β1 Integrin and α-dystroglycan binding sites are localized to different laminin-G-domain-like (LG) modules within the laminin α5 chain G domain

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Laminins are a group of extracellular-matrix proteins important in development and disease. They are heterotrimers, and specific domains in the different chains have specialized functions. The G domain of the α5 chain has now been produced in transfected mammalian cells as single modules and two tandem arrays, α5LG1–3 and α5LG4–5 (LG is laminin G domain-like). Using these fragments we produced specific polyclonal antibodies functional in immunoblotting and immunofluorescence studies and in solid-phase assays. Both α5LG tandem arrays had physiologically relevant affinities for sulphated ligands such as heparin and sulphatides. Cells adhered to these fragments and acquired a spread morphology when plated on α5LG1–3. Binding of integrins $\alpha3\beta1$ and $\alpha6\beta1$ was localized to the α5LG1–3 modules, and α-dystroglycan binding was localized to the α5LG4–5 modules, thus locating these activities to different LG modules within the laminin α5 G domain. However, both these activities were of relatively low affinity, indicating that integrin-mediated cell adhesion to the laminin 10/11 α5G domain depends on contributions from the other chains of the heterotrimer and that high-affinity α-dystroglycan binding could be dependent on specific Ca^{2+}-ion-co-ordinating amino acids absent from α5LG4–5.

Key words: basement membrane, cell–matrix interaction, protein module, receptor, recombinant protein.

INTRODUCTION

The basement membrane is a specialized form of extracellular matrix (ECM) underlying epithelial and endothelial cells and surrounding muscle, peripheral nerve and fat cells. Its diverse functions include supporting and separating cells from the underlying stromal matrix and influencing cell activities such as adhesion, migration, proliferation, differentation and apoptosis through the interaction with specific receptors [1].

Laminins are composed of three distinct chains termed α, β and γ, and they play an important role in the formation, architecture and stability of basement membranes [2]. To date, 11 laminin chains forming at least 15 different heterotrimers have been described [2,3]. The α5 chain-containing isoforms, laminin-10 ($\alpha5\beta1\gamma1$), laminin-11 ($\alpha5\beta2\gamma1$) and laminin-15 ($\alpha5\beta2\gamma3$), are newly discovered laminins [3,4]. The α5 chain is widely distributed in embryonic as well as adult tissues [4]. Previously, it has been demonstrated that laminin α5 chain is required for normal development during embryogenesis [5]. These gene-targeting experiments, together with the broad distribution of laminin α5 chain, suggest distinct biological functional roles which have not yet been extensively studied. The adhesion of laminin-10/11 to different cells has also been studied, and integrin receptors involved have been identified as integrins $\alpha3\beta1$ [6], $\alpha6\beta1$ [7], $\alpha6\beta4$ [8] and the extracellular splice variant X1 of integrin $\alpha7\beta1$ [9]. Another integrin that has been reported to bind to the N-terminal domain IVa of laminin α5 chain is $\alpha V\beta3$ [10]. Other non-integrin receptors binding to laminin 10/11 are Lu/B-CAM [11] and α-dystroglycan [12].

Like most ECM proteins, laminin chains consist of several domains, and specific domains have special functions. N-terminal domains VI in α, β and γ chains connect laminin molecules to each other, forming one of the two self-assembling networks of basement membranes. The other is formed by collagen IV [13]. The laminin γ1III4 domain connects these networks to each other through its binding to nidogen-1 [14] and possibly nidogen-2 [15], which in their turn bind collagen IV [15,16]. In the C-terminal ends of the α chains there are globular G domains. These are composed of five similar, but not identical, laminin-G-domain-like (LG) modules [17]. Receptor-binding sites have been mapped to several domains on laminins [2]. However, integrins $\alpha3\beta1$ and $\alpha6\beta1$, as well as α-dystroglycan, identified as laminin 10/11 receptors, are known to bind the G domains of laminin α1 and/or α2 chains [18–20].

Structural studies of laminin α1 and α2 LG domains have led to a model for α-dystroglycan binding, predicting that it should not bind to laminin α5 [21], whereas recombinant laminin α5LG1–5 produced in bacteria weakly bind α-dystroglycan [22]. We therefore undertook to produce, by recombination, the different α5LG modules 1–5, as well as the tandem modules α5LG1–3 and α5LG4–5 in mammalian cells in order to clarify this discrepancy and to determine the receptor-binding repertoire of the laminin α5 chain G domain.

Using these fragments we were able to localize integrin and α-dystroglycan binding to different LG modules within the laminin α5 G domain. However, both these activities showed relatively low affinity, indicating that integrin-mediated cell adhesion to laminin 10/11 depends on contributions from the other chains of the heterotrimer and that high-affinity α-dystroglycan binding may be dependent on specific Ca^{2+}-ion-co-ordinating amino acids absent from α5LG4–5.

EXPERIMENTAL

Vector construction and recombinant protein production

Mouse liver RNA was used as a template to amplify sequences encoding the five laminin α5 chain LG modules individually or

Abbreviations used: DMEM, Dulbecco's modified Eagle's medium; ECM, extracellular matrix; FBS, fetal bovine serum; LG, laminin-G-domain-like; RT-PCR, reverse-transcription PCR; WGA, wheat-germ agglutinin.

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in combinations, by reverse-transcription (RT)-PCR using Superscript II (Gibco Life Technologies) and Vent polymerase (Clontech) following the manufacturers' instructions. The 5′ end primers used were: GTCAGCTAGCGTCCATGAAGTTCAAAATGGGC (LG1 and LG1–3), GTCAGCTAGCCAAGGCCACCGGTGACCCAT (LG2), GTCAGCTAGCCCTGCTAGTGGGACGCAC (LG3), GTCAGCTAGCACCAGCCCAACTCATCGAG (LG4 and LG4–5), and GTCAGCTAGCTGTCTCAGGCCCCCTGGAA (LG5).

The 3′ end primers used were: GTCACTCGAGCTAGGCCTTGGAGCGAGCACA (LG1), GTCACTCGAGCTATAGCAGGTCAGCGGTGCAGCC (LG2), GTCACTCGAGCTATGAGGTCTCGATGAGTTGGG (LG3 and LG1–3), GTCACTCGAGCTAGCCTGAGACACAGGGTGTG (LG4), and GTCACTCGAGTCAATGCCAAAGTAGCGGGG (LG5 and LG4–5).

In addition to the coding sequences, these primers introduced a stop codon and single *Nhe*I and *Xho*I restriction sites in order to allow in-frame insertion of the cDNA distal to the BM-40 signal peptide sequence present in the episomal expression vector pCEP-Pu [23]. The PCR products were initially ligated into plasmid pUC18 (Pharmacia), and sequences were verified by sequencing. PCR products were cut out by *Nhe*I (Fermentas AB, Vilnius, Lithuania) and *Xho*I (Fermentas AB) digestion and ligated into plasmid pCEP-Pu.

HEK-293 cells, which constitutively express the EBNA-1 protein from Epstein–Barr virus (293-EBNA; Invitrogen) were transfected with the constructs and transfectants were selected with 0.5 μg/ml puromycin (Sigma) and 250 μg/ml G418 (Gibco Life Technologies). Transfected cells were washed extensively with PBS and grown in serum-free Dulbecco's modified Eagle's medium (DMEM)/F12 medium (Gibco Life Technologies) for 2 days, after which the medium was harvested and replaced by new serum-free medium for another 2 days.

Purification of proteins

Conditioned serum-free medium (1 litre) was dialysed against 50 mM Tris/HCl (pH 7.4)/0.5 mM PMSF (Sigma)/0.5 mM *N*-ethylmaleimide (Sigma). Recombinant proteins were isolated on a Hi-prep heparin–Sepharose column (16/10; Amersham Pharmacia Biotech) in 50 mM Tris/HCl, pH 7.4, and eluted with a salt gradient (0–0.5 M NaCl). They were then further purified on a Superose 12 column (HR 16/50; Amersham Pharmacia) equilibrated in 0.2 M ammonium acetate, pH 6.8.

