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Identification of α -Dystroglycan Binding Sequences in the Laminin α 2 Chain LG4-5 Module

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Abstract

The biological activities of the laminin α 2 chain LG4-5 module result from interactions with cell surface receptors, such as heparan sulfate proteoglycans and α -dystroglycan. In this study, heparin and α -dystroglycan binding sequences were identified using 42 overlapping synthetic peptides from the LG4-5 module and using recombinant LG4-5 protein (rec- α 2LG4-5). Physiological activities of the active peptides were also examined in explants of submandibular glands. Heparin binding screens showed that the A2G78 peptide (GLLFYMARINHA) bound to heparin and prevented its binding to rec- α 2LG4-5. Furthermore, alanine substitution of the arginine residue in the A2G78 site on rec- α 2LG4-5 decreased heparin binding activity. When α -dystroglycan binding of the peptides was screened, two peptides, A2G78 and A2G80 (VQLRNGFPYFSY), bound α -dystroglycan. A2G78 and A2G80 also inhibited α -dystroglycan binding of rec- α 2LG4-5. A2G78 and A2G80 specifically inhibited end bud formation of submandibular glands in culture. These results suggest that the A2G78 and A2G80 sites play functional roles as heparan sulfate- and α -dystroglycan-binding sites in the module. These peptides are useful for elucidating molecular mechanisms of heparan sulfate- and/or α -dystroglycan-mediated biological functions of the laminin α 2 chain.

Keywords

laminin; synthetic peptide; basement membrane; heparin; α -dystroglycan

1. Introduction

Basement membranes are extracellular matrices (ECMs) that are critical for early embryonic development and tissue formation, as well as for maintenance of mature tissues (Miner and

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Yurchenco, 2004). All basement membranes contain type IV collagen, nidogens, perlecan, and laminins.

Laminins are multifunctional trimeric glycoproteins. At present, 5 α (α 1- α 5), 3 β (β 1- β 3), and 3 γ (γ 1- γ 3) chains have been identified and 16 isoforms can be formed by various combinations of each subunit (Aumailley et al., 2005; Yan and Cheng, 2006). Laminin isoforms show tissue- and/or developmental stage-specific expression and play important roles in the structures and functions of basement membranes (Miner and Yurchenco, 2004). The laminin α 2 chain, a component of laminins-211, -221, and -213 (Aumailley et al., 2005), is expressed in skeletal muscle, peripheral nerves, brain, capillaries and submandibular glands (SMG). Mutational analyses of patients have revealed that some types of mutations in the laminin α 2 chain underlie congenital muscular dystrophy (Helbling-Leclerc et al., 1995). Furthermore, mice lacking the laminin α 2 chain die after birth due to muscular dystrophy and peripheral nerve defects (Kuang et al., 1998; Miyagoe et al., 1997).

Integrins, α -dystroglycan (α DG), and proteoglycans (PGs) such as syndecans, and sulfatides have been identified as major cellular receptors for laminins. Several integrins, including α 3 β 1, α 6 β 1, and α 7 β 1, interact with the laminin α 2 chain. One of the non-integrin receptors for the laminin α 2 chain is α DG, a highly glycosylated cell surface protein involved in the interaction of muscle cells with the α 2 chain in muscle basement membranes. Other non-integrin receptors for the laminin α 2 chain are the cell surface PGs. These bind to extracellular matrix proteins and are important for cell adhesion and spreading (Couchman, 2003).

The interaction of laminins with α DG and PGs is essential for proper morphogenesis of the SMG (Kadoya and Yamashina, 2005; Patel et al., 2007). The expression of the laminin α 2 chain is increased in the end bud basement membranes of SMGs in the late-stages of mouse embryonic development (embryonic day 17-newborn). In contrast, there is no expression in the duct basement membranes (Kadoya et al., 1998). A similar expression pattern is observed for α DG in the end bud basement membranes in late stage embryos (Durbeej et al., 2001; Kadoya and Yamashina, 2005), which suggests that the laminin α 2 chain exerts its biological functions through interactions with α DG in the end buds. Cell surface PGs are also critically involved in SMG development, not only through cell adhesion and spreading, but also through binding and activating growth factors such as fibroblast growth factors (FGFs) (Patel et al., 2007; Patel et al., 2006).

The laminin α chains contain 5 tandem laminin G domain-like modules (LG1-5). The C-terminal LG4-5 module of the α 2 chain possesses heparin- and α DG-binding activity (Talts et al., 1999). A previous study using structure-based mutagenesis analysis identified heparin- and α DG-binding sites in the α 2 chain LG4-5 module. They also demonstrated that these binding sites required several basic residues (Wizemann et al., 2003). However, the binding sequences of heparin- and α DG-binding in this module have not yet been identified.

Recently, we identified several laminin-receptor binding sequences from the laminin α chain LG4-5 modules, using recombinant proteins and large sets of synthetic peptides (Hozumi et al., 2009a; Suzuki et al., 2005). From these analyses, we identified peptides AG73 (RKRLQVQLSIRT, mouse laminin α 1 chain: 2719-2730), EF-1 (DYATLQLQEGRHLHFMFDLG, mouse laminin α 1 chain: 2747-2765), A3G756 (KNSFMALYLSKGRLVFALG, human laminin α 3 chain: 1411-1429), A4G82 (TLFLAHGRLVFM, mouse laminin α 4 chain: 1514-1525), and A5G77 (LVLFLNHGHFVA, mouse laminin α 5 chain: 3307-3318) (Hozumi et al., 2009a; Nomizu et al., 1995; Okazaki et al., 2002; Suzuki et al., 2003b; Utani et al., 2001). AG73, A3G756, and A4G82 bind to syndecans and promote various biological activities, including cell migration, neurite outgrowth, and tumor growth and metastasis (Hoffman et al., 1998; Kato et al., 2002; Kim et

al., 1998; Okazaki et al., 2002; Richard et al., 1996; Utani et al., 2003). EF-1 specifically interacts with integrin $\alpha 2\beta 1$ and induces cell spreading, focal contacts, and production of actin stress fibers (Suzuki et al., 2003b). Our mutagenesis analyses showed that these sequences were active for biological functions through interaction with cell surface proteins (Hozumi et al., 2006; Utani et al., 2001; Yamaguchi et al., 2000). Some of these active peptides have been used as ECM mimetics, such as peptide-conjugated chitosan membranes (Hozumi et al., 2009b). These studies are useful in developing tissue engineering and therapeutic reagents based on cell-ECM interactions.

