Crystal structure and cell surface anchorage sites of laminin α1LG4-5

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Abstract

The laminin G-like (LG) domains of laminin-111, a glycoprotein widely expressed during embryogenesis, provide cell anchoring and receptor binding sites that are involved in basement membrane assembly and cell signaling. We now report the crystal structure of the laminin α1 LG4-5 domains and provide a mutational analysis of heparin, α-dystroglycan and galactosyl-sulfatide binding. The two domains of α1LG4-5 are arranged in a V-shaped fashion similar to that observed with laminin α2 LG4-5, but with a substantially different interdomain angle. Recombinant α1LG4-5 binding to heparin, α-dystroglycan and sulfatides was dependent upon both shared and unique contributions from basic residues distributed in several clusters on the surface of LG4. For heparin, the greatest contribution was detected from two clusters, 2719RKR and 2791KRK. Binding to α-dystroglycan was particularly dependent on basic residues within 2719RKR, 2831RAR, and 2858KDR. Binding to galactosyl-sulfatide was most affected by mutations in 2831RAR and 2768KGRTK, but not in 2719RKR. The combined analysis of structure and activities reveal differences in LG domain interactions that should enable dissection of biological roles of different laminin ligands.

INTRODUCTION

Laminin-111, recently renamed from laminin-1 to better reflect its α1β1γ1 subunit composition, is one of the first of two laminins to be expressed during embryonic development, appearing in the peri-implantation period in the basement membrane of the embryonic plate along with laminin-511 (laminin-10), and in the absence of other laminins in Reichert’s membrane (1,2). Later in development, laminin-111 is strongly expressed in placenta, liver, kidney and testis where it is thought to play a role in organogenesis (3). In the adult, the laminin α1 chain has very limited expression and is largely supplanted by the laminin α5 chain. Targeted inactivation of the LAMA1 gene coding for the α1 chain was found to result in a failure of Reichert’s membrane with developmental arrest by embryonic day 6.5 in the mouse (4). In-frame deletion of the mouse laminin α1 exons corresponding to laminin G-like (LG) domains 4 and 5 was found to result in similar stage lethality.

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The atomic coordinates and structure factors (code 2JD4) have been deposited in the Protein Data Bank (http://www.rcsb.org).
accompanied by defective epiblast differentiation without loss of basement membrane (5), the last possibly a result of the partially redundant expression of laminin-511 (4).

The α1 subunit provides most of the unique characteristics of laminin-111. The N-terminal LN domain participates in polymerization by interacting with the LN domains of the β1 and γ1 chains. The C-terminal LG domains, LG1-3, bind to the α4β1 integrin, while LG4-5 bind to heparin, sulfated glycolipids, and α-dystroglycan (α-DG). The polymerization and cell-anchoring activities are thought to act in concert to assemble a functional basement membrane on a cell surface (6).

Our earlier understanding of the laminin α1 LG1-5 structure was based on crystal structures of LG4 and LG4-5 from the related α2 chain (~40% sequence identity). The LG domain fold was revealed as a multi-stranded β-sandwich with one bound calcium ion. In the LG4-5 pair the two domains are connected in a V-shaped arrangement, in which LG5 is disulfide-bonded to the linker preceding LG4 (7,8). While the structure of LG1-3 remains to be elucidated, it is thought that these three domains form a closed arrangement with similar angles between domains and separated from the LG4-5 pair by a hinge-like region (9).

Analysis of contributions of α1LG4-5 to laminin interactions with Schwann cells, myotubes and developing epithelia has led to a model in which these domains provide the major anchoring activity of laminin, an initiating event of basement membrane assembly that leads to alterations of the cell cytoskeleton accompanied by signaling (6,10,11). Basic residues within the LG4-5 pair of several laminins mediate key interactions with three types of molecules: heparan sulfate chains that are attached to perlecan, agrin, collagen and syndecan core proteins of basement membranes and cell surface; the glycoprotein α-DG that is a component of a larger transmembrane and sub-membrane complex associated with dystrophin and utrophin; and sulfated glycolipids, in particular the sulfatides, that can be present in the outer leaflet of the cell plasma membrane. Earlier mutagenesis analyses of recombinant laminin α1 and α2 LG fragments revealed that there are binding similarities and differences between the two laminins. Heparin, representing the highly sulfated regions of glycosaminoglycan chains, α-DG and galactosyl-3-sulfate ceramide (galactosyl sulfatide) all bind to an extensive basic surface region between the calcium sites of the laminin α2 LG4-5 domain pair (12,13). In contrast, a smaller topographical region confined to LG4 appears to bind the same cell surface components in the laminin α1 chain (12,14).

The present study describes the crystal structure of laminin α1 LG4-5 in conjunction with a site-directed mutagenesis and in vitro analysis of the binding sites for heparin, α-DG and sulfatides. We report that the backbone of the α1LG4-5 domain pair is very similar to that of α2LG4-5, but there are significant differences in the distribution of charged residues on the protein surface. Binding of α1LG4-5 to heparin, α-DG and sulfatides was found to be dependent upon partially overlapping basic amino acid residue clusters. Our results have led, we believe, to an improved understanding of the amino acid residues involved in each type of interaction and will aid in defining the biological roles of different laminin ligands.