A rat schwannoma cell line, RT4, was used as a source of α-dystroglycan [24]. RT4-cell-culture supernatant (0.8 litre) was circulated over 10 ml of wheat-germ agglutinin (WGA)–Sepharose (Amersham) at 4 °C overnight in the presence of 0.5 M NaCl. The WGA–Sepharose was washed with 50 mM Tris/HCl, pH 7.4, containing protease inhibitors (0.75 mM benzamidine, 0.1 mM PMSF, 0.7 μM pepstatin A, 76.8 nM aprotinin and 1.1 μM leupeptin) (buffer A) containing 0.5 M NaCl. Bound material was eluted with buffer A containing 0.3 M *N*-acetylglucosamine. A 1 ml portion of mouse laminin-1-coupled Sepharose was blocked with buffer A containing 1 mM $CaCl_2$, 1 mM $MgCl_2$ and 3% BSA at 4 °C for 2 h, and washed with buffer A containing 1 mM $CaCl_2$ and 1 mM $MgCl_2$. The *N*-acetylglucosamine eluate from WGA–Sepharose was incubated with laminin-1–Sepharose at 4 °C overnight in the presence of 1 mM $CaCl_2$ and 1 mM $MgCl_2$. After washing with buffer A containing 1 mM $CaCl_2$ and 1 mM $MgCl_2$, α-dystroglycan was eluted with buffer A containing 10 mM EDTA.

Antibodies and other reagents

Laminin-1 and laminin-10/11 were from a commercial source (Gibco Life Technologies). Recombinant α2LG2, α2LG1–3, α2LG4–5, α4LG1–3, α4LG4–5 were produced as described in [25,26]. BSA-coupled heparin (heparin–BSA) and sulphatides were from a commercial source (Sigma).

The following commercial antibodies were used: CD 29 hamster anti-rat mAb Ha2/5 against β1-integrin subunit (Pharmingen), CD 49f rat anti-human mAb GoH3 recognizing the α6 integrin subunit (BD Biosciences, Erembodegem, Belgium), and mouse anti-human mAb P1B5 recognizing the α3 integrin subunit (Chemicon International Inc). Anti-α-dystroglycan (clone IIH6) was kindly given by Professor Kevin Campbell, Department of Physiology and Biophysics, and Howard Hughes Memorial Institute, University of Iowa, Iowa City, IA, U.S.A.

Rabbit antisera were generated against laminin α5LG1–3 and α5LG4–5 by two injections of 50–100 μg in complete Freund's adjuvant. Antisera were then passed over columns filled with CNBr-activated Sepharose coupled with recombinant fragments α5LG1–3 and α5LG4–5 respectively. Columns were prewashed with 20 ml of PBS, followed by the rabbit antisera. After passage of the antiserum, columns were washed with 20 ml of PBS until the A_{280} was below 0.02–0.03. Antibodies were then displaced by elution with 5 ml of 1 M acetic acid, followed by 3 ml of 0.05 M HCl/0.15 M NaCl. The acidic eluates were immediately neutralized by addition of 4 M Tris base and dialysed against PBS [27].

Cell-adhesion assays

A human lung simian-virus-40-transformed epithelial cell line WI-26 VA4 (A.T.C.C. no. CCL-95-1) was grown in DMEM containing 10% fetal bovine serum (FBS). The human Wilm's tumour cell line WCCS-1 with some epithelial features [28] was grown in Eagle's minimal essential medium containing Earle's salts and 10% FBS. The mouse multipotent haematopoietic FDCP-Mix cell line [29] was maintained in Iscove's Modified Dulbecco's Medium ('IMDM') supplemented with 20% (v/v) horse serum and recombinant interleukin-3 (PeproTech EC Ltd, Hammersmith, London, U.K.). The human U-937 cell line was grown in RPMI 1640 medium containing 10% FBS. Microtitre plates (96-well; Greiner, Nürtingen, Germany) were coated with recombinant protein dissolved in PBS at 4 °C overnight and then blocked with PBS containing 1% BSA for another 1 h at the same temperature. Cells were trypsin-treated and suspended in serum-free medium at a density of 1×10^5 cells/ml; then 0.1 ml of the cell suspension was added to each well of the plates, followed by incubation at 37 °C for 1 h. The attached cells were fixed with 96% ethanol and stained with 0.1% Crystal Violet in distilled water. After washing with distilled water, the attached cells were photographed, lysed with 0.2% Triton X-100 and incubated for 1 h at 37 °C. Absorbance was measured at 595 nm in a DigiScan microtiter plate reader (LabVision, Stockholm, Sweden).

For the adhesion-inhibition assays, the cells were preincubated with monoclonal antibodies against different types of integrins and α-dystroglycan, polyclonal antisera against α5LG domains, or recombinant α5LG tandem fragments at 4 °C for 1 h. U-937 cells were in addition treated with 5 ng/ml PMA for 4 h before the assay. The preincubated cells were transferred on to plates coated with laminin 10/11 or recombinant protein and then incubated at 37 °C for 1 h. After staining with Crystal Violet, the attached cells were quantified as described above.

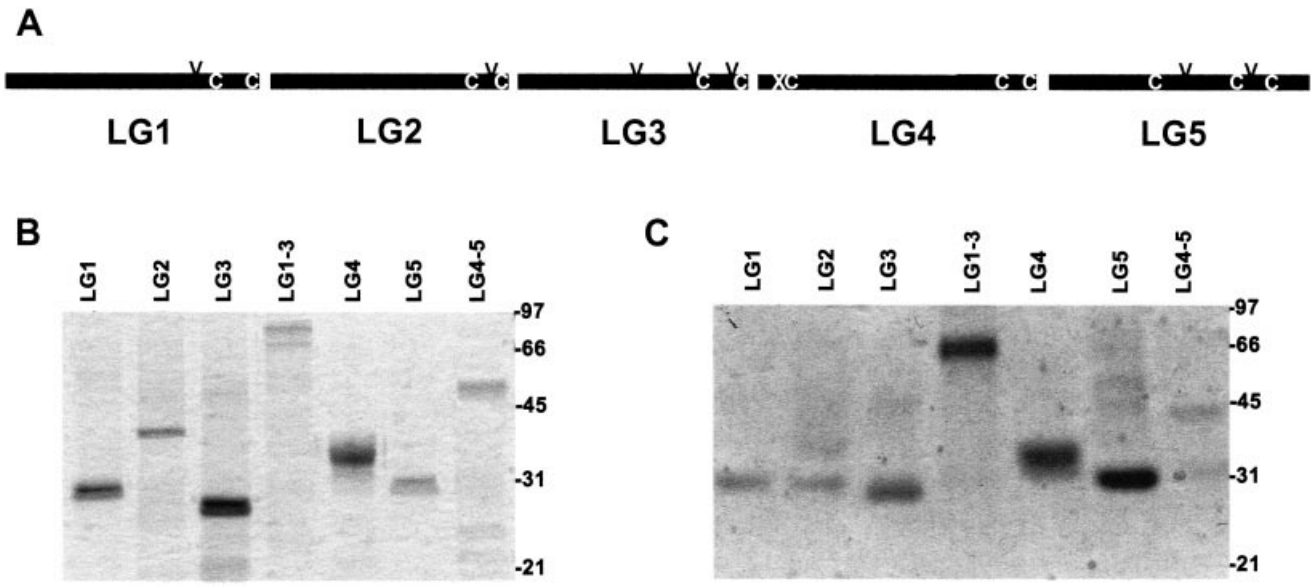

Figure 1 Sequence borders and structural properties of recombinant LG modules of the mouse laminin α5 chain

(**A**) Modular composition of the laminin α5 G domain. Black bars represent LG modules, where the positions of the cysteine residues are indicated by C, the putative furin-protease cleavage site RRSR in the beginning of LG4 is indicated by X and potential N-glycosylation sites are marked by V. (**B**) SDS/PAGE of purified recombinant fragments from G domain of laminin α5 chain. Purified proteins were electrophoresed on 12%-(w/v)-polyacrylamide gels under reducing conditions. Proteins were stained with Coomassie Brilliant Blue. Molecular markers are denoted in kDa to the right. (**C**) SDS/PAGE of purified recombinant fragments from G domain of laminin α5 chain after incubation with endoglycosidase H. Lanes and conditions are as in (**B**).