In the present study, we screened heparin- and α DG-binding sequences from the laminin $\alpha 2$ chain LG4-5 module, using a recombinant LG4-5 protein and 42 synthetic peptides that covered the entire module. We demonstrated that A2G78 (GLLFYMARINHA, mouse laminin $\alpha 2$ chain: 2796-2807) binds heparin and α DG, whereas A2G80 (VQLRNGFPYFSY, mouse laminin $\alpha 2$ chain: 2812-2823) specifically binds α DG. These sequences are useful tools for investigating heparan sulfate- and α DG-mediated biological functions and their mechanisms.

2. Results

2.1. Heparin Binding Activity of the Laminin $\alpha 2$ Chain LG4-5 Module

A recombinant mouse laminin $\alpha 2$ chain LG4-5 module (rec- $\alpha 2$ LG4-5) was prepared as a fusion protein with a human IgG Fc portion, as previously described (Suzuki et al., 2003b; Utani et al., 2001). At first, heparin-binding activity of rec- $\alpha 2$ LG4-5 was tested using heparin-Sepharose beads. The rec- $\alpha 2$ LG4-5 protein bound to the heparin-Sepharose beads in the presence of 100 mM NaCl (Fig. 1A, lane none) and 150 mM NaCl (data not shown), and was eluted with 200 mM NaCl (data not shown). The heparin binding activity was similar to that reported previously (Talts et al., 1999; Wizemann et al., 2003).

2.2. Identification of Peptides that Inhibit Heparin Binding to the rec- $\alpha 2$ LG4-5 Protein

To identify heparin-binding sequences from the laminin $\alpha 2$ chain LG4-5 module, the sequences were screened using rec- $\alpha 2$ LG4-5 and overlapping synthetic peptides. Forty-two overlapping peptides (A2G72-A2G113) covering the entire laminin $\alpha 2$ chain LG4-5 module were prepared (Table 1). Each peptide was designed to be approximately 12 residues in length and to overlap with neighboring peptides by 4 residues. Peptide A2G96 was insoluble in an aqueous solution, and was not used for further experiments. The ability of the 41 soluble peptides to inhibit rec- $\alpha 2$ LG4-5 binding to heparin-Sepharose beads was evaluated (Table 1, Fig. 1A). Only one peptide, A2G78 (GLLFYMARINHA, mouse laminin $\alpha 2$ chain: 2796-2807), significantly inhibited heparin binding to rec- $\alpha 2$ LG4-5, while the other 40 soluble peptides did not. The A2G78 peptide inhibited heparin binding to rec- $\alpha 2$ LG4-5 in a dose-dependent manner (Fig. 1B). A scrambled peptide, A2G78S (LFGLYMARHAIN), had minor inhibitory activity at high concentrations (Fig. 1B). These results indicate that the inhibitory effect of A2G78 on the heparin binding of rec- $\alpha 2$ LG4-5 was sequence-specific.

2.3. Amino Acid Substitution Analyses of A2G78: Inhibitory Effect on Heparin Binding of the rec- $\alpha 2$ LG4-5 Protein

Alanine-substituted A2G78 peptides were prepared and their ability to inhibit heparin binding to rec- $\alpha 2$ LG4-5 was tested (Table 2). A2G78-1GA and A2G78-11HA inhibited heparin binding to rec- $\alpha 2$ LG4-5 in a similar manner to A2G78, while A2G78-2LA, -4FA, -6MA, and -10NA only weakly inhibited heparin binding. A2G78-3LA, -5YA, -8RA, and -9IA had no effect on heparin binding to rec- $\alpha 2$ LG4-5. These results suggest that 8 residues are important for the interaction of A2G78 with heparin and that the L²⁷⁹⁸, Y²⁸⁰⁰, R²⁸⁰³, and I²⁸⁰⁴ residues, in particular, play critical roles in the activity of A2G78.

2.4. Mutation Analysis of the rec- α 2LG4-5 Protein for Heparin Binding

To examine the significance of these 4 residues (L²⁷⁹⁸, Y²⁸⁰⁰, R²⁸⁰³, and I²⁸⁰⁴) of the A2G78 site in the laminin α 2 chain LG4-5 module, we performed a mutagenesis analysis of the 4 residues within the module for heparin binding. We prepared 4 different mutant rec- α 2LG4-5 proteins (3LA, 5YA, 8RA, and 9IA) that had an alanine substitution in each of those residues and tested their heparin-binding activity using heparin-Sepharose (Fig. 2). Only 8RA did not bind to heparin-Sepharose, while the others (3LA, 5YA, and 9IA) showed comparable binding to that of the wild type rec- α 2LG4-5 protein. These results suggest that the R²⁸⁰³ residue is critical for heparin binding of the LG4-5 module. The other 3 residues (L²⁷⁹⁸, Y²⁸⁰⁰, and I²⁸⁰⁴) may have minor effects on heparin binding of rec- α 2LG4-5, or may not be important for the binding of rec- α 2LG4-5, although they are critically involved in heparin binding activity of the A2G78 peptide.

2.5. Amino Acid Deletion Analysis of A2G78: Inhibitory Effect on Heparin Binding of the rec- α 2LG4-5 Protein

Truncated peptides of A2G78 were also prepared and their inhibitory effects on heparin binding to rec- α 2LG4-5 were tested (Table 3). When the N-terminal glycine was deleted (A2G78N-1: LLFYMARINHA), A2G78 no longer inhibited heparin binding. A2G78C-3 (GLLFYMARI), a C-terminal truncated peptide, still inhibited heparin binding, whereas A2G78C-4 (GLLFYMAR), which had a deletion of the C-terminal isoleucine from A2G78C-3, lost its inhibitory activity. These results indicate that the 9-amino acid sequence (GLLFYMARI) is critical for the inhibitory effect on heparin binding to rec- α 2LG4-5.

