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Binding of the G domains of laminin α1 and α2 chains and perlecan to heparin, sulfatides, α-dystroglycan and several extracellular matrix proteins

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The C-terminal G domain of the mouse laminin α2 chain consists of five lamin-type G domain (LG) modules (α2LG1 to α2LG5) and was obtained as several recombinant fragments, corresponding to either individual modules or the tandem arrays α2LG1-3 and α2LG4-5. These fragments were compared with similar modules from the laminin α1 chain and from the C-terminal region of perlecan (PGV) in several binding studies. Major heparin-binding sites were located on the two tandem fragments and the individual α2LG1, α2LG3 and α2LG5 modules. The binding epitope on α2LG5 could be localized to a cluster of lysines by site-directed mutagenesis. In the α1 chain, however, strong heparin binding was found on α1LG4 and not on α1LG5. Binding to sulfatides correlated to heparin binding in most but not all cases. Fragments α2LG1-3 and α2LG4-5 also bound to fibulin-1, fibulin-2 and nidogen-2 with K_d = 13–150 nM. Both tandem fragments, but not the individual modules, bound strongly to α-dystroglycan and this interaction was abolished by EDTA but not by high concentrations of heparin and NaCl. The binding of perlecan fragment PGV to α-dystroglycan was even stronger and was also not sensitive to heparin. This demonstrated similar binding repertoires for the LG modules of three basement membrane proteins involved in cell–matrix interactions and supramolecular assembly.

Keywords: basement membranes/binding assays/cell–matrix interaction

Introduction

Most extracellular matrix proteins have a multidomain structure in which individual modules have specific functions in cell–matrix interactions or supramolecular assembly. The laminin-type G domain (LG) modules consist of ~190 residues and have been identified as >60 variants (Bork *et al*., 1996). They occur in 5-fold tandem arrays (LG1 to LG5) at the C-terminus of the laminin α1 to α5 chains (Timpl, 1996a) and in different arrangements in the basement membrane proteoglycans perlecan (Noonan *et al*., 1991) and agrin (Patthy and Nikolics, 1993). They also exist in various cellular receptors such as neurexins (Ushkaryov *et al*., 1992) and developmentally regulated *Drosophila* genes (Patthy, 1992), as well as in several extracellular ligands (Joseph and Baker, 1992; Manfioletti *et al*., 1993). Despite having only a limited sequence identity (20–40%), the different LG modules may have evolved related functions, such as binding to cellular receptors and sulfated ligands.

Functional studies of LG modules have mainly used the proteolytic laminin-1 fragments E8 (containing α1LG1-3) and E3 (α1LG4-5). Fragment E8 was identified as a major ligand for cell adhesion, mediated through α6β1, α7β1 or α9β1 integrins (Aumailley *et al*., 1996). Fragment E3, however, was shown to provide major binding sites for heparin, heparan sulfate chains of perlecan, sulfatides and fibulin-1 (Timpl, 1996b; Sasaki *et al*., 1998), and for the cellular receptor α-dystroglycan (Gee *et al*., 1993; Smalheiser, 1993; Brancaccio *et al*., 1995). Splice variants of two LG modules of agrin were previously shown to be important for its acetylcholine receptor clustering activity (McMahan *et al*., 1992; Patthy and Nikolics, 1993) but not for its high-affinity binding to α-dystroglycan (Gesemann *et al*., 1998). The latter activity and heparin binding are associated with different LG modules of agrin (Gesemann *et al*., 1996; Hopf and Hoch, 1996). Perlecan domain V, which consists of three LG modules separated by smaller spacers, was also shown to be cell-adhesive through β1 integrins, and to bind to heparin and the extracellular matrix proteins nidogen-1 and fibulin-2 (Brown *et al*., 1997). Another extracellular protein, Gas6, which is related to the coagulation factor S (Manfioletti *et al*., 1993), was recently shown to be a ligand for the receptor tyrosine kinases Rse and Axl, binding through its two LG modules (Mark *et al*., 1996).

The functions of the G domain of the laminin α2 chain, which is shared by laminin-2 and -4, have not yet been extensively studied. Corresponding recombinant fragments have recently been produced in insect (Rambukkana *et al*., 1997) and mammalian cells (Talts *et al*., 1998). This has demonstrated binding of mycobacterium leprae to the entire G domain, which could be important for the neural targeting of the pathogen (Rambukkana *et al*., 1997). It was also demonstrated that the absence of α2 chains in two mutant mouse strains causes severe muscular dystrophies, presumably because of interference with cell–matrix interactions (Xu *et al*., 1994; Miyagoe *et al*., 1997). Other indications for potential functions of α2LG modules came from previous studies with laminin-2 and -4 which demonstrated cell adhesion through β1 integrins and heparin binding (Brown *et al*., 1994) and a distinct interaction with α-dystroglycan (Yamada *et al*., 1994, 1996; Pall *et al*., 1996). Binding of α-dystroglycan to various laminins and agrin was also shown to differ in sensitivity to inhibition by salt and heparin (Gee *et al*., 1993, 1994; Yamada *et al*., 1994, 1996; Brancaccio *et al*., 1995; Pall *et al*., 1996; McDearmon *et al*., 1998), prompting the question of whether the binding epitopes for heparin and α-dystroglycan are related.

In the present study we used a set of six recombinant fragments (Talts *et al*., 1998) and two mutants to localize the binding sites for heparin, sulfatides, α-dystroglycan and some potential protein ligands in the G domain of the laminin α2 chain. In addition, the heparin- and sulfatide-binding epitope of α2LG5 was mapped by site-directed mutagenesis to a basic sequence region. A comparison with similar LG modules of the laminin α1 chain and perlecan showed distinct differences in epitope localizations, binding strengths and other binding parameters.