Unpaired Student *t* tests with Welch correction were performed to statistically evaluate differences in cell adhesion.

Ligand-binding assays

Solid-phase assays were carried out with α-dystroglycan, sulphatides or heparin–BSA coated on to the plastic surface of microtitre wells at 4 °C and by using a previously described procedure [19]. Coated wells were blocked at rom temperature (2 h) with 0.05 M Tris (pH 7.4)/0.15 M NaCl (TBS), 1 % BSA and 5 mM $CaCl_2$, then washed and incubated with soluble ligands serially diluted in the same buffer for 1 h. After washing, bound ligands were detected with specific rabbit antisera, which were diluted to give a maximum absorbance of ≈ 0.8 at 490 nm. After a further wash, bound antibodies were detected by addition of horseradish-peroxidase-conjugated goat anti-rabbit IgG (Bio-Rad) followed by addition of 1 mg/ml 5-amino-2-hydroxybenzoic acid (Sigma) and 0.01 % H_2O_2. Heparin binding was assayed by coating with 10 μg/ml heparin–BSA (Sigma) and incubating for 3 h with soluble ligands. Coating with sulphatides (Sigma) dissolved in methanol (0.2 mg/ml) was achieved by drying at room temperature (20 °C) overnight.

Immunohistochemistry

Cryosections were washed in PBS and blocked with 1 % BSA in PBS for 30 min at room temperature. Antibody solutions were applied at 0.1 μg/ml, and incubated for 1 h at room temperature. Slides were washed in PBS/BSA and secondary antibodies coupled to a fluorochrome (Cy3; Jackson ImmunoResearch Laboratories Inc., West Grove, PA, U.S.A.) were used for detection.

Analytical methods

Edman degradation was performed with a process protein sequencer (Applied Biosystems), following the manufacturer's instructions. SDS/PAGE involved standard protocols. For immunoblotting, proteins were transferred to PVDF filters (Amersham), which were incubated with primary antibodies, washed and allowed to react with horseradish peroxidase-conjugated secondary antibodies (Bio-Rad). Protein was detected with the Amersham ECL® (enhanced chemiluminescence) kit. Digestion with endoglycosidase H (New England Biolabs Inc.) to remove oligosacharides from N-linked glycoproteins was performed according to the manufacturer's instructions.

RESULTS

Recombinant production of laminin α5 G domain modules

The G domain of mouse laminin α5 was prepared in the form of two recombinant fragments, α5LG1–3 (residues Ser^{2654}–Ala^{3215}) and α5LG4–5 (residues Gln^{3216}–His^{3634}), as well as in the form of single LG modules (LG1 Ser^{2654}–Ser^{2852}, LG2 Lys^{2853}–Asp^{3038}, LG3 Leu^{3039}–Ala^{3215}, LG4 Gln^{3216}–Gly^{3430}, and LG5 Pro^{3431}–His^{3634}), following strategies previously used for the laminin α2 and α4 chains [25,26]. The boundaries chosen were outside the predicted β-sandwich structure of the LG modules [21], and the border between LG3 and LG4 was placed at the beginning of a long link region (Figure 1A). All fragments were produced and the yields were good for the larger fragments (0.4–0.8 mg/l). Because of their strong affinity for heparin, all fragments could be purified by a two-step chromatographic procedure, as shown by electrophoresis (Figure 1B).

Fragment α5LG1–3 migrated as a doublet with molecular masses of 70 and 84 kDa. Both had the N-terminal sequence APLASMK, where APLA is derived from the BM-40 signal

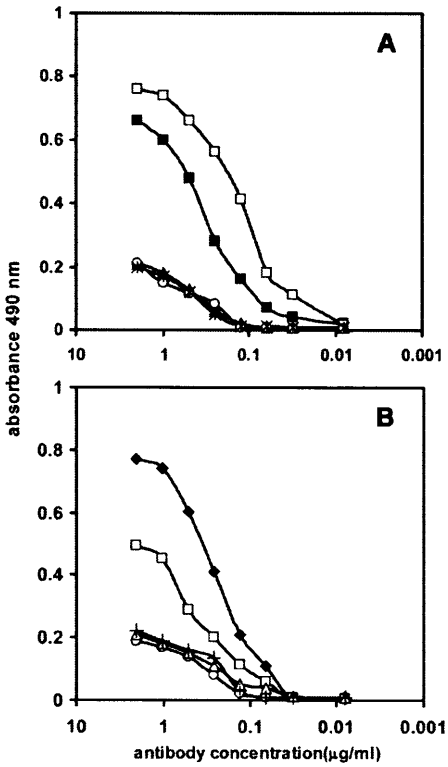

Figure 2 Titration of affinity-purified antibodies against α5LG1-3 (A) and α5LG4–5 (B) in ELISAs

Antigens used were mouse laminin 1 (○), human laminin 10/11 (□), recombinant proteins α5LG1–3 (■), α5LG4–5 (◆), α4LG1–3 (▲), α4LG4–5 (△), α2LG1–3 (∗), α2LG4–5 (+).

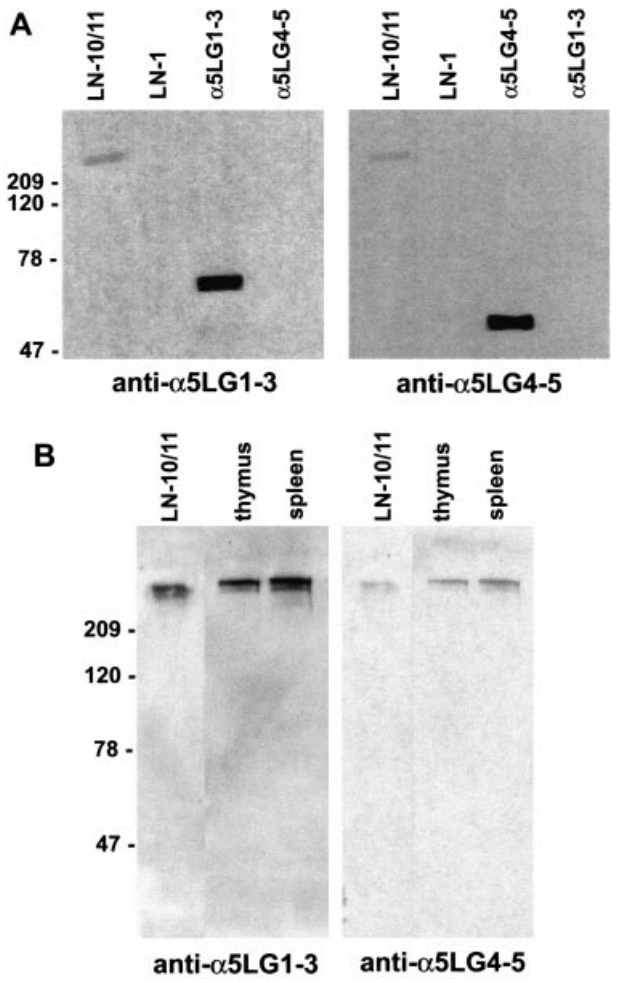

Figure 3 Immunoblotting analysis of purified recombinant laminin fragments α5LG1–3 and α5 LG4–5 (A) and laminin expression in tissues (B)

(**A**) Recombinant proteins were subjected to SDS/12%-PAGE under reducing conditions. Proteins were then transferred on to PVDF membranes, followed by immunodetection with polyclonal antibodies against α5LG1–3 and α5LG4–5. The gel was loaded with recombinant fragments and commercial laminin preparations. (**B**) Protein extracts from 6-day-old-mouse thymus and spleen were subjected to SDS/PAGE using 3–12% gradient gels under reducing conditions. Proteins were then transferred on to PVDF membranes, followed by immunodetection with polyclonal antibodies against α5LG1–3 and α5LG4–5. Human laminin (LN) 10/11 was loaded as a control.