2.6. Heparin Binding Activity of Peptides

Next, direct heparin-binding activities of the 41 soluble peptides were examined using peptide-coated plates and biotinylated heparin (Table 1). In a solid-phase binding assay, biotinylated heparin was added to the peptide-coated plates, and the bound heparin was detected by SA-HRP. A2G74, A2G84, A2G104, A2G110, and A2G111 strongly bound to heparin. Moderate heparin-binding was detected with A2G73, A2G78, A2G83, A2G89, A2G95, A2G103, and A2G109. The remaining peptides did not bind to heparin. These results suggest that the LG4-5 module has multiple heparin-binding sites, as previously reported (Wizemann et al., 2003).

Since only A2G78 strongly inhibited heparin binding to rec- α 2LG4-5 (Fig. 1), we analyzed the heparin binding activity of A2G78 in more detail. A2G78 bound to heparin in a dose-dependent manner (Fig. 3A). The C-terminal truncated A2G78C-3 peptide also showed heparin binding in a dose-dependent manner similar to A2G78, while A2G78S, the control scrambled peptide of A2G78, did not bind to biotinylated heparin (Fig. 3A). We further tested whether heparin and HS inhibited heparin binding of A2G78. Both heparin and HS inhibited the binding in a dose-dependent manner (Fig. 3B,C). These results demonstrate that A2G78 and A2G78C-3 directly bind to heparin and suggest that the A2G78 site may serve as one of the major heparin/HS binding sites in the laminin α 2 chain LG4-5 module.

2.7. α DG Binding Activity of Peptides

The laminin α 2 chain LG4-5 module possesses heparin binding activity and also α DG binding activity. The α DG binding activity is crucial for the physiological functions of the laminin α 2 chain. Therefore, the α DG binding activities of the 41 soluble peptides were examined. α DG was partially purified from C2C12 mouse myoblast cells using wheat germ agglutinin (WGA)-agarose beads, and was used to examine the interaction with peptide-conjugated beads (Table 1, Fig. 4). α DG strongly bound to A2G78 and A2G80 (VQLRNGFPYFSY, mouse laminin α 2 chain: 2812-2823). Weak α DG binding to A2G84, A2G97, A2G103, A2G104, A2G109, A2G110, and A2G111 was detected when longer time exposures of chemiluminescence

detection were performed in Western blotting (Table 1). These results indicate that there are several α DG-binding sites in the module, as previously reported (Wizemann et al., 2003), and suggest that the A2G78 and A2G80 sites are prominent binding sites in the module.

We next prepared a further purified recombinant α DG protein that was glycosylated by co-expression with the LARGE glycosyltransferase in cultured cells (rec- α DG). Multiple protein bands had been detected by silver staining of pull-down materials of the peptide-beads using the partially purified α DG from the C2C12 cells (data not shown). We therefore tested whether rec- α DG binds to A2G78- and A2G80-conjugated beads. Using the substantially purified protein, we confirmed that rec- α DG bound to both A2G78- and A2G80-conjugated beads (Fig. S1). These results suggest that the A2G78 and A2G80 sites directly interact with α DG.

2.8. Inhibitory Effect of Peptides on α DG Binding to the rec- α 2LG4-5 Protein

We next tested the inhibitory effects of A2G78 and A2G80 on α DG binding to rec- α 2LG4-5 in a pull-down assay. We first incubated rec- α 2LG4-5 and α DG, then rec- α 2LG4-5 was precipitated using Protein G-agarose beads. The α DG bound to the precipitated rec- α 2LG4-5 was then detected by Western blotting. An α DG band was detected in the presence, but not in the absence, of rec- α 2LG4-5 (Fig. 5A). The α DG binding of rec- α 2LG4-5 was inhibited by EDTA (Fig. 5A), indicating that the interaction requires divalent cations, such as Ca^{2+} and Mg^{2+} , as previously described (Talts et al., 1999; Wizemann et al., 2003). Heparin also inhibited the binding in a dose-dependent manner (Fig. S2), suggesting that the α DG binding sites may overlap with heparin binding sites. When the effect of peptides on the α DG binding to rec- α 2LG4-5 was evaluated, A2G78 and A2G80 were found to inhibit the binding (Fig. 5A,B). The inhibitory effect of A2G78S (29.9 ± 3.0) on α DG binding to rec- α 2LG4-5 was reduced compared with that of A2G78 (66.4 ± 10.2) (Fig. 5B). A2G77, whose C-terminal 4 residues (GLLF) overlap with the N-terminal 4 residues of A2G78, did not inhibit the binding (Fig. 5B). Furthermore, A2G80 also inhibited the α DG binding to rec- α 2LG4-5 (62.9 ± 16.3) (Fig. 5A,B). These results suggest that the A2G78 and A2G80 sequences play critical roles in the α DG binding of the module.

2.9. Effect of Peptides on Branching Morphogenesis of Mouse SMGs

The binding of laminins to α DG and HSPGs is critical for SMG morphogenesis and inhibition of the laminin α 2- α DG interaction with anti-DG antibodies decreases morphogenesis (Durbecq et al., 2001; Kadoya and Yamashina, 2005; Patel et al., 2007). Therefore, A2G78 and A2G80 were added to SMG organ culture and compared with AG73 from the laminin α 1 chain LG4 module, which was previously reported to reduce branching morphogenesis, (Hoffman et al., 2001; Kadoya et al., 1998; Suzuki et al., 2003a). In the presence of AG73, the gland appeared smaller in size and had a reduced number of end buds (Fig. 6A,B). Treatment with A2G78 or A2G80 also significantly reduced the number of end buds, but the sizes of the rudiments were similar to controls (Fig. 6A,B). The control peptide, A2G78S, had no effect on branching morphogenesis (Fig. 6A,B). These peptides did not affect the expression level of DG, but the expression of the laminin α 2 chain was increased in the presence of AG73, A2G78, and A2G80 compared with the control peptide, A2G78S (Fig. 6C). This suggests that there may be feedback from the peptide- α DG interaction to the transcript of the laminin α 2 chain. The A2G78 and A2G80 sequences in the laminin α 2 chain LG4 module influence SMG morphogenesis.