**EXPERIMENTAL PROCEDURES**

**Laminin α1 LG4-5 vector constructs**

An expression construct containing the wild-type (WT) mouse laminin α1 LG4-5 sequence (coding for residues 2666LHREH...PGPEP3060 of the mature laminin α1 chain, i.e. the numbering scheme used here omits the 24-residue signal peptide of SwissProt entry P19137) was created by amplifying cDNA from laminin α1 pCIS (15) utilizing three successive PCR reactions. Three overlapping 5′ sense primers were used to place a 5′ terminal Nhel site followed by the 5′ UTR and signal sequence of human BM-40 (cleaved by the signal
peptidase) and a FLAG epitope tag (DYKDDDDK), while the 3′ primer placed a KpnI site downstream of the STOP codon at the 3′ terminus of the amplified product (oligonucleotide sequences are listed in Supplementary Table 1). Pfx polymerase (Invitrogen) was used along with a PTC-100 thermal cycler (MI Research) to amplify the DNA which was then purified after each reaction (UltraClean PCR DNA purification kit, MoBio). The NheI and KpnI sites were used to clone the PCR product into the analogous sites in the pcDNA3.1+/zeo vector (Invitrogen). The mutated α1LG4-5 DNAs were constructed by strand overlap extension PCR using the same three 5′ upstream sense primers and downstream 3′ antisense oligonucleotide in conjunction with internal primers introducing the desired mutations. E. coli DH5α cells (Invitrogen) were transformed with the plasmids and plasmid DNA was purified by alkaline lysis and spin columns (UltraClean Standard Mini Plasma Prep Kit, MoBio). All generated plasmids were completely sequenced.

Recombinant α1LG4-5 protein production and purification

Expression constructs were linearized with BglII (New England Biolabs) and transfected into the human kidney fibroblast cell line 293 (ATCC) using Lipofectamine 2000 (Invitrogen) and stable clones selected under zeocin for secretion of α1LG4-5 protein. Cells were grown in DMEM with high glucose (Invitrogen) supplemented with 10% FBS, 50 units/ml penicillin-G, 50 μg/ml streptomycin, and 100 μg/ml zeocin (Invitrogen). Once cells had reached confluency the growth medium was replaced with fresh medium minus zeocin and then collected 72 hours later. The genomic DNA was isolated from the cells after media harvesting (Exact-N-Amp kit; Sigma) and sequenced to verify the identity of the various α1LG4-5 proteins. The conditioned media were passed through a gravity column (BioRad) packed with anti-FLAG M2 agarose resin (Sigma) and recombinant α1LG4-5 proteins eluted with FLAG peptide (Sigma) in 90 mM NaCl, 1 mM CaCl₂, 50 mM Tris-HCl, pH 7.4 (TBS50/Ca) at 4°C. The eluted proteins were then loaded onto a heparin 5PW column (TosoHass) on an Äkta FPLC system (Pharmacia), where the FLAG peptide (unbound component) was recovered for re-use and the α1LG4-5 proteins eluted using a 1M NaCl gradient. The eluted α1LG4-5 proteins were concentrated and the buffer exchanged into TBS50/Ca at room temperature via centrifugal filtration with Amicon Ultra spin filters (Millipore). The recombinant proteins were further dialyzed against TBS50/Ca buffer with several buffer changes for two days using dialysis cassettes (Slide-A-Lyzer, Pierce). Deglycosylated WT α1LG4-5 protein was produced either by isolating WT α1LG4-5 from the medium of stably transfected cell lines grown in 2 μg/ml tunicamycin (Sigma) for 24 hours or by treating 50 μg of purified α1LG4-5 with 1000 units of PNGase F (New England Biolabs) in TBS50/Ca for 1 hour at 37°C.

Laminin α1 LG4-5 vector construct for crystallography

DNA coding for residues 2682 QPELC...PGPEP3060 of the mature mouse laminin α1 chain was obtained by PCR amplification from laminin α1 pCIS (15). The PCR primers added NotI and NheI sites at the 5′ end and a STOP codon followed by XhoI and BamHI sites at the 3′ end. The PCR product was cloned into pBluescript II KS+ using NotI and BamHI and four mutations (N2714Q, N2811K, N2900Q, C3014S) were introduced by strand overlap extension PCR. The sequence-verified insert was cloned into the pCEP-Pu vector (16) using NheI and XhoI. After cleavage of the BM-40 sequence signal, a vector-derived APLA sequence remains at the N-terminus of the secreted recombinant α1LG4-5 protein.