peptide region of the pCEP-Pu vector [23]. Fragments α5LG1, α5LG2, α5LG3, α5LG4, α5LG5, and α5LG4–5 migrated as bands of 30, 41, 26, 35, 30 and 51 kDa respectively (Figure 1B). They also showed the expected single N-terminal amino acid sequences after Edman degradation. However, some microheterogeneity was apparent in several fragments. To determine if this was due to differential N-glycosylation, fragments were incubated with endoglycosylase H and separated by SDS/PAGE. After removal of N-linked oligosacharides, all fragments run as single bands (Figure 1C). Fragment α5LG1–3 now migrated as a band of 66 kDa, α5LG4–5 as a band of 44 kDa and α5LG2 migrated as a band of 29 kDa. For the other fragments, the decrease in size was minimal. There is a putative furin protease cleavage site in the linker region between α5LG3 and α5LG4 [17] (furin is a ubiquitous subtilisin-like proprotein convertase). However, no evidence was found for proteolytic processing in the recombinantly produced fragments α5LG4–5 or α5LG4.

Production and specificity of antisera against laminin α5 recombinant proteins

In order to measure binding in solid-phase assays and to further characterize tissue forms of laminin α5 chain, rabbit antisera were generated against the two tandem domains α5LG1–3 and α5LG4–5. The antibodies were purified by passing them over Sepharose columns coupled with the tandem domains α5LG1–3 and α5LG4–5 respectively, and the purified antibodies were titrated against laminin-1, human laminin 10/11 and recombinant proteins of the LG modules from laminin α2, α4 and α5. Titration curves showed the antisera to be specific for the respective LG domains. Moreover, both antisera, especially the one against mouse α5LG1–3, cross-reacted with human laminin 10/11 containing the α5 chain (Figure 2).

Immunoblotting of recombinant α5LG domains, laminin-1 and laminin 10/11 with these antisera demonstrated specific reactions with the recombinant fragments at the expected molecular masses of 70 kDa (α5LG1–3) and 51 kDa (α5LG4–5) respectively (Figure 3A). Both antisera reacted with single bands of approx. 350–400 kDa in the human laminin 10/11 preparation. No reactions were observed in the laminin-1 lanes, demonstrating the specificity of these antisera (Figure 3A).

Immunoblotting was also performed with tissues from newborn mice (Figure 3B). Both antisera reacted with a doublet

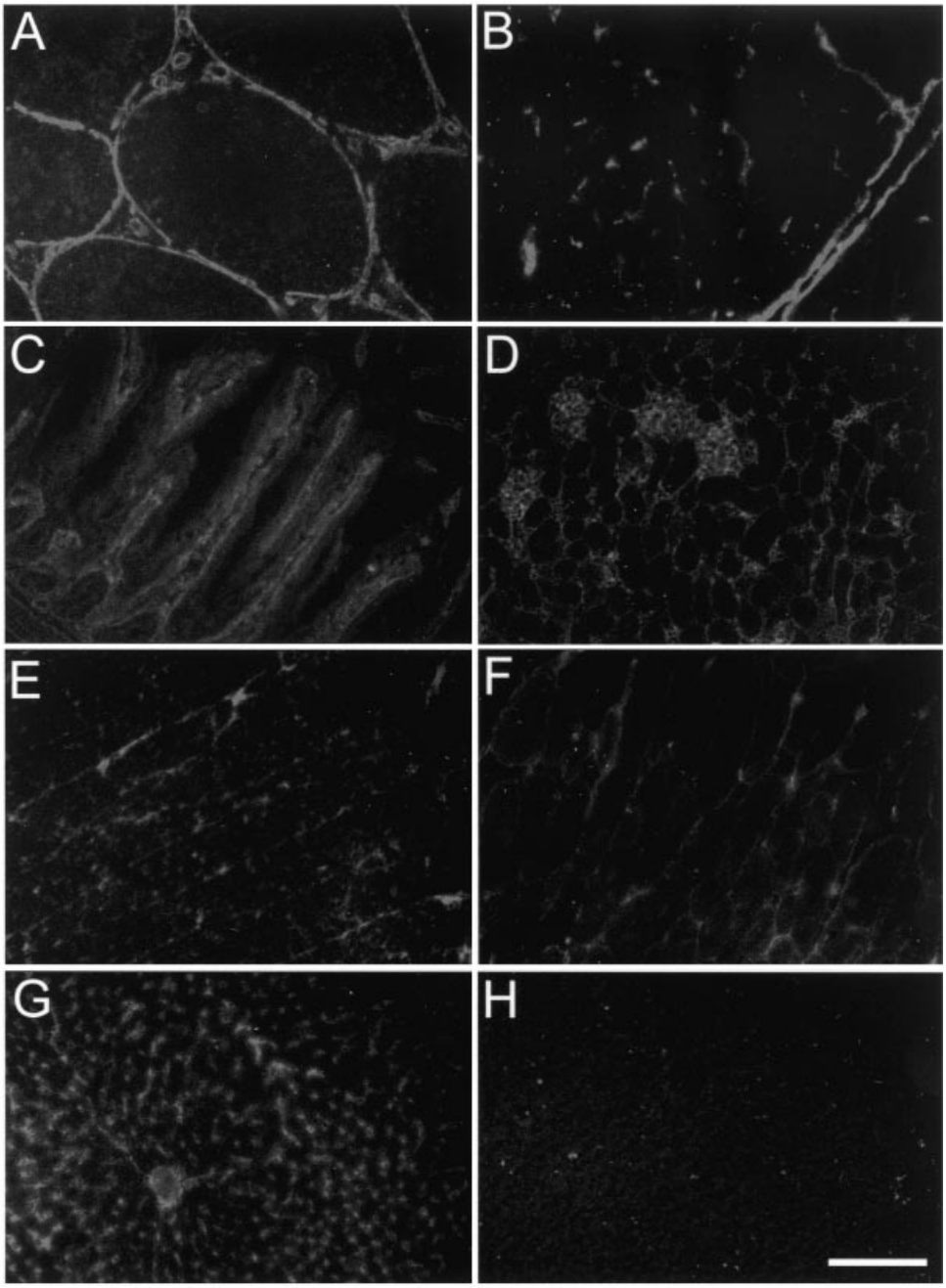

Figure 4 Localization of laminin α5 polypeptides by immunofluorescent staining of adult mouse tissues

Adult testis (**A**), brain (**B**), intestine (**C**), kidney (**D**), cardiac muscle (**E**), skeletal muscle (**F**), liver (**G** and **H**) were stained with polyclonal antibodies against α5LG1–3 (**A**–**C**), polyclonal antibodies against α5LG4–5 (**D**–**G**) or normal rabbit serum (**H**). In all tissues, blood vessels were strongly stained. In some tissues epithelial and muscle basement membranes were also stained. The horizontal bar represents 50 μm.

migrating at approx. 350–400 kDa in thymus and spleen, suggesting that this was intact laminin α5 chain. Furthermore, this doublet suggests differentially glycosylated isoforms or limited proteolytic processing. However, as no proteolytic processing was seen in recombinantly produced α5LG domains, and doublets were detected with both antisera, any proteolytic processing is suggested to occur in the N-terminal end of the laminin α5 chain.

Immunofluorescence of adult tissues

Several tissues from adult mice were examined with the two antisera. Immunofluorescent staining of testis, brain, intestine, kidney, heart muscle, skeletal muscle and liver was performed (Figure 4). In all tissues tested, an identical expression pattern was seen with both antisera, and they reacted with a pattern expected for antibodies against laminin α5 chain [4,10,30–32].

Table 1 Relative affinities of the LG modules of the laminin α5 chain for heparin and sulphatides

Heparin binding was measured by affinity chromatography in 0.05 M Tris/HCl, pH 7.4, and the NaCl concentrations required for elution are recorded. Solid-phase binding assays with immobilized heparin or sulphatides were performed in physiological buffer and recorded as the concentrations of soluble ligands required for half-maximal binding. Abbreviations: ND, not determined; nb, no binding.