3. Discussion

Laminin α chain LG modules interact with cell surface PGs, α DG, and carbohydrate chains of the cell surface proteins, and these interactions are important in establishing and maintaining the associations between cells and basement membranes (Miner and Yurchenco, 2004). In the present study, two functional sequences were identified by peptide screening of the laminin

α 2 chain LG4-5 module. These were A2G78 (GLLFYMARINHA) as the heparin- and α DG-binding sequence, and A2G80 (VQLRNGFPYFSY) as the α DG-specific binding sequence. Since A2G78 and A2G80 inhibited α DG binding to rec- α 2LG4-5 and A2G78, but not to A2G80, and inhibited heparin-binding of rec- α 2LG4-5, the mechanisms of the inhibitory activities of these two peptides are likely different. These peptides may therefore be useful for elucidating the HS- and/or α DG-mediated biological functions of laminins.

Glycosylated cell surface receptors syndecans and dystroglycan, and integrins, are major receptors for laminins. Syndecans have negatively charged HS and chondroitin sulfate chains in their ectodomains. α DG has uncommon sialyl *O*-mannosyl glycans (SiaA α 2-3Gal β 1-4GlcNAc β 1-2Man) in its mucin domain. Several studies suggest that sialic acids and/or unknown anionic sugars containing α -linked GlcNAc oligosaccharides in α DG mediate its interactions with laminins (Combs and Ervasti, 2005; Yamada et al., 1996). These anionic carbohydrate chains of syndecans and α DG play a role in binding to basic residues of laminins.

A2G78 has an arginine residue (R²⁸⁰³). The alanine-substitution analysis of A2G78 revealed that R²⁸⁰³ is important for the interaction with heparin. Furthermore, the mutation of rec- α 2LG4-5 at R²⁸⁰³ (8RA) decreased its capacity for heparin binding. Interestingly, the 8RA mutation also reduced binding activity to α DG binding (data not shown). These results are consistent with a previous report (Wizemann et al., 2003) and indicate that R²⁸⁰³ of the A2G78 site is a critical residue for the interaction with both heparin and α DG. Further alanine substitutions in the A2G78 peptide revealed that other residues (L²⁷⁹⁸, Y²⁸⁰⁰, and I²⁸⁰⁴) were also important for heparin binding. However, mutations at these residues in rec- α 2LG4-5 did not inhibit its capacity for heparin binding. Therefore, unlike the case for the A2G78 peptide, these 3 residues have minor involvement in the heparin binding of rec- α 2LG4-5, most probably because of protein conformation.

The crystal structure of each LG module of laminin α chains consists of 14 β strands (A-N strands) (Hohenester et al., 1999). A2G80 is located in the connecting loop region between the E and F strands. Our previous studies demonstrated that the connecting loop region in the laminin α chain LG4 modules plays an important role in interactions with cell surface receptors (Suzuki et al., 2003b). The EF-2 peptide (DFGTVQLRNGFPFFSYDLG) was previously identified from the loop region of the laminin α 2 chain and was found to promote cell adhesion through syndecan-2 (Suzuki et al., 2003b). A2G80 is the middle sequence of EF-2 (lacking 4 N-terminal and 3 C-terminal amino acids of EF-2) and primarily covers the loop region. These results suggest that this loop region of the laminin α 2 chain LG4 module is important for binding to cellular receptors, such as α DG and syndecans. Our preliminary data showed that an alanine substituted mutation of the arginine residue (R²⁸¹⁵) in the A2G80 site did not affect α DG binding activity of rec- α 2LG4-5 (data not shown). This result indicates that another residue or multiple residues in the A2G80 site may be critical for this binding.

Previously, Wizemann et al. found several basic amino acids important for heparin (K²⁸⁷⁰, K²⁸⁷¹, K²⁹⁵³, K³⁰³⁰, K³⁰⁸⁸, K³⁰⁹¹, and K³⁰⁹⁵) and α DG (R²⁸⁰³, K²⁸⁷⁰, K²⁸⁷¹, K²⁹⁵³, and K³⁰⁹¹) binding by the alanine-substituted mutagenesis of the LG4-5 module. In the present study, A2G73, A2G74, A2G78, A2G83, A2G84, A2G89, A2G95, A2G103, A2G104, A2G109, A2G110, and A2G111 exhibited direct heparin-binding in the solid phase assay, while only A2G78 inhibited heparin binding of rec- α 2LG4-5. Furthermore, not only A2G78 and A2G80, but also A2G84, A2G97, A2G103, A2G104, A2G109, A2G110, and A2G111, weakly bound to α DG. A2G95 (KAVGGFKVGLDL, residues 2947-2958), A2G104 (AKKIKNRLELVV, residues 3026-3037), and A2G111 (IRSLKLTGKTGKP, residues 3084-3096) contain K²⁹⁵³, K³⁰³⁰, and K³⁰⁸⁸, K³⁰⁹¹, and K³⁰⁹⁵, respectively. Taken together,

these results suggest that multiple sites are involved in both heparin- and α DG-binding of the module.

Laminins and DG are essential molecules for proper development and maintenance of many tissues. Previous studies have shown that DG is important for kidney, lung, and SMG branching morphogenesis, and that DG is mainly expressed at the end bud area (Durbeej et al., 2001; Williamson et al., 1997). The expression pattern of the laminin α 2 chain and α DG throughout SMG development is similar (Durbeej et al., 2001; Kadoya and Yamashina, 2005). The α DG function-blocking antibody, IIH6, reduced the number of end buds in the SMGs (Durbeej et al., 2001). The α DG-binding peptides from the laminin α 2 chain, A2G78 and A2G80, reduced the number of end buds but did not affect stalk elongation. These results suggest that A2G78 and A2G80 may compete for the interaction between α DG and the laminin α 2 chain and disrupt α DG functions. On the other hand, exogenous heparin, heparin binding peptides, and heparitinase I treatment inhibited SMG branching, indicating that HSPGs are also important for branching morphogenesis (Patel et al., 2006).