Protein production for crystallography

The laminin α1LG4-5 quadruple mutant (N2714Q/N2811K/N2900Q/C3014S) was purified from the conditioned medium of episomal transfected 293-EBNA cells. Cells were maintained in DMEM/10% FCS (Invitrogen), transfected using Fugene reagent (Roche), and selected with 1 μg/ml puromycin (Sigma). Serum-free conditioned medium (1.5 litres) was
dialyzed against 50 mM Na-HEPES, pH 7.5 and loaded onto a 2 x 5 ml heparin HiTrap column (GE Healthcare). Bound proteins were eluted using a linear NaCl gradient (0-1 M). The α1LG4-5 mutant protein eluted as a sharp single peak at ~0.5 M NaCl and was further purified by size exclusion chromatography using a 24 ml Superdex 200 column (GE Healthcare) run at 0.5 ml/min in 20 mM Na-HEPES pH 7.5, 150 mM NaCl. The final yield was 4 mg of pure protein.

Crystal structure determination

The α1LG4-5 quadruple mutant protein was concentrated to 19 mg/ml in 10 mM Na-HEPES, pH 7.5. Crystals were obtained by hanging drop vapor diffusion using 20% PEG8000, 100 mM Tris-HCl, pH 8.5, 200 mM MgCl₂ as precipitant. Crystals were frozen in liquid nitrogen in mother liquor supplemented with 20% glycerol. Diffraction data to 1.9 Å resolution were collected at 100 K on beamline 9.6 at the SRS Daresbury (λ = 0.87 Å). The crystals belong to space group P2₁, a = 70.53 Å, b = 55.81 Å, c = 100.99 Å, β = 98.48°. There are two α1LG4-5 molecules in the asymmetric unit, resulting in a solvent content of ~45%. The diffraction data were processed with MOSFLM (http://www.mrc-lmb.cam.ac.uk/harry/mosflm) and programs of the CCP4 suite (17). The structure was solved by molecular replacement with PHASER (18), using the laminin α2LG4-5 structure (8) as a search model; the LG domains had to be placed individually to obtain a solution. The structure was rebuilt with O (19) and refined with CNS (20) without non-crystallographic symmetry restraints. Data collection and refinement statistics are summarized in Table 1. The figures were made with PYMOL (http://www.pymol.org).

Heparin binding of laminin α1 E3 fragment and recombinant α1LG4-5

The laminin-111 E3 fragment was prepared from laminin-111 purified from EHS tumor (21). E3 fragment, recombinant α1LG4-5, α1LG4-5 treated with enterokinase to remove the FLAG tag, and mutant α1LG4-5 proteins were loaded onto a Tosohass Heparin 5PW column in TBS50/Ca at 4°C on an Åkta FPLC system, eluted with a 0-1 M NaCl salt gradient, and the NaCl concentration required for elution determined.

Analysis of α1LG4-5 binding to α-dystroglycan

α-DG was purified from rabbit muscle as described (22). Equal aliquots of α-DG (1 μg) were loaded into the slots of SDS-acrylamide gels and electrophoresed under reducing conditions. The protein bands were then electroeluted onto nitrocellulose membranes, blocked in PBS containing 5% nonfat dry milk for 1 h at room temperature, and assessed for binding to each α1LG4-5 protein (1 μg/ml) using a previously described overlay assay (23). Binding of the α1LG4-5 proteins was detected with 1 ml of 1.1 μg/ml horseradish peroxidase coupled anti-FLAG antibody M2 (Sigma) per 3.5 cm² of blot membrane. The solid phase assay was performed in 96 well microtiter plates with α-DG bound to the plate (100 μl of 1 μg/ml per well) and incubated with various concentrations of α1LG4-5 proteins as previously described (24), except that HRP-linked monoclonal FLAG antibody M2 (100 μl of 1.1 μg/ml per well) was used for detection followed by color development with 3,3',5,5'-tetramethylbenzidine (Bio-Rad Laboratories). Color development was quantitated at 655 nm using a Molecular Dynamics Spectramax 340UV/Vis microplate reader (25). We verified that the signal readout was linear to OD > 3 by collecting kinetic data on color development. Estimates of half-maximal binding (apparent dissociation constant, K_D) and binding capacity (B_max) were determined by curve fitting of the binding data of WT LG4-5 using a single-site model (fitted values = B_max * L/(K_D + L), where L is the molar ligand concentration), with the calculated B_max value used for subsequent determinations of all other half-maximal binding, an approach employed to minimize the errors inherent in estimating binding from plots that are low and near-linear over the concentration range evaluated.
Analysis of α1LG4-5 binding to galactosyl sulfatide and other lipids