	Heparin		Sulphatides
Soluble ligand	[NaCl] required for elution (M)	[NaCl] required for half-maximal binding (nM)	[NaCl] required for half-maximal binding (nM)
α5LG1	0.16	ND	ND
α5LG2	0.15	ND	ND
α5LG3	0.14	ND	ND
α5LG1–3	0.30	70	80
α5LG4	0.19	50	80
α5LG5	0.16	nb	nb
α5LG4–5	0.26	20	20

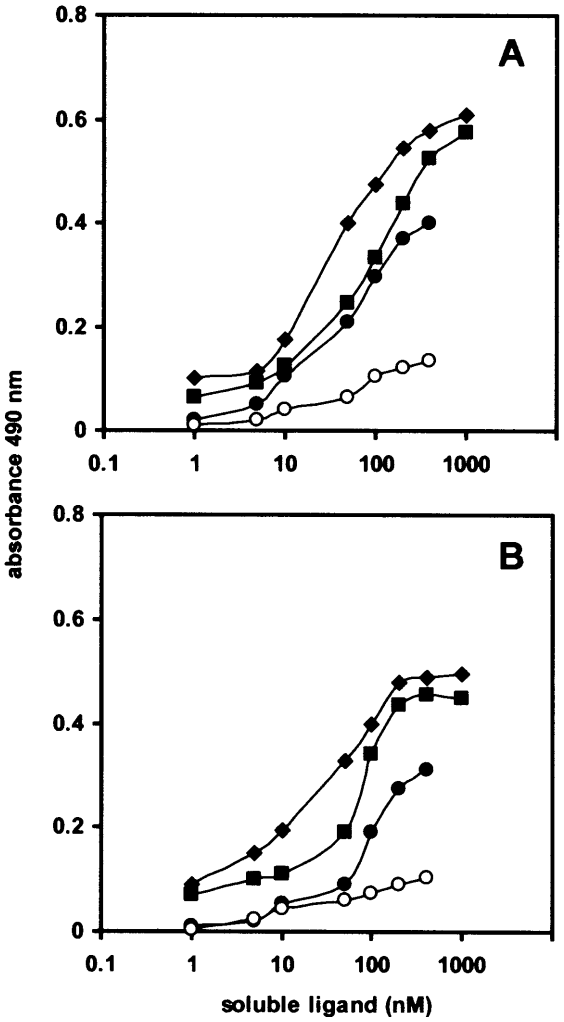

Figure 5 Profiles of solid-phase binding of recombinant laminin α5 G domain fragments to immobilized heparin–albumin (A) and sulphatides (B)

Soluble ligands were fragments α5LG1–3 (■), α5LG4–5 (◆), α5LG4 (●) and α5LG5 (○).

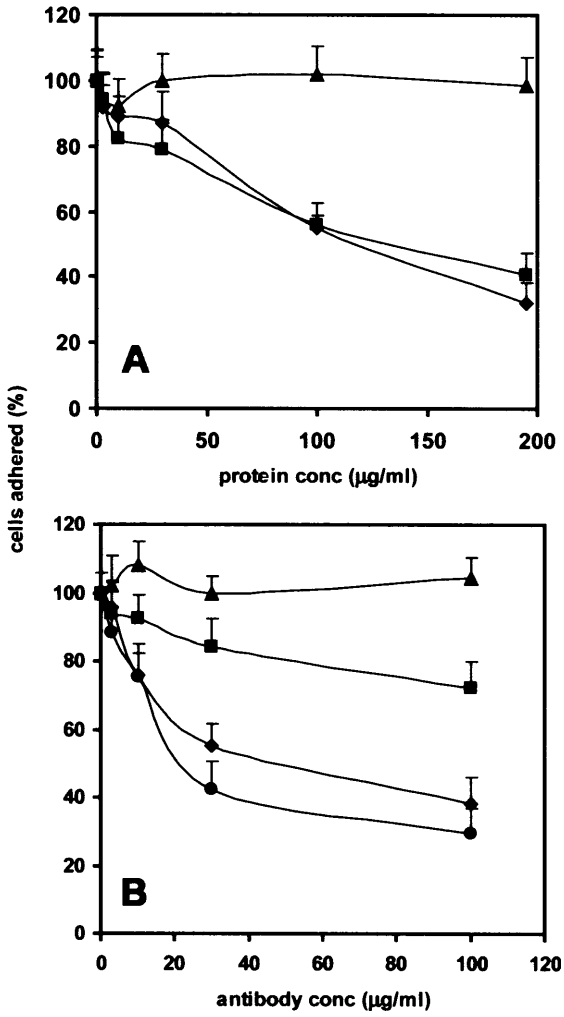

Figure 6 Inhibition of cell binding to laminin 10/11 by antibodies or recombinant proteins

(**A**) Cell binding assays on laminin 10/11 using WI26VA4 cells were performed on untreated controls or in the presence of recombinant α2LG2 (▲), α5LG1–3 (◆) or α5LG4–5 (■). (**B**) Cell binding assays on laminin 10/11 using WI26VA4 cells were performed on untreated controls or in the presence of normal rabbit serum (▲), anti-α5LG1–3 (◆), anti-α5LG4–5 (■) or combinations (●). Each value represents the mean for three separate determinations. Duplicate experiments gave similar results.

In testis, the walls of seminiferous tubules as well as blood vessels in the connective-tissue septa were positive for laminin α5 chain (Figure 4A). Staining of brain showed laminin α5 expression in both larger and smaller blood vessels as well as in the meninges (Figure 4B). In small intestine, the blood vessels of microvilli and epithelial basement membranes stained positive for laminin α5. The strongest epithelial staining was seen in the crypts of Lieberkühn, whereas epithelial staining in the villi was fainter (Figure 4C). In kidney, laminin α5 staining was apparent in glomerular basement membranes as well as in tubular and blood-vessel basement membranes (Figure 4D). Basement membranes of both cardiac and skeletal muscle showed faint staining of laminin α5 (Figures 4E and 4F). In skeletal muscle, the perimysium stained much stronger than the endomysium (Figure 4F). In liver the collecting veins in the centre of liver lobules and the sinusoids expressed the α5 chain (Figure 4G). A control