Cell surface HSPGs are involved in cell adhesion and spreading, and in binding and activating FGFs, which are heparin-binding growth factors and play an important role in SMG development (Patel et al., 2007; Rebustini et al., 2007). The A2G78 peptide bound to both α DG and heparin, suggesting that A2G78 may also inhibit cellular interactions with the laminin α 2 chain and potentially FGFs by disrupting their interaction with HS chains during SMG branching morphogenesis. AG73, the peptide from the laminin α 1 chain that binds syndecan-1 through its HS, reduced not only the number of end buds, but also the size and branching of the glands. During SMG development, the laminin α 1 chain is expressed earlier than the laminin α 2 chain and is found throughout the basement membrane surrounding the epithelium (Kadoya and Yamashina, 2005). Increased expression of the laminin α 2 chain was observed in the presence of A2G78, A2G80, and AG73. This may be a compensatory effect due to interference with α DG and/or HSPG binding to laminins by the peptides. These data suggest that downstream signaling from the laminin α 2- α DG interaction results in transcriptional regulation of the laminin α 2 chain, although the signaling pathways remain to be elucidated. These biologically active peptides possess laminin α chain-specific inhibitory effects in this complex *ex vivo* model of salivary gland development.

In conclusion, we identified a heparin- and α DG-binding peptide, A2G78, and α DG-binding peptide, A2G80, from the laminin α 2 chain. A2G80 is the first peptide that is identified to specifically bind to α DG. These peptides may be useful for analysis of the interaction of laminins with HS or DG and for application for tissue engineering and development of therapeutic agents for treatment of laminin-related disease.

4. Experimental Procedures

4.1. Preparation of Wild-Type and Mutant Recombinant Laminin α 2 Chain LG4-5 Proteins

The expression plasmid (MO90) encoding the mouse laminin α 2 chain LG4-5 module (mouse laminin α 2 chain: 2741-3106) with the human laminin γ 2 chain signal peptide and the human IgG Fc portion was prepared as previously described (Suzuki et al., 2003b; Utani et al., 2001). The PCR primers with restriction enzyme sites (forward primer: *Avr* II; reverse primer: *Bam*H I) were as follows: 8158-9372 nt of the laminin α 2 chain, 5'-GAGCCTAGGGACCATGGTGCATGGCCCTTG-3' (forward) and 5'-GAGGGATCCCCGTTCCAGGGCCTTGCAAAATTAACC-3' (reverse). The recombinant α 2 chain LG4-5 module (rec- α 2LG4-5) was expressed in 293T cells by transfection using a Ca-P transfection kit (Invitrogen). The transfected cells were incubated in Dulbecco's modified Eagle's medium (DMEM) (Invitrogen) containing 0.5% fetal bovine serum (FBS) (HyClone) for 4 days and the conditioned medium was collected every 24 hours. The rec- α 2LG4-5 protein

was purified from the conditioned medium using a Protein A-Sepharose column (GE Healthcare Bio-Sciences Corp.) and a heparin-Sepharose column (GE Healthcare Bio-Sciences Corp.). The eluted fraction was dialyzed with 20 mM Tris-HCl (pH 7.5) containing 150 mM NaCl (buffer A). Protein concentration was determined with the BCA assay kit (Thermo Scientific).

For preparation of mutant rec- α 2LG4-5 proteins, we subcloned the laminin α 2 chain LG4-5 region from the MO90 wild type rec- α 2LG4-5 expression vector to the *Xho* I and *Bam*H I sites of pCRII TOPO vector (Invitrogen) (pCRII-1). Then, site-directed mutagenesis was performed with pCRII-1 using the QuickChange Site-Directed Mutagenesis Kit (Stratagene). The following amino acid residues of the laminin α 2 chain LG4-5 module were substituted with alanine (L²⁷⁹⁸, Y²⁸⁰⁰, R²⁸⁰³, and I²⁸⁰⁴) within the A2G78 site. The mutant α 2LG4-5 fragments were ligated into the *Xho* I and *Bam*H I sites of the MO90 vector. All mutations were verified by DNA sequencing. These expression vectors were transfected into COS-7 cells using FuGENE6 (Roche Applied Science). The transfected cells were incubated in DMEM containing 0.5% FBS for 4 days and the conditioned medium was collected every 24 hours. Mutant proteins were purified from conditioned media using a Protein A-Sepharose column.

4.2. Preparation of Synthetic Peptides

All peptides were prepared as previously described (Nomizu et al., 1998). Amino acid derivatives and resins were purchased from Novabiochem. The respective amino acids were condensed manually in a stepwise manner using 4-(2', 4'-dimethoxyphenyl-Fmoc-aminomethyl)-phenoxy resin. Dimethylformamide (DMF) was used as a solvent during the synthesis. For condensation, diisopropylcarbodiimide/N-hydroxybenzotriazole was employed, and for deprotection of N-Fmoc groups, 20% piperidine in DMF was employed. The following side chain protecting groups were used: for asparagine, glutamine, and histidine, trityl; for aspartic acid, glutamic acid, serine, threonine, and tyrosine, tert-butyl; for arginine, 2,2,5,7,8-pentamethylchroman-6-sulfonyl; and for lysine, tert-butoxycarbonyl. The resulting protected peptide resins were deprotected and cleaved from the resin using trifluoroacetic acid-thioanisole-m-cresol-ethanedithiol-H₂O (80:5:5:5:5, v/v/v/v/v) for 3 hours at room temperature. The crude peptides were precipitated and washed with diethyl ether, then purified by reverse-phase high performance liquid chromatography (HPLC) using a Mightysil RP-18 column (Kanto Chemical Co., Inc.) with a gradient of water/acetonitrile containing 0.1% trifluoroacetic acid. Only one peptide (A2G96) was insoluble in aqueous solutions and could not be purified by reverse-phase HPLC. Identity of the peptides was confirmed by an analytical HPLC and an electrospray ionization mass spectrometer at the Central Analysis Center, Tokyo University of Pharmacy and Life Sciences.