The ammonium salt of HSO₄⁻3Galβ1-1’Ceramide (brain sulfatides, Avanti Polar Lipids) was dissolved in methanol and 10 μg added per immulon-1B microtiter plate well (ThermoLabsystems). The plate was dried at 37°C for 2 hours and the wells washed four times with 200μl of ELISA Wash Buffer (EWB; 1% BSA in TBS50/Ca) at room temperature. The wells were then blocked for 1 hour at room temperature with 200 μl of EWB, followed by three 200 μl washes of EWB. α1LG4-5 proteins in varying concentrations in EWB were added to each well and incubated for 1.5 hours at room temperature. The wells were then washed four times with 200ul of EWB and HRP-linked monoclonal FLAG antibody (Sigma) in EWB added. After 1 hour at room temperature the wells were washed four times with EWB and 150 μl of substrate solution (4 mM o-phenylenediamine (Sigma), 50 mM citric acid, 100 mM Na₂HPO₄, 0.012% H₂O₂) added. The developing color reaction was stopped after 2-10 minutes by the addition of 60 μl of 2 M H₂SO₄, followed by 50 μl of ethanol and the plates read in a TECAN SpectraFluor microtiter spectrophotometer at 492 nm. Samples with OD > 2 were diluted and remeasured to correct for any deviation from linearity. A molecular weight of 44.3 kDa was used to calculate molar α1LG4-5 concentrations. Inhibition studies were performed in the presence of either 10 μg/ml low molecular weight heparin (Sigma) or 5mM EDTA. The assay was also performed with several other lipids: galactosyl ceramide (Avanti Polar Lipids and Sigma), sphingomyelin (APL), phosphatidic acid (APL), cholesterol-3-sulfate (Sigma) and GM1 ganglioside (APL). Half-maximal binding was estimated in the same manner as described for α-DG.

Electron microscopy

Pt/C rotary shadowing of proteins was performed by deposition of 0.9 nm metal at an 8º angle as previously described (21). Images are shown with reversed contrast.

RESULTS

Crystal structure of laminin α1LG4-5

The C-terminal LG domain pair of the laminin α1 chain contains an unpaired cysteine (Cys³⁰¹⁴) and three predicted N-linked glycosylation sites (Asn²⁷¹⁴, Asn²⁸¹¹, Asn²⁹₀₀). Because we found that WT α1LG4-5 preparations always contained a small fraction of disulfide-linked dimers (data not shown), we mutated Cys³⁰₁⁴ to serine. An α1LG4-5 C3014S construct with an N-terminal His-tag failed to crystallize, as did several other constructs with additional mutations of asparagine residues modified by glycosylation. Eventually, crystals could be obtained of an untagged α1LG4-5 quadruple mutant (N2714Q/N2811K/N2900Q/C3014S) devoid of any N-linked carbohydrate. The crystal structure of this mutant, hereafter termed simply α1LG4-5, was refined at 1.9 Å resolution to R_free = 0.261 (Table 1).

The asymmetric unit of the crystals contains two crystallographically independent α1LG4-5 molecules, A and B. We observed clear electron density for both molecules, with the exception of residues 2987-2990, 3032-3034 and 3060 of molecule A, and residues 2682-2684 and 3060 of molecule B. Molecules A and B are very similar in their LG4 and LG5 domain structures (r.m.s. deviation 0.36 Å and 0.58 Å, respectively, for all Ca atoms), but differ substantially in their respective domain arrangements. When the molecules are superimposed on their LG4 domains, a rotation by 14.5º is required to bring their LG5 domains into superposition; the pivot point of this rotation is in the interdomain linker, near Tyr²₈⁷¹ (Fig. 1A). The following description of the structure is based upon the more complete molecule B.
The α1LG4-5 structure consists of two canonical LG domains (9), LG4 and LG5, connected by a short linker and interacting through a small interface near the domain termini (Fig. 1B). Each LG domain folds into a curved β-sandwich built from two anti-parallel sheets and contains a single disulfide bond near the C-terminus. A third disulfide bond tethers the segment preceding LG4 to an α-helical turn in LG5. The interface between LG4 and LG5 is water-filled and predominantly polar, and the different conformations of molecules A and B are likely to be due to the paucity of specific interactions in the LG4-LG5 interface.

Both LG4 and LG5 contain one bound metal ion, located on the rim of the β-sandwich opposite the interdomain linker. These ions have been modeled as magnesium, given their coordination geometry and the high magnesium concentration in the crystals, but we assume that the binding sites are occupied by calcium under physiological conditions (7). Magnesium ion 1 is coordinated octahedrally by the side chains of Asp\(^{2747}\) and Asp\(^{2816}\), the main chain carbonyl oxygens of residues 2764 and 2814, and two water molecules; the average metal-ligand distance is 2.17 Å. Magnesium ion 2 is coordinated octahedrally by the side chains of Asp\(^{2923}\) and Asp\(^{2996}\), the main chain carbonyl oxygens of residues 2940 and 2994, and two water molecules; the average metal-ligand distance is 2.15 Å. The unpaired cysteine of α1LG4-5, Cys\(^{3014}\), is located in the convoluted loop that occupies most of the concave face of LG5. Two predicted N-linked glycosylation sites are located in LG4 (Asn\(^{2714}\) and Asn\(^{2811}\)) and one in LG5 (Asn\(^{2900}\)). Asn\(^{2811}\) is close to the metal ion binding site in LG4 (Fig. 1B).