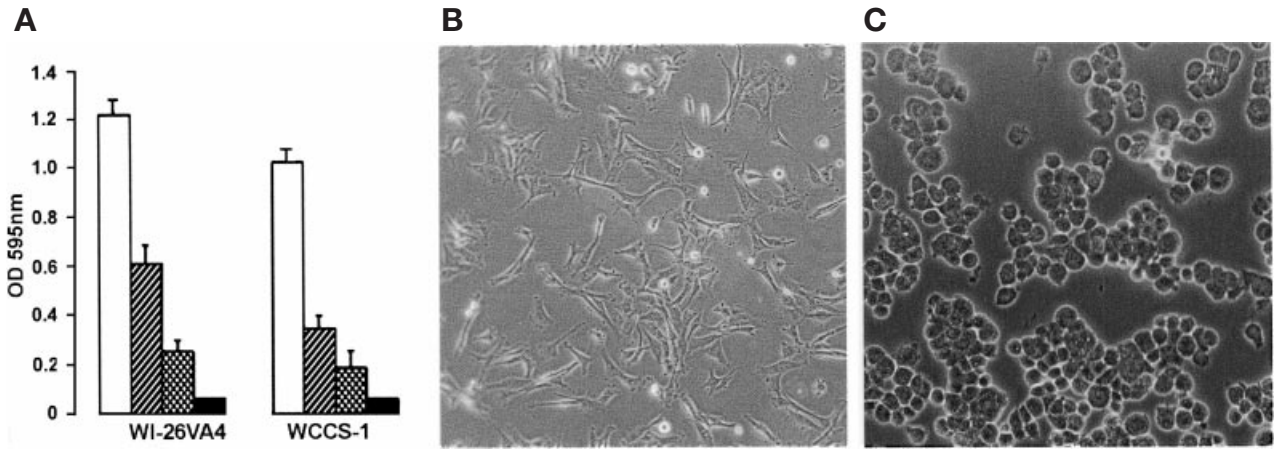

Figure 7 Cell binding activity of recombinant laminin α5LG modules

(**A**) Plates (96-well) were coated with recombinant laminin α5LG fragments and α5 chain-containing laminin 10/11. Two different epithelial cell lines, WCCS-1 and WI26VA4, were added to the wells and allowed to adhere at 37 °C for 60 min. Crystal Violet staining was used to assess the number of attached cells. Attachment to laminin-10/11 is shown as white bars, to α5LG1–3 as hatched bars, to α5LG4–5 as cross-hatched bars and to BSA as solid black bars. Each value represents the mean for three separate determinations. Duplicate experiments gave similar results. Concentrations used for coating the wells were: for laminin 10/11, 5 μg/ml (6.4 nM); for α5LG1–3, 10 μg/ml (158 nM); and for α5LG4–5, 10 μg/ml (212 nM). OD 595 nm = A_{595}. (**B**) Spreading of WI26VA4 cells on surfaces coated with recombinant laminin α5LG1–3. (**C**) Absence of spreading of the same cells adhering to α5LG4–5 tandem modules.

staining with normal rabbit serum showed no positive staining (Figure 4H).

Binding to sulphated ligands

The first activities assigned to laminin α1LG modules were binding to heparin [33] and sulphatides [34]. This has been confirmed for LG modules from both α2 chain [19] and α4 chain [26]. All α5 chain LG modules were similar in this context. They bound quantitatively to an analytical heparin affinity column (Table 1). However, only the tandem arrays α5LG1–3 and α5LG4–5, as well as the single α5LG4 module, needed NaCl concentrations larger than physiological ones to be displaced. The binding activities of these fragments and α5LG5 for a heparin–albumin conjugate and for sulphatides were examined in solid-phase assays (Figure 5). For α5LG1–3 these activities were 70 and 80 nM respectively, which is slightly higher than for α4LG1–3 and slightly lower than for α2LG1–3 [26]. For α5LG4–5, half-maximal binding to heparin–BSA was 20 nM. This activity was higher than for α4LG4–5 and approximately the same as for α1LG4–5 and α2LG4–5 [26,35]. Half-maximal binding of α5LG4–5 to sulphatides was 20 nM, indicating a binding activity stronger than for α4LG4–5, approximately the same as for α1LG4–5, but distinctly lower than for α2LG4–5 [26,35]. Solid-phase assays using the α5LG4 and α5LG5 modules located the heparin- and sulphatide-binding sites to the α5LG4 module (half maximal binding at 50 and 80 nM respectively).

Identification of laminin α5 G-domain as a cell adhesive region

Human laminin-10/11 was previously shown to promote strong adhesion of the two epithelial cell lines WI26VA4 and WCCS-1 [36]. These cell lines were then used to analyse the adhesive properties of various recombinant LG modules of the α5 chain. Both cell lines adhered maximally to laminin 10/11 at a coating concentration of 5 μg/ml (6.4 nM), whereupon adhesion was followed by distinct cell spreading. First we used the recombinant fragments produced in the present study, α5LG1–3 and α5LG4–5, to inhibit cell adhesion to laminin 10/11. Adhesion of the epithelial cell line WI26VA4 could be inhibited by recombinant α5LG1–3 [from 14 % ($P < 0.05$) at 30 μg/ml to 72 % ($P < 0.0001$) at 200 μg/ml] and by recombinant α5LG4–5 [from 19 % ($P < 0.05$) at 10 μg/ml to 62 % ($P < 0.0001$) at 200 μg/ml], whereas recombinant α2LG2 was inactive (Figure 6A).

With the same experimental set-up, using the antisera produced in the present study, adhesion of WI26VA4 cells could be inhibited with the antiserum against α5LG1–3 [from 25 % ($P < 0.005$) at 10 μg/ml to 65 % ($P < 0.0001$) at 100 μg/ml], whereas the antiserum against α5LG4–5 only inhibited cell adhesion by 17 % ($P < 0.05$) at 30 μg/ml and 29 % ($P < 0.005$) at 100 μg/ml. A normal rabbit serum used as control had no effect on cell adhesion. A combination of the two antisera only marginally increased the inhibitory effect on cell adhesion. However, this increase in inhibition was significant compared with incubation with only anti-α5LG1–3 ($P < 0.05$) at 30 and 100 μg/ml (Figure 6B). These experiments indicated that most of the adhesive activity of laminin 10/11 was located in the α5LG1–3 region and also showed a small, but important, contribution from the α5LG4–5 region.

We tested cell adhesion on the recombinant fragments with two epithelial cell lines. Significant adhesion was seen on all substrates ($P < 0.01$ compared with BSA, except WCCS-1 on α5LG4–5, where $P < 0.05$). When we allowed cells to adhere to α5LG1–3, maximal adhesion was obtained with a coating concentration of 10 μg/ml (158 nM), and this adhesion was 30 (WCCS-1)–48 % (WI26VA4) of adhesion to laminin-10/11 (Figure 7A). The cells also acquired spread morphology after 60 min, as shown for WI26VA4 (Figure 7B). For α5LG4–5, maximal adhesion was obtained with a coating concentration of 10 μg/ml (212 nM), and this adhesion was 13 (WCCS-1)–16 % (WI26VA4) of adhesion to laminin-10/11 (Figure 7A). However, when coated on α5LG4–5, the cells did not spread (Figure 7C).

Identification of cellular receptors for the laminin α5 G-domain

Bivalent cations are required for binding of laminins to receptors such as integrins. Adhesion of cells to recombinant α5LG1–3 could be abolished by addition of 5 mM EDTA, whereas adhesion to α5LG4–5 could not, indicating a dependence on

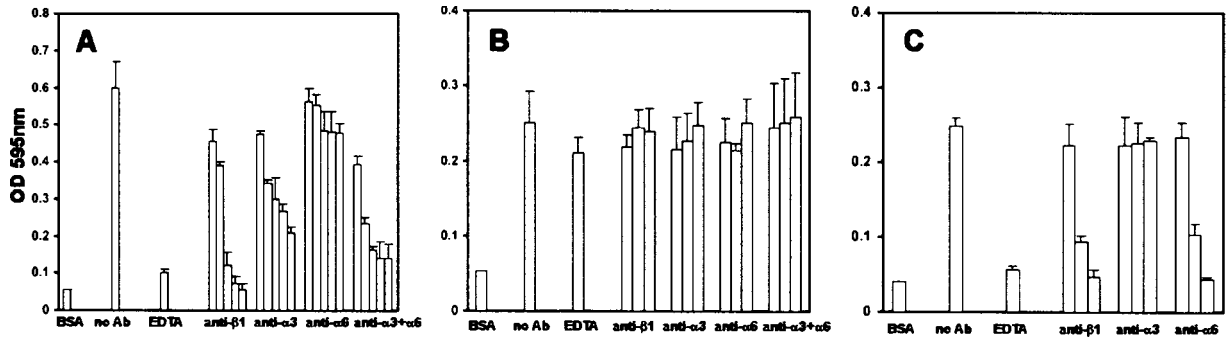

Figure 8 Inhibition of cell binding to recombinant laminin α5LG1–3 and α5LG4–5 tandem modules by integrin antibodies

(**A**) Cell binding assays on laminin α5LG1–3 using WI26VA4 cells were performed on untreated controls or in the presence of EDTA, anti-(β1 integrin), anti-(α3 integrin) and anti-(α6 integrin) monoclonal antibodies at 0.3, 1, 3, 10 and 30 μg/ml. (**B**) Cell binding assays on laminin α5LG4–5 using WI26VA4 cells were performed on untreated controls or in the presence of EDTA, anti-(β1 integrin), anti-(α3 integrin) and anti-(α6 integrin) monoclonal antibodies at 3, 10 and 30 μg/ml. (**C**) Cell binding assays on laminin α5LG1–3 using FDCP-Mix cells were performed on untreated controls or in the presence of EDTA, anti-(β1 integrin), anti-(α3 integrin) and anti-(α6 integrin) monoclonal antibodies at 3, 10 and 30 μg/ml. Each value represents the mean for three separate determinations. Duplicate experiments gave similar results. OD 595 nm = A_{595}.