4.3. Heparin Binding Assay of rec- α 2LG4-5 Proteins Using Heparin-Sepharose Beads

Heparin binding to rec- α 2LG4-5 and inhibition of the binding using synthetic peptides were carried out as previously described (Suzuki et al., 2003a). The rec- α 2LG4-5 protein (0.1 μ g) and heparin-Sepharose beads (1 mg) (GE Healthcare Bio-Sciences Corp.) were mixed in 70 μ l of 10 mM Tris-HCl (pH 7.4) containing 100 mM NaCl (buffer B). After 1 hour incubation, the beads were pelleted by centrifugation. The supernatant was removed, and the beads were washed twice with buffer B. Then, the rec- α 2LG4-5 protein bound to the beads was eluted by a stepwise gradient of NaCl. Each fraction was analyzed by SDS-PAGE under reducing conditions using an 8% polyacrylamide gel. After the electrophoresis, the rec- α 2LG4-5 protein was transferred to a nitrocellulose membrane (Bio-Rad Laboratories). The blotted membrane was blocked with 5% skim milk in 10 mM Tris-HCl (pH 7.5) containing 150 mM NaCl (buffer C). After the blocking, the rec- α 2LG4-5 protein was detected by a biotinylated goat anti-human IgG Fc (Jackson ImmunoResearch Laboratories) (1:1000 dilution) and a streptavidin-

conjugated horseradish peroxidase (SA-HRP) (Sigma) (1:2000 dilution) using an ECL kit (GE Healthcare Bio-Sciences Corp.).

For inhibition assays using synthetic peptides, peptide (20 μ g) was incubated with the rec- α 2LG4-5 protein and heparin-Sepharose beads in buffer B. After the incubation, the rec- α 2LG4-5 protein bound to the beads was extracted with SDS-PAGE sample buffer, and then the eluted sample was analyzed by Western blotting, as described above. The relative amount (%) of rec- α 2LG4-5 bound to the heparin-Sepharose beads was assessed using NIH image 1.63 software.

For heparin binding of mutant proteins, the proteins were mixed with heparin-Sepharose in the presence of 150 mM NaCl. The proteins bound to heparin-Sepharose were detected as above.

4.4. Solid Phase Heparin Binding Assay Using Biotinylated Heparin

A solid phase heparin binding assay using biotinylated heparin was performed as previously described (Suzuki et al., 2003a). Various amounts of peptides (for screening: 0.5 μ g) in Milli-Q water (50 μ l) were coated onto 96-well ELISA plates (AGC Techno Glass) and dried overnight at room temperature. Then, the wells were blocked with 3% bovine serum albumin (BSA) (Sigma) in PBS at room temperature for 2 hours. After the blocking, 10 ng of biotinylated heparin (Celsus Laboratories Inc.) in 50 μ l of 0.05% Tween 20 in PBS was added to the wells and incubated at 37°C for 1 hour. After the incubation, the biotinylated heparin bound to the peptides was detected by SA-HRP, and then 3,3',5,5'-tetramethyl-benzidine (TMB) solution (Sigma) was added to the wells and incubated for 30 minutes at room temperature. After addition of 0.5 N H₂SO₄ to stop the colorimetric reaction by HRP, the optical density at 450 nm was measured using a microplate reader (Safire, Tecan Ltd.).

For the inhibition assays by heparin and HS, each peptide (1 μ g/well) was coated. After blocking, 10 ng of biotinylated heparin was added with various amounts of heparin or HS. Then, biotinylated heparin bound to peptides was detected as above.

4.5. Preparation of Partially Purified α DG from Lysate of C2C12 Mouse Myoblasts

For preparation of cell lysates of mouse C2C12 cells, the cells were cultured in DMEM containing 10% FBS, 100 units/ml penicillin, and 100 μ g/ml streptomycin (Invitrogen) at 37°C in a humidified 5% CO₂, 95% air atmosphere. The cultured C2C12 cells were collected into buffer C using a cell scraper. The collected cells were centrifuged at 3,000 rpm for 5 minutes at 4°C. The pellets were washed with buffer C and centrifuged again. The pellets were homogenized in 2% Triton X-100 (Sigma) and Complete EDTA-free (Roche Applied Science) in buffer C (buffer D) for 2 hours on ice with occasional vortexing. After centrifugation at 15,000 rpm for 30 minutes at 4°C, the supernatant was collected.

WGA-agarose (Vector Laboratories Inc.) was washed with 0.1% Triton X-100 and Complete EDTA-free in buffer C (buffer E). The lysate from C2C12 cells was added to the WGA-agarose and rotated for 16 hours at 4°C. WGA-agarose was washed with buffer E and then eluted with buffer E containing 300 mM N-acetylglucosamine (Sigma). The eluted fraction was dialyzed with buffer C. The concentration was determined with the BCA assay kit.

4.6. α DG Binding Assay of rec- α 2LG4-5

The rec- α 2LG4-5 protein (0.15 μ g) and partially purified α DG from C2C12 cells (15 μ g) were mixed in 60 μ l of buffer C containing 1 mM CaCl₂ and 1 mM MgCl₂ (buffer F). After a 1 hour incubation at 4°C, Protein G-agarose beads (7.5 μ l) (Thermo Scientific) in 15 μ l of buffer F were added into the mixture to precipitate rec- α 2LG4-5. The samples were mixed every 15 minutes during a 1 hour incubation at 4°C. The beads were pelleted by centrifugation. The

supernatant was removed, and the beads were washed three times with buffer F. Then, the α DG bound to rec- α 2LG4-5 was eluted with SDS-PAGE sample buffer. Each sample was analyzed by SDS-PAGE under reducing conditions using a 4-12% polyacrylamide Bis-Tris gel (Invitrogen). After electrophoresis, α DG was transferred to a PVDF membrane (Invitrogen). The blotted membrane was blocked with 5% skim milk in buffer C. Then, α DG was detected by IIH6, mouse anti- α DG antibody (kindly contributed by Dr. Kevin P. Campbell) (1:1000 dilution), and a goat anti-mouse IgM antibody-conjugated horseradish peroxidase (Thermo Scientific) (1:1000 dilution) using SuperSignal West Pico Chemiluminescent Substrate (Thermo Scientific).