**Structural comparison of α1LG4-5 and α2LG4-5**

Mouse laminin α1LG4-5 is 41% identical to the homologous region of the mouse α2 chain (α2LG4-5) (Fig. 2A), whereas the sequence identity to the α3-5 chains is substantially lower (<30%). A structural comparison of α1LG4-5 and α2LG4-5 (8) reveals only a few notable differences at the level of individual LG domains. LG4 of laminin α1 and α2 can be superimposed with an r.m.s. deviation of 0.91 Å for 148 C\(^{α}\) atoms; the major differences are concentrated in the spatially adjacent B-C and L-M loops, and in the edge β-strand J, which is irregular in α1LG4-5 (Fig. 2B). The LG5 domains can be superimposed with an r.m.s. deviation of 0.59 Å for 153 C\(^{α}\) atoms; the major differences are again concentrated in the B-C and L-M loops (Fig. 2C).

The relative arrangement of LG4 and LG5 in α1LG4-5 and α2LG4-5 is also similar, with α2LG4-5 more closely resembling molecule B than molecule A of α1LG4-5 (not shown). In terms of their interdomain angles, the two crystallographically independent α1LG4-5 molecules are, in fact, more different than α2LG4-5 is from molecule B of α1LG4-5. Notably, only a few contacts in the LG4-LG5 interface are conserved in the two laminin isoforms. Near the pivot point of interdomain flexibility, an aromatic side chain (Tyr\(^{2871}\) in α1LG4-5) stacks against a proline (Pro\(^{3056}\) in α1LG4-5). Further away from the hinge, a conserved leucine in LG4 (Leu\(^{2703}\) in α1LG4-5) makes a van der Waals contact with a proline in LG5 (Pro\(^{3052}\) in α1LG4-5). Finally, a conserved glutamine (Gln\(^{2700}\) in α1LG4-5) points its side chain into the water-filled cavity within the inter-domain interface (not shown).

The coordination of the two magnesium ions in α1LG4-5 is identical to that of the two calcium ions in α2LG4-5, with equivalent protein residues acting as metal ligands (Fig. 2A) and water molecules observed in equivalent positions in both structures. Thus, we expect that calcium would readily occupy the metal sites of α1LG4-5 under more physiological conditions than those used for crystallization.
Characterization of recombinant laminin α1LG4-5 proteins

To evaluate the contributions of basic residues to ligand binding by laminin α1 LG4-5, we prepared WT α1LG4-5, as well as six mutants, in which selected basic residues within LG4 were replaced by alanine (Table 2). For comparison, we also prepared the proteolytic E3 fragment from mouse EHS tumor laminin-111, which has the same sequence as the recombinant WT α1LG4-5 construct (26). Recombinant α1LG4-5 proteins were purified from the conditioned medium of transfected 293 cells by anti-FLAG and heparin affinity chromatography. The typical yield of recombinant α1LG4-5 was greater than 4 μg/ml of harvested culture media. Little or no degradation was detected by SDS-PAGE (Fig. 3A) and the FLAG tag could be cleaved by enterokinase treatment (Figs 3B-D). Rotary shadow electron micrographs were prepared of representative recombinant proteins (WT and mutants A and G) and showed a similar monomeric appearance to that of the E3 fragment (Fig. 3E). A doublet structure, thought to reflect the LG domain pairs, was frequently appreciated in the images (Fig. 3E, insets). Size exclusion chromatography of the recombinant proteins showed a single peak eluting at the same position as E3 (data not shown). Laminin α1 LG4-5 has three potential N-linked glycosylation sites. Treatment of secreted protein with PNGaseF (an amidase that cleaves the glycosidic bond between the modified asparigine residue and the first GlcNAc moiety) or treatment of transfected cells with tunicamycin (an inhibitor of N-acetylglucosamine transferase) produced similar increases in migration on SDS-PAGE, reflecting loss of N-linked carbohydrate mass (data not shown).

Elution of α1LG4-5 proteins from a heparin affinity column

To determine relative binding affinities for heparin, we evaluated the NaCl elution behavior of the different α1LG4-5 proteins from a heparin affinity column. Fragment E3, recombinant WT α1LG4-5, enterokinase-treated WT α1LG4-5, and deglycosylated WT α1LG4-5 all eluted at 0.25 M NaCl (Fig. 4 and data not shown). All mutant α1LG4-5 proteins eluted at lower NaCl concentrations, in the following order: WT>J>I>D>A≈G>A2. We note that the elution value of 0.25 M NaCl, which holds identically for recombinant and native α1LG4-5 protein, corresponds to an apparent KD of 22 nM as determined by the method of affinity co-electrophoresis (27).

Dystroglycan binding

The ability of the α1LG4-5 proteins to bind α-DG was evaluated using two different assay formats. In a blot overlay assay (Fig. 5A), all α1LG4-5 mutants showed reduced binding to immobilized α-DG compared to wild type protein, with mutants A and J showing no detectable binding. A very similar result was obtained using an ELISA-based solid phase assay with immobilized α-DG (Fig. 5B). All mutations decreased α-DG binding, in the following order: WT>A2≈G>D≈I≈A≈J; mutants A and J had very low α-DG binding activity. The finding that A2 bound α-DG with higher affinity than A was surprising given the greater loss of charge of the former compared to the latter. The sequences of A and A2 were reconfirmed by PCR of genomic DNA isolated from cells secreting the two laminin fragments (data not shown). We cannot offer a mechanistic explanation for the unexpected behavior of mutants A and A2 in the α-DG binding assays.

