain and show a similar integrin requirement: they more or less all interact with integrins α3β1 and α6β1 [Colognato and Yurchenco, 2000]. Nevertheless, the biological effect greatly differs among the laminin isoforms [Fujiwara et al., 2001]. This implies that different ligands produce different effects even if the receptor is identical. The differential effects of laminins seem to

depend at least in part on the difference in the N-terminal structures of the three subunits. Our present study clearly shows that small structural changes in the G3 domain of laminin α3 chain differently affect cell adhesion and migration on LN5, presumably inducing different intracellular signals without apparent change in the integrin requirement. This strongly suggests that the G3 domain, or LG3 module, of laminin α3 chain plays an central role in the expression of the unique biological activities of LN5. More generally the structure of G3 domain in the five laminin α chains may be an important determinant for the laminin isoform-specific internal signals.

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