For inhibition assays using synthetic peptides, peptide (7.5 μ g) was incubated with the rec- α 2LG4-5 protein and partially purified α DG in buffer F. Then, α DG bound to rec- α 2LG4-5 was extracted with SDS-PAGE sample buffer, and the eluted sample was analyzed by Western blotting as described above. The relative amount (%) of α DG bound to rec- α 2LG4-5 was assessed using Image J software.

4.7. α DG Binding Assay of Peptides Using Peptide-Conjugated Sepharose Beads

Synthetic peptides were coupled to cyanogen bromide (CNBr)-activated Sepharose 4B (GE Healthcare Bio-Sciences Corp.) as previously described (Nomizu et al., 1998). Peptide solutions (66.7 μ l, 1 mg/ml in Milli-Q H₂O) were mixed with 5 mg of the CNBr-activated Sepharose beads. The peptide-conjugated beads were incubated with partially purified α DG from C2C12 cells (15 μ g) in buffer F at 4°C overnight. The beads were pelleted by centrifugation and the supernatant was removed. The beads were then washed three times with buffer F and the α DG bound to the beads was eluted with SDS-PAGE sample buffer. Each sample was analyzed by SDS-PAGE and Western blotting to detect α DG, as described above.

4.8. Ex Vivo SMG Organ Culture

SMG rudiments dissected from embryonic day 12.5 ICR mice were cultured on polycarbonate track-etch filters (13 mm, 0.1- μ m pore size, Sterlitech Corp.) at the air/medium interface (Hoffman et al., 2001). The filters were floated on 240 μ l of DMEM/F-12 in 50-mm glass-bottom microwell dishes (MatTek). The medium was supplemented with 100 units/ml penicillin, 100 μ g/ml streptomycin, 150 μ g/ml vitamin C, and 50 μ g/ml transferrin. Gland rudiments were cultured on each filter in the presence of 100-150 μ g/ml of peptides at 37°C in a humidified 5% CO₂, 95% air atmosphere. Glands were photographed after 2 hours and 40 hours, and the number of end buds was counted and expressed as a ratio (the number of end buds at 40 hours/number at 2 hours).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Fig. 1. Inhibitory effect of peptides on heparin binding to rec-α2LG4-5. (A) The rec-α2LG4-5 protein, peptide, and heparin-Sepharose beads were incubated in 10 mM Tris buffer (pH 7.4) containing 100 mM NaCl. After washing the beads, the rec-α2LG4-5 protein bound to heparin-Sepharose beads was analyzed by Western blotting as described in Materials and Methods. (B) Various amounts of A2G78 and A2G78S were tested in the same assay. The relative amount (%) of rec-α2LG4-5 bound to heparin-Sepharose beads was assessed using NIH image 1.63 software. Triplicate experiments produced similar results. *, Inhibition of heparin binding to rec-α2LG4-5 by A2G78.

Fig. 2. Effect of alanine substitutions on heparin binding to rec- α 2LG4-5. Mutant rec- α 2LG4-5 proteins that have an alanine substitution in the A2G78 site (L²⁷⁹⁸, Y²⁸⁰⁰, R²⁸⁰³, and I²⁸⁰⁴) were prepared. These are named 3LA, 5YA, 8RA, and 9IA, respectively. The same amounts of the proteins were incubated with heparin-Sepharose beads in the presence of 150 mM NaCl. After washing the beads, proteins bound to heparin-Sepharose were detected by Western blotting as described in Materials and Methods.

Fig. 3.

Solid phase heparin binding assay of peptides. (A) Binding of biotinylated heparin to peptide-coated plates was evaluated. ELISA plates with 96 wells were coated with various amounts of peptides. After blocking with 3% BSA, biotinylated heparin was added, incubated and washed, and the biotinylated heparin bound to the peptides was detected by SA-HRP. (B), (C) Inhibitory effect of heparin (B) or HS (C) on binding of biotinylated heparin to the A2G78- and A2G78S-coated wells (1 μ g/well) was examined. Black bar: A2G78, white bar; A2G78S. Triplicate experiments gave similar results. $p < 0.01 = *$.

Fig. 4.

α DG binding assay of peptides by peptide-affinity chromatography. A total of 41 peptide-conjugated beads were prepared using CNBr-activated Sepharose. The eluate of WGA-agarose from the C2C12 cell lysate was added to the suspension of peptide-conjugated beads in the presence of 1 mM CaCl_2 and MgCl_2 . After incubation, α DG bound to the peptides was detected by an anti- α DG antibody, I1H6. Weak α DG binding of A2G84, A2G97, A2G103, A2G104, A2G109, A2G110, and A2G111 was detected with longer time exposures of chemiluminescence detection (data not shown). Duplicate experiments gave similar results.

Fig. 5.

Inhibitory effect of peptides on α DG binding to rec- α 2LG4-5. (A) α DG, rec- α 2LG4-5, and each peptide were mixed and incubated in the presence of 1 mM CaCl_2 and MgCl_2 . After the incubation, Protein G-agarose beads were added to collect rec- α 2LG4-5. After washing the beads, α DG bound to rec- α 2LG4-5 was analyzed by Western blotting as described in Materials and Methods. (B) The relative amount (%) of α DG bound to rec- α 2LG4-5 was assessed using Image J software. Triplicate experiments gave similar results. $p < 0.03 = *$.

Fig. 6.