Sulfatide binding

Binding of α1LG4-5 proteins to immobilized galactosyl sulfatide was also examined. The interaction appeared to be specific for lipids bearing a sulfated sugar residue (Fig. 6A) since no binding was detected with the non-sulfated galactosyl-ceramide, or with lipids bearing sulfated charges in the absence of a sugar moiety (sulfated cholesterol) or bearing phosphate (e.g. phosphatidyl serine) or sialic acid (GM1 ganglioside) moieties. Recombinant α1LG4-5
and its mutants bound to immobilized sulfatide with different half-maximal binding values (Fig. 6B), reflecting substantial differences in affinities, in the following order: WT ≈ A > A2 ≈ G > J > D > I. Mutant I had almost no sulfatide binding activity. We fitted the sulfatide binding data with the simplest (i.e., a single-site) model to obtain the values listed in Table 2, but noted that the fits seen for the strongest binders (WT α1LG4-5 and mutants A and G) were not good. We suspect that the poor fits are a reflection of the inherent difficulties in analyzing protein-lipid interactions in a solid phase binding assay, in which a large protein binds to a relatively small ligand presented as a densely packed array. A two-site model improved the fits (not shown), perhaps by allowing for some degree of negative cooperativity resulting from steric hindrance between proteins that limits access to the lipid head groups as saturation is approached. We suspect that such a phenomenon of dense sulfatide packing may also exist on cell surfaces that assemble basement membranes, e.g. Schwann cells.

**DISCUSSION**

Interactions of the distal LG4-5 portion of the laminin α1 chain with cellular receptors (heparan sulfate proteoglycans and α-DG) and plasma membrane components (sulfatides) are responsible for laminin anchorage during basement membrane assembly, as well as for cell signaling (6,10,11). Previous mutagenesis studies have identified basic amino acid residues within LG4 contributing to ligand binding (14,28), but, in the absence of a α1LG4-5 structure, the precise structure of binding sites remained unknown. We now have determined the crystal structure of α1LG4-5 and analyzed the binding properties of this laminin α1 portion.

An electrostatic surface representation of the α1LG4-5 structure reveals a large, contiguous surface area of positive potential extending over both LG domains (Fig. 7A). Basic residues previously implicated in receptor binding (14,28) are clustered around the metal ion binding site in LG4 (Fig. 7B). A particularly striking feature is the spatial proximity of two basic sequences, 2719RKR and 2791KRK, which are located, respectively, at the start of β-strand C and in the H-I turn (Fig. 7C). Five of the six basic side chains are fully surface-exposed and available for receptor binding. The only exception is Arg2792, which donates two hydrogen bonds to main chain carbonyl groups in the long J-K loop, which in turn makes rather loose contacts with the body of the domain. Two other basic motifs, 2766KGRTK and 2831RAR, are located closer to the cleft between the LG4 and LG5 domains.

Regarding heparin binding, we found similar elution behaviour to the earlier study of Andac et al. (14) for those mutants evaluated in common (Supplementary Table 2) Hozumi et al. (28) used an energy-minimized homology model of α1LG4-5 to gain insight into the interactions of LG4 with heparin/heparan sulfate proteoglycans and integrin α2β1, the latter a cryptic activity in intact laminin. These authors found heparin binding to LG4 to depend upon contributions from 2719RKR and 2791KRK, and to a lesser extent from 2766KGRTK, in full agreement with our heparin binding data.

Regarding the interactions with α-DG and sulfatide, there are some notable discrepancies between our data and those of Andac et al. (14) (the interaction with these molecules were not analyzed by Hozumi et al. (28)). We found α-DG binding to 2766KGRTK (mutant D) and 2791KRK (mutant G) was only moderately reduced, whereas the same mutations led to a complete loss of α-DG in the study of Andac et al. (14). Conversely, we observed strong effects on sulfatide binding when we mutated 2831RAR (mutant I) and 2858KDR (mutant J), unlike Andac et al. (14). There are several possible reasons for these different results. First, we expressed α1LG4-5 proteins bearing mutations within LG4, because the tight structural linkage between the two LG domains argues that the domain pair behaves as a single
structural unit. In contrast, the earlier study (14) looked at interactions of isolated α1LG4 proteins. Second, there were methodological differences that may have contributed to the observed differences. We found that drying and rehydrating the protein led to loss of activity and that it was preferable to detect an N-terminal epitope tag located away from the residues of interest rather than use an E3-specific antibody that could act as a blocking agent. Finally, a different α-DG preparation was used in our study compared with Andac et al. (14), and it is known that tissue-specific α-DG modifications can have an effect on laminin binding.