bivalent cations and a possible role of integrin receptors in cell adhesion to α5LG1–3 (Figure 8). Of the suggested laminin10/11 receptors, these cells express integrins α3β1 and α6β1 and α-dystroglycan, but not integrins αVβ3 and α6β4. The WI26VA4 cells also express α1β1 and α2β1, but are known to utilize integrins α3β1 and α6β1 for adhesion to laminin 10/11 [12]. To test whether some of the integrins involved in cell adhesion to laminin 10/11 [7,8,12,37] and present on these cells, were also active in adhesion to the recombinantly produced LG modules, an antibody-inhibition assay was performed. An antibody against β1 integrin inhibited WI26VA4 cell binding to α5LG1–3 in a dose-dependent manner ($P < 0.05$), abolishing adhesion completely (no significant difference from BSA) at 3 μg/ml (Figure 8A). Moreover, adhesion of cells to α5LG1–3 could also be inhibited in a dose-dependent manner by an antibody against the integrin α3 subunit (Figure 8A), although this inhibition was not complete [from 49% inhibition at 1 μg/ml ($P < 0.05$) to 73% inhibition at 30 μg/ml ($P < 0.001$)]. An antibody against integrin α6 had only minor effect on WI26VA4 cell adhesion to α5LG1–3, giving 24% inhibition at 30 μg/ml ($P < 0.05$) (Figure 8A). However, a combination of anti-α3 and anti-α6 antibodies could increase the inhibition of cell adhesion to α5LG1–3 seen with anti-α3 antibodies alone. This increase was significant at antibody concentrations 0.3 ($P < 0.005$), 1 ($P < 0.001$), and 10 μg/ml ($P < 0.05$). It was not quite significant at antibody concentrations 3 and 30 μg/ml, owing to considerable differences in S.D. (Figure 8A). None of the integrin antibodies were able to inhibit cell binding to α5LG4–5, even at high concentrations (Figure 8B).

We concluded that integrin α3β1 functioned as a receptor for α5LG1–3. However, the evidence for involvement of the α6β1 integrin was somewhat weak. It is known that blocking one integrin function can affect the activity of others. Therefore, we utilized a cell line, FDCP-Mix, known to attach to laminin 10/11 exclusively through this receptor [7]. These cells adhered to α5LG1–3, but not to α5LG4–5. Moreover, the adhesion was inhibitable by EDTA and antibodies against integrins α6 ($P < 0.0005$) and β1 ($P < 0.0005$), at antibody concentrations of 10 and 30 μg/ml, but not with antibodies against integrin α3 (Figure 8C). Both results support the data obtained with WI26VA4 cells indicating that integrin α6β1 also functions as a receptor for α5LG1–3.

It has been predicted that α-dystroglycan would not bind to laminin α5 [17,21]. However, it has been reported that a bacterially produced laminin α5 chain G domain could bind α-dystroglycan [22]. To analyse whether we could repeat this result with α5 chain G domain fragments produced in mammalian cells, we purified rat α-dystroglycan. A single band of 120–140 kDa corresponding to α-dystroglycan was seen after separation on a polyacrylamide gel and silver staining or immunoblotting with an antibody directed against α-dystroglycan (Figure 9).

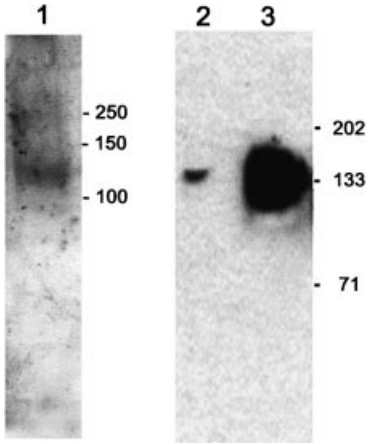

Figure 9 Analysis of purified α-dystroglycan

Conditioned medium from RT4 rat schwannoma cells (lane 2) and α-dystroglycan purified from this conditioned medium (lanes 1 and 3) were subjected to SDS/PAGE using 7% gels under reducing conditions. The gel was then silver stained to detect total proteins (lane 1) or protein transferred on to PVDF membranes, followed by immunodetection with the monoclonal antibody IIH6 detecting α-dystroglycan (lanes 2 and 3).

Intact laminin 10/11 bound to α-dystroglycan with a half-maximal binding concentration of 150 nM [12]. Therefore we tested recombinant fragments α2LG2, α2LG1–3, α5LG1–3 and α5LG4–5, as well as human laminin 10/11 binding to α-dystroglycan, in a solid-phase assay (Figure 10A). Fragment α2LG1–3 showed, as expected, a distinct binding profile [19], where a concentration of 40 nM was required for half-maximal binding. Laminin fragments α2LG2 and α5LG1–3 did not bind at all at the concentrations tested (maximum 500 nM and 1 μM

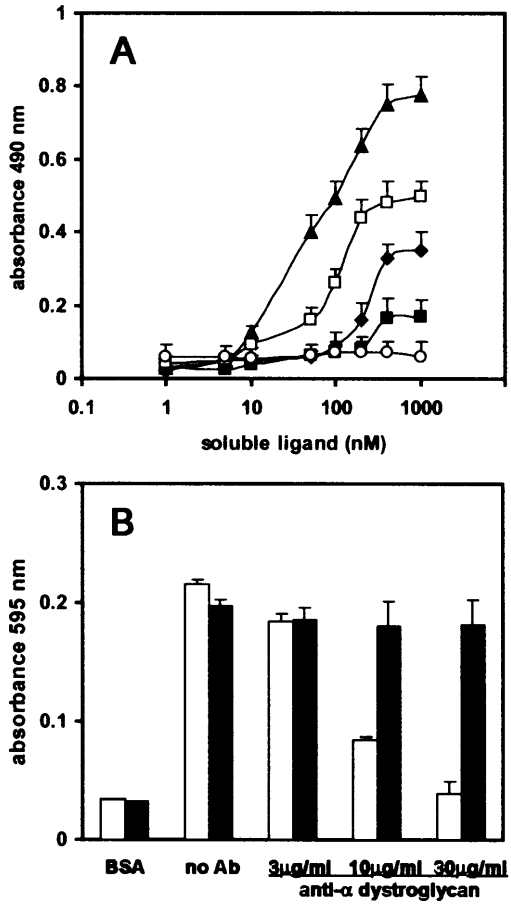

Figure 10 Binding to laminin α5LG4–5 tandem modules through α-dystroglycan

(**A**) Solid-phase assays of recombinant laminin G domain fragments and laminin 10/11 binding to α-dystroglycan purified from rat schwannoma cell line RT4. Soluble ligands were fragments α2LG2 (○), α2LG1–3 (▲), α5LG1–3 (■), α5LG4–5 (◆) and human laminin 10/11 (□). (**B**) Cell binding assays on laminin α5LG4–5 using WI26VA4 cells (white bars) or U-937 cells (black bars) were performed on untreated controls or in the presence of anti-α-dystroglycan monoclonal antibodies at 3, 10 and 30 μg/ml.

respectively), whereas α5LG4–5 showed a weak but distinct binding with half-maximal binding at a concentration of 250 nM. This interaction was completely inhibited by EDTA (not shown), indicating a dependence on bivalent cations also for α-dystroglycan–laminin-10/11 interactions. To test whether this interaction was localized to a single LG module, as in laminin-1, or dependent on several LG modules, as seen for other ECM proteins [19], we tested fragments α5LG4 and α5LG5 for α-dystroglycan binding activity. However, at the maximum concentrations tested (400 nM respectively) no binding was apparent.