The peptides reduce branching morphogenesis of mouse SMGs in organ culture. (A) SMG organ culture is described under Materials and Methods. Peptides were added to the culture media and the glands were cultured for 40 hours. (B) Branching morphogenesis is quantitated by counting the number of terminal epithelial buds at 2 hours and 40 hours. Each value represents the mean of five separate determinations $\pm$ S.D. Triplicate experiments gave similar results. $p < 0.01 = *$. (C) Expression levels of mRNA for DG (Dag1) and the laminin $\alpha 2$ chain (Lama2) were evaluated after peptide treatment. After a 40 hour incubation, RNA was extracted from the cultured glands and quantitative RT-PCR was carried out. Ribosomal protein S29 was used as a standard. Triplicate experiments gave similar results.

Table 1Biological activities of synthetic laminin $\alpha 2$ chain LG4-5 module peptides.

Peptide	Sequence	Heparin binding		α DG binding
		Inhibition of ^{a)} rec- α 2LG4-5	heparin ^{b)} binding	α DG ^{c)} binding
A2G72	VAESEPALLTGSK	–	–	–
A2G73	LTGSKQFGLSRN	–	+	–
A2G74	LSRNSHIAIAFD	–	++	–
A2G75	IAFDDTKVKNRL	–	–	–
A2G76	KNRLTIELEVRT	–	–	–
A2G77	LEVRTEAESGLLF	–	–	–
A2G78	GLLFYMARINHA	+	+	++
A2G79	INHADFATVQLR	–	–	–
A2G80	VQLRNGFPYFSY	–	–	++
A2G81	YFSYDLGSGDTS	–	–	–
A2G82	GDTSTMIPTKIN	–	–	–
A2G83	TKINDGQWHKIK	–	+	–
A2G84	HKIKIVRVKQEG	–	++	+/-
A2G85	KQEGILYVDDAS	–	–	–
A2G86	DDASSQTISPKK	–	–	–
A2G87	SPKKADILDVVG	–	–	–
A2G88	DVVGILYVGGLP	–	–	–
A2G89	GGLPINYTTRRI	–	+	–
A2G90	TRRIGPVTYSLDG	–	–	–
A2G91	VRNLHMEQAPVDLD	–	–	–
A2G92	VDLDQPTSSFHVGT	–	–	–
A2G93	FANAESGTYFDG	–	–	–
A2G94	YFDGTGFAKAVG	–	–	–
A2G95	KAVGGFKVGLDL	–	+	–
A2G96	GLDLLVEFEFRT	ND	ND	ND
A2G97	FEFRTTRPTGVLL	–	–	+/-
A2G98	GVLLGVSSQKMDG	–	–	–
A2G99	KMDGMGIEMIDEK	–	–	–
A2G100	IDEKLMFHVDNG	–	–	–
A2G101	HVDNGAGRFTAI	–	–	–
A2G102	RFTAIYDAEIPGHM	–	–	–
A2G103	NGQWHKVTAKKI	–	+	+/-
A2G104	AKKIKNRLELVV	–	++	+/-
A2G105	RLELVVDGNQVDAQ	–	–	–
A2G106	VDAQSPNSASTS	–	–	–
A2G107	ASTSADTNDPVF	–	–	–
A2G108	DPVFVGGFPGGL	–	–	–

Peptide	Sequence	Heparin binding		α DG binding
		Inhibition of ^{a)} rec- α 2LG4-5	heparin ^{b)} binding	α DG ^{c)} binding
A2G109	GFPGGLNQFGLTTN	–	+	+/-
A2G110	NQFGLTTNIRFRG	–	++	+/-
A2G111	IRSLKLTGKTGKP	–	++	+/-
A2G112	TGKPLEVNFAKAL	–	–	–
A2G113	AKALELRGVQPVS	–	–	–

^{a)} Inhibitory effect of peptides on heparin binding of rec- α 2LG4-5 was evaluated as described in Materials and Methods. +, inhibition; –, no inhibition.

^{b)} Heparin binding activities of peptides were performed by a solid phase binding assay as described in Materials and Methods. The activities were evaluated compared to AG73 as a positive control peptide: ++, binding comparable to that on AG73; +, weak binding compared with that on AG73; –, no binding.

^{c)} α DG binding activities of peptides were performed by peptide-affinity chromatography as described in Materials and Methods: ++, intense bands by western blotting; +/-, weak bands were detected with longer time exposures by western blotting; –, no band was detected by Western blotting. Active peptides are shown in bold.

Table 2

Alanine substituted peptides of A2G78 and their inhibitory effect on heparin binding of rec- α 2LG4-5.

Peptide	Sequence	Inhibition ^{a)}
A2G78	GLLFYMARINHA	++
A2G78-1GA	A LLFYMARINHA	++
A2G78-2LA	G A LFYMARINHA	+
A2G78-3LA	GL A FYMARINHA	–
A2G78-4FA	GLL A YMARINHA	+
A2G78-5YA	GLLF A MARINHA	–
A2G78-6MA	GLLFY A ARINHA	+
A2G78-8RA	GLLFYMA A INHA	–
A2G78-9IA	GLLFYMAR A NHA	–
A2G78-10NA	GLLFYMAR I AHA	+
A2G78-11HA	GLLFYMARIN A A	++

^{a)} Activity was scored on the following subjective scale: ++, inhibitory effect on heparin binding of rec- α 2LG4-5 comparable to that on A2G78; +, weak inhibitory effect compared with that on A2G78; –, no inhibition. Bold A represent a substitution mutation.

Table 3

Truncated peptides of A2G78 and their inhibitory effect on heparin binding of rec- α 2LG4-5.

Peptide	Sequence	Inhibition ^{a)}
A2G78	GLLFYMARINHA	+
A2G78N-1	LLFYMARINHA	–
A2G78N-2	LFYMARINHA	–
A2G78N-3	FYMARINHA	–
A2G78N-4	MARINHA	–
A2G78N-5	ARINHA	–
A2G78C-1	GLLFYMARINH	+
A2G78C-2	GLLFYMARIN	+
A2G78C-3	GLLFYMARI	+
A2G78C-4	GLLFYMAR	–
A2G78C-5	GLLFYMA	–

^{a)} Activity was scored on the following subjective scale: +, inhibitory effect on heparin binding of rec- α 2LG4-5 comparable to that on A2G78; –, no inhibition.