Summarizing all of the binding data in the light of our new crystal structure, we can safely assign the heparin/heparan sulfate binding site of laminin α1 LG4 to the basic patch made up of RKR and KRK; of the KGRTK sequence, only the side chain of Lys\(^{2766}\) is positioned to contribute to heparin binding (Figs 7B,C). The α-DG binding site appears to be formed by a larger, semi-circular arrangement of basic side chains, with our new results assigning a key role to residues located away from the heparin binding site (i.e., 2831RAR and 2858KDR). The metal ion bound to LG4 (probably calcium under physiological conditions) is likely to be essential for α-DG, given that mutation of calcium ligands in LG4 of the related laminin α2 chain abolished α-DG binding (13) and that equivalent calcium sites in the LG domains of neurexin (29) and agrin (30) are critical for biological function. Finally, sulfatide binding in our hands is most strongly affected by mutations of basic residues on the upper face (view of Fig. 7B) of LG4. Two asparagine residues in laminin α1 LG4, Asn\(^{2714}\) and Asn\(^{2811}\), carry bulky glycan modifications, and the glycosylation status of these residues might influence ligand binding. However, whether and how glycosylation of laminin affects its interaction with cell surfaces has not been studied.

Laminin α2LG4-5 binds to the same cell surface molecules as α1LG4-5. Using a similar approach as in the present study, Wizemann et al. (13) found that heparin and sulfatide binding to α2LG4-5 were most strongly affected by mutation of KK in LG4 and KLTKGTGK in LG5, whereas α-DG binding was particularly dependent upon Arg\(^{2803}\) and KK, as well as upon the calcium ion in LG4. Remarkably, neither of these critical sequences correspond to the binding sites identified in α1LG4-5 (Fig. 2A and Table 2). The lack of conservation of functionally important residues in two closely related proteins is unusual. We speculate that the predominance of electrostatic interactions in ligand binding by the laminin LG4-5 portion accounts for the poor conservation of binding sites. Evidently, it is sufficient to maintain the general basic character of the binding surfaces.

Our earlier findings do not support a role of heparin/heparan sulfates in basement membrane anchorage, but instead argue for a prominent role of sulfated glycolipids with a signaling rather than an anchorage contribution arising from α-DG (11). Analysis of general and tissue-specific DG knockouts has revealed an essential role of DG for Reichert’s membrane, but not muscle, peripheral nerve and other basement membranes where it may function primarily as a signaling receptor (31). A recent analysis of cultured breast epithelial cells (32) revealed an anchoring activity for DG, raising the possibility that some cells employ DG as an anchor in a manner similar to sulfated glycolipids. In this regard, it will be informative to analyze cell surface assembly of heterotrimeric laminins bearing mutations that distinguish between α-DG and sulfated glycolipid binding, i.e. A (RKR to AKA) and I (RAR to AAA). Based on the results presented here, we would predict reduced anchorage only of the latter mutant, whereas both mutants could be compromised in their ability to signal through α-DG.

**Supplementary Material**

Refer to Web version on PubMed Central for supplementary material.
Acknowledgments

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2 The abbreviations used are

| Abbreviation | Description |
|--------------|-------------|
| LG           | laminin G-like domain |
| DG           | dystroglycan |
| WT           | wild-type (native sequence) |

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FIGURE 1. Structure of laminin α1LG4-5

A, Superposition of the two α1LG4-5 molecules in the asymmetric unit. Molecules A (light brown) and B (blue) were superimposed on their LG4 domains. The position of Tyr\textsuperscript{2871} (see text) is indicated. B, Cartoon drawing of molecule B (LG4, cyan; LG5, green). The N- and C-termini are labelled. Disulfide bonds are shown as yellow ball-and-stick models. Metal ions are shown as purple spheres. The positions of Asn\textsuperscript{2714} and Asn\textsuperscript{2811} (N-linked glycosylation sites) and Cys\textsuperscript{3014} (unpaired cysteine) are indicated. The third glycosylation site at Asn\textsuperscript{2900} is located at the back of LG5.
FIGURE 2. Comparison of α1LG4-5 and α2LG4-5

A, Sequence alignment of α1LG4-5 and α2LG4-5. Identical residues are shaded yellow, cysteines are shaded black, and metal ion ligands are shaded purple. The sequence numbering and β-strands of mouse α1LG4-5 are indicated above the alignment, and the sequence numbering of mouse α2LG4-5 is indicated below the alignment. Residues implicated in receptor binding to α1LG4-5 (this work) and α2LG4-5 (13) are indicated in red. B, Superposition of α1LG4 (this work) and α2LG4 (8). A total of 148 Ca atoms were superimposed with an r.m.s. deviation of 0.91 Å. C, Superposition of α1LG5 (this work) and α2LG5 (8). A total of 153 Ca atoms were superimposed with an r.m.s. deviation of 0.59 Å.
FIGURE 3. Characterization of recombinant α1LG4-5 proteins