To examine whether WI26VA4 cell adhesion to α5LG4–5 (Figure 7) was due to an interaction with α-dystroglycan, we tested whether this interaction could be inhibited by antibodies against α-dystroglycan (Figure 10B). This was found to be the case. Significant inhibition was seen at antibody concentrations 10 and 30 μg/ml ($P < 0.0001$). Moreover, we examined the adhesion of a cell line (U-937) not expressing α-dystroglycan (M. Durbeej, personal communication) to α5LG4–5. These cells were able to adhere to α5LG4–5, but this adhesion could not be inhibited with anti-α-dystroglycan antibodies (Figure 10B), indicating that receptors other than α-dystroglycan could be important for adhesion to fragment α5LG4–5. Results with both cell lines support a role for laminin α5LG4–5 fragment in α-dystroglycan-mediated adhesion.

DISCUSSION

Recombinant production of several laminin α5 chain G domain fragments allowed us to perform several functional biological studies. Proteolytic cleavage of the α5 chain of laminin 10/11 has been suggested to occur *in vivo*, but it is not known exactly where in the α5 chain this processing takes place [4]. Other investigators have found a more restricted or negligible proteolytic processing [30]. Laminin G domains of the α2 and α4 chains have been reported to undergo endogenous proteolytic processing [25,26] and such processing has been predicted for the α3 and α5 chain G domains [17,38]. We started by investigating the proteolytic processing of the produced α5 LG modules. However, no evidence for such processing was found in the recombinantly produced fragments. To investigate whether such processing occured *in vivo*, we made immunoblots of tissue extracts from newborn mice. We obtained doublets with both polyclonal antibodies against α5LG1–3 and α5LG4–5 indicating that laminin α5 chain G domain is not proteolytically cleaved at the putative α5LG4 furine protease cleavage site in spleen and thymus.

To ascertain further that no proteolytic processing occurred we used the antisera produced in the present study to stain tissue sections. For laminin α4, it has been clearly shown that significant proteolysis occurs. The α4LG4–5 fragment is cleaved off and cannot be detected at all in tissues with antibodies directed against this fragment [26]. We therefore stained tissues with antisera against both α5LG1–3 and α5LG4–5. However, no differences in staining patterns with these two antibodies were detected in any of the tissues tested. Moreover, a nearly identical laminin α5 chain expression pattern as has been described previously, with a polyclonal antiserum against bacterial fusion proteins [4] reacting with the N-terminal domain IVa, or the monoclonal antibody 4G6 [30], was found in the tissues examined.

The major differences from results obtained with monoclonal antibody 4G6 [30] were meningeal staining in brain, stronger staining of liver sinusoids, and staining of the crypts of Lieberkühn in small intestine with the antibodies produced in the present study. Monoclonal antibody 4G6 has also been used by others to stain adult intestine [32], where it was found that basement-membrane staining occurred from the upper parts of the crypts to the villus tip. This discrepancy can perhaps be explained by the higher sensitivity of our polyclonal antisera or that, in certain tissues, the epitope that 4G6 reacts with is masked.

Heparin and sulphatide affinity could be important for cellular interactions and also for interactions with heparan sulphate side chains of ECM proteoglycans, as shown for the laminin α1LG4 modules interaction with perlecan [39,40]. Measurements of heparin affinity have been performed previously on recombinant α5LG4, α5LG5 and α5LG4–5 fusion proteins, locating the major heparin-binding site to the α5LG4 module [41]. This is in accordance with our results using native LG domains. Our measured relative affinities for heparin–BSA were 50 nM (α5LG4) and 20 nM (α5LG4–5), whereas Nielsen et al. [41] obtained values of 10 nM and 46.2 nM (α5LG4) and 3 nM and 1.7–2 nM (α5LG4–5) in two different assays. A possible explanation for the differing binding affinities could be that their fusion protein LG modules are stabilized by the introduction of

the laminin γ-chain. The physiological relevance of such a putative stabilization has yet to be determined.

The biological effects of the laminins are to a large extent mediated by cell-surface receptors linking laminin to intracellular signalling pathways. Two types of receptors have so far been well described: integrins and dystroglycan [42,43]. Dystroglycan may provide an important complement to the role of integrins. Dystroglycan consists of an extracellular laminin-binding subunit (α-dystroglycan) plus a transmembrane subunit (β-dystroglycan). Both integrins and dystroglycan can form links between laminin in the ECM and the cytoskeleton.

Here we show that laminin α5 chain binds both α-dystroglycan and integrins $\alpha3\beta1$ and $\alpha6\beta1$ through its G domain. Moreover, we could localize the α-dystroglycan binding to domains LG4–5, whereas no binding was seen with single LG modules. This indicates that the α-dystroglycan binding site spans at least two LG modules in a manner analogous to the interaction of laminin α2LG1–3 and α2LG4–5 with α-dystroglycan [19], whereas the interaction of laminin α1 with α-dystroglycan is dependent on the single module α1LG4 [19,35]. The half-maximal binding of α5LG4–5 to α-dystroglycan was 250 nM, whereas affinities of the corresponding fragments α1LG4–5 and α2LG4–5 were 200 nM and 50 nM respectively [19]. One possibility is that high-affinity α-dystroglycan binding is dependent on specific Ca^{2+} ion coordinating amino acids absent in α5LG4–5 [17,21]. However, Ca^{2+} ions appear to be in some way involved in the interaction between α-dystroglycan and laminin 10/11 through α5LG4–5, since binding could be abolished by EDTA. Another possibility is that α5LG4–5 binds to α-dystroglycan in a completely different way from α2LG4–5.

In laminin α2, interactions with integrins $\alpha6\beta1$ and $\alpha3\beta1$ probably solely reside in the α2LG1–3 domain, as cells bind equally well to this fragment as to laminin 2/4. However, for laminin-1 it has been shown that the laminin α1 chain is not sufficient for cell adhesion through integrin $\alpha6\beta1$ [44]. Here a contribution from either or both $\beta1$ and $\gamma1$ seems necessary for binding. The situation is analogous to that for laminin-8 ($\alpha4\beta1\gamma1$), which is known to bind cells via integrin $\alpha6\beta1$ [45,46]. The laminin α4LG1–3 fragment weakly supports cell adhesion, and cell binding to laminin-8 can be almost completely inhibited by antibodies towards integrin subunits α6 and β1 as well as by antibodies against α4LG1–3 [26]. In the present study we could map integrin $\alpha3\beta1$ and $\alpha6\beta1$ binding to α5LG1–3. However, cells bound laminin-10/11 far better than α5LG1–3, indicating additional cell-adhesion sites in other domains of laminin-10/11. This was also indicated by incomplete inhibition (68%) of cell adhesion to laminin 10/11 by a polyclonal antiserum against α5LG1–3. One possibility is that much of the non-LG-domain cell-binding activity resides in domains IVa and VI and their interactions with integrin $\beta1$ [10,47]. Another possibility is that integrin binding to α5LG1–3 is as dependent on tertiary structure as is integrin binding to laminin α1LG1–3.

Both integrins and α-dystroglycan are expressed on cancer cells, and binding to laminin-10/11 may therefore be important for cell movement through endothelial and epithelial basement membranes and thus for metastasis. These quantitative binding studies, together with previous studies on other laminin α chain G domains, will facilitate further studies into the molecular mechanisms of normal and malignant cell attachment to basement membranes.

This work was supported by grants from Kungliga Fysiografiska Sällskapet i Lund (The Royal Physiographic Society in Lund), Crafoordska stiftelsen (Crafoord Foundation), Harald Jeanssons stiftelse samt Harald och Greta Jeanssons stiftelse (Harald Jeansson's Foundation, and Harald and Greta Jeansson's Foundation), Kungl. Vetenskapsakademien (The Royal Swedish Academy of Science) and Naturvetenskapliga forskningsrådet (The Swedish Natural Science Research Council).

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Received 26 September 2002/3 January 2003; accepted 8 January 2003
Published as BJ Immediate Publication 8 January 2003, DOI 10.1042/BJ20021500