A, Reducing 12% SDS-PAGE gel stained with Coomassie blue of recombinant α1LG4-5 proteins (WT, wild-type; A-I, mutants as defined in Table 2). The E3 fragment purified from EHS tumor laminin-111 is included for comparison. Recombinant α1LG4-5 proteins were purified by anti-FLAG affinity chromatography from the conditioned medium of 293 cell lines. The molecular masses of marker proteins are indicated on the left. B, Coomassie blue-stained SDS-PAGE gel of WT α1LG4-5 before (right lane) and after incubation (left lane) with enterokinase to remove the FLAG tag. C, Western blot with anti-FLAG-HRP of gel in panel B. D, Western blot with mouse α1LG4-5 specific polyclonal antibody after stripping of blot in panel C. E, Rotary shadow electron micrographs, contrast reversed, of E3 and selected recombinant LG4-5 proteins. Insets show selected molecules enlarged 2.5 fold.
FIGURE 4. Heparin affinity elution of α1LG4-5 proteins
The indicated proteins were loaded on a heparin-5PW column in 50mM Tris-HCl, 1 mM CaCl₂, pH 7.4 at 4°C and eluted with a 0-1 M NaCl gradient. The peak values were used to estimate relative affinities.
FIGURE 5. Binding of α1LG4-5 proteins to α-dystroglycan

A, Blot-immobilized α-DG overlaid with recombinant α1LG4-5 proteins. B, Microtiter plate wells coated with α-DG were incubated with increasing concentrations of the indicated α1LG4-5 proteins. In each assay (A and B), bound proteins were detected with HRP-linked anti-FLAG monoclonal antibody. Values are presented as mean ± S.D. (n = 3) and were fitted for single-site binding.
FIGURE 6. Lipid binding of α1LG4-5 proteins

Binding of WT α1LG4-5 (0.4 mg/ml) to the indicated lipids (10 μg/well) immobilized in microtiter plates. B, Binding of WT and mutant α1LG4-5 proteins to immobilized galactosyl-sulfatide. Values are presented as mean ± S.D. (n = 4) and were fitted for single-site binding.
FIGURE 7. Functionally important sites in α1LG4-5

A, Electrostatic surface representation of the α1LG4-5 structure. Positive and negative potential are indicated by blue and red coloring, respectively. B, Mapping of functionally important residues (compare Fig. 2A and Table 2) onto a surface representation of α1LG4-5. The view direction is the same as in A. The locations of the metal ion binding site and two asparagines modified by glycosylation are also shown (DG, dystroglycan; hep, heparin; S, sulfatide). C, Atomic details of the major basic region in LG4. A magnesium ion and selected side chains are shown as ball-and-stick models. Hydrogen bonds are indicated by dashed lines. Selected β-strands are labelled. Asn^{281} carries an N-linked glycan in WT α1LG4-5.
### Table 1

**Crystallographic statistics**

Numbers in parentheses refer to data in the highest resolution shell.

| **Data collection and reduction** |  |
|----------------------------------|---|
| Space group                      | P2$_1$ |
| Unit cell dimensions             | a = 70.53 Å, b = 55.81 Å, c = 100.99 Å, $\beta$ = 98.48° |
| Resolution range (Å)             | 20.0 (2.00) - 1.90 |
| Unique reflections               | 59682 |
| Multiplicity                     | 2.6 (2.1) |
| Completeness (%)                 | 97.3 (91.5) |
| Mean I/σ(I)                      | 10.2 (2.2) |
| $R_{merge}$                      | 0.070 (0.333) |

**Refinement**

| Reflections (working set/test set) | 56655/3015 |
| Atoms (protein/solvent)            | 5781/334   |
| $R_{cryst}$/$R_{free}$             | 0.230/0.262 |
| R.m.s. deviations                 |  |
| Bond lengths (Å)                   | 0.006     |
| Bond angles (°)                    | 1.4       |
| B-factors (Å$^2$)                  | 2.8       |
| Ramachandran plot (%)             | 86.9/11.9/0.8/0.5 |

1. Difference in B-factors of covalently bonded atoms.

2. Residues in most favored, additionally allowed, generously allowed, and disallowed regions (33). In both crystallographically independent molecules, two residues assume unfavourable main chain conformations: Lys$^{2791}$, which is part of the heparin binding site, and Arg$^{2896}$, whose peptide carbonyl oxygen receives a hydrogen bond from a buried lysine.
### Summary of binding data

Heparin binding is expressed as the molar NaCl concentration required for elution from a heparin affinity column. α-DG and sulfatide binding is expressed as the apparent dissociation constant ± S.E. obtained from fitting a single-site binding curve to the experimental data.

| α1LG4-5 protein | Symbol | Mutations | Heparin, M NaCl | α-DG, nM | Sulfatide, nM |
|-----------------|--------|-----------|-----------------|---------|--------------|
| Wild-type       | WT     | none      | 0.252           | 34 ± 3.4| 93 ± 6.7     |
| 271 RKR         | A      | AKA       | 0.154           | 387 ± 357| 38 ± 6.5     |
| 271 RKR         | A2     | AAA       | 0.137           | 93 ± 15 | 382 ± 53     |
| 286 KGRTK       | D      | AGATA     | 0.199           | 145 ± 164| 1627 ± 147   |
| 281 KRK         | G      | AAA       | 0.150           | 81 ± 18 | 382 ± 43     |
| 283 RAR         | I      | AAA       | 0.216           | 257 ± 33| 6729 ± 2377  |
| 285 KDR         | J      | ADA       | 0.234           | 315 ± 35| 512 ± 28     |