Results

The five LG modules from the G domain of the mouse laminin α2 chain have previously been prepared in the form of six recombinant fragments (Talts *et al*., 1998). These include the tandem arrays α2LG1-3 and α2LG4-5 (53 kDa) and the individual modules α2LG1, α2LG2, α2LG4 and α2LG5 (26–33 kDa). Fragment α2LG1-3 was proteolytically processed at a single basic site to 60 and 26 kDa components, which remained non-covalently associated. We therefore prepared a triple mutant α2LG3M (see Materials and methods), which abolished the protease-sensitive site (Talts *et al*., 1998) and allowed us to obtain an intact fragment (35 kDa) with good yields. These recombinant fragments were now used to examine binding activities for several extracellular matrix and cellular ligands. In several cases, a comparison was made with homologous structures from the mouse laminin α1 chain, including the proteolytic fragment E3 (equivalent to α1LG4-5), its individual modules and fragment E8, containing the α1LG1-3 tandem, as well as with the recombinant perlecan fragment V (Brown *et al*., 1997). The latter consists of three LG and four EG modules.

Binding to heparin and sulfatides

Binding to these ligands was indicated from previous studies with laminin α1 chain fragments (Ott *et al*., 1982; Taraboletti *et al*., 1990; Yurchenco *et al*., 1993) and the partial binding of laminin-2 and -4, which contain the α2 chain, to a heparin column (Brown *et al*., 1994). The recombinant fragments were therefore used on an analytical scale in affinity chromatography on a heparin HiTrap column at low ionic strength in order to determine the NaCl concentrations required for displacement (Table I). This demonstrated efficient binding of α2LG1-3, α2LG3M, α2LG4-5 and α2LG5 (>90%) and the need for salt concentrations above physiological levels (0.19–0.36 M NaCl) for elution. Lower salt concentrations were required for α2LG1 and α2LG4 displacement while no binding was observed for α2LG2. A similarly high level of binding and concentration of salt required for elution was observed for proteolytic laminin-1 fragments containing either the modules α1LG4-5 or α1LG1-3. Recombinant fragment α1LG4 showed only a slightly reduced strength in binding while no binding was observed for α1LG5 (Table I). Heparin binding has also been shown previously for perlecan fragment V, which required 0.2 M NaCl for displacement (Brown *et al*., 1997). Thus, quite a large variety of LG modules may be involved in heparin interactions.

These interactions were confirmed in a solid-phase assay carried out at physiological ionic strength with immobilized heparin–albumin and various concentrations of soluble laminin ligands. Binding was dose-dependent as shown by typical saturation profiles (Figure 1A), which were evaluated by determining the concentrations required for half maximal binding (Table I). These concentrations showed a good inverse correlation with the NaCl concentrations required for displacement in affinity chromato-

Table I. Relative affinities of the LG modules of the laminin α2 and α1 chains for heparin and sulfatides

Soluble ligands	Heparin		Sulfatides nM NaCl
	M NaCl[a]	nM[b]	
α2LG1-3	0.36	20	35
α2LG1	0.14	150	250
α2LG2	NB	NB	NB
α2LG3M	0.19	75	65
α2LG4-5	0.23	45	5.5
α2LG4	0.04	NB	40
α2LG5	0.19	25	25
α1LG4-5	0.26	30	65
α1LG1-3[c]	0.22	n.d.	n.d.
α1LG4	0.21	24	33
α1LG5	NB	NB	NB

Heparin binding was measured by affinity chromatography in 0.05 M Tris–HCl pH 7.4[a] and the NaCl concentrations required for elution are recorded. [b]Solid-phase binding assays with immobilized heparin or sulfatides were performed in physiological buffer and recorded as the concentrations of soluble ligands required for half-maximal binding (see Figure 1). NB denotes no significant binding above background up to 500 nM. n.d., not determined. [c]Used in the form of proteolytic fragment E8.

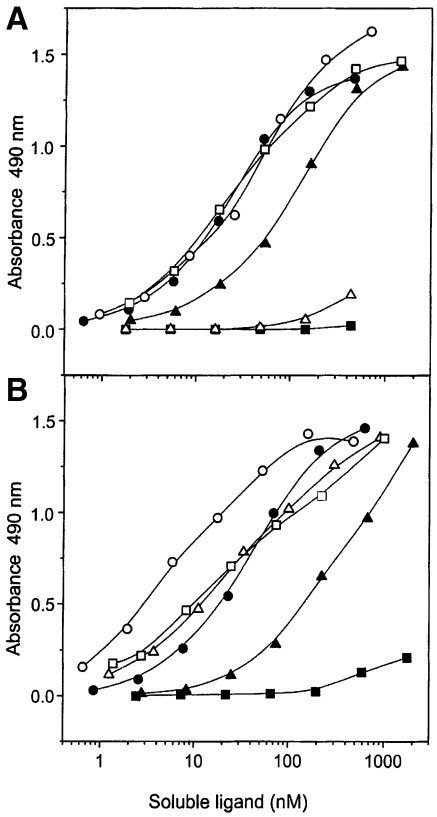

Fig. 1. Solid-phase binding profiles of recombinant laminin α2 chain fragments with immobilized heparin–albumin (**A**) and sulfatides (**B**). Soluble ligands were fragments α2LG1-3 (●), α2LG1 (▲), α2LG2 (■), α2LG4-5 (○), α2LG4 (△) and α2LG5 (□).

Table II. Kinetic analysis of the binding of fibulins and nidogens to G domain fragments of the laminin α2 chain by surface plasmon resonance

Immobilized ligand	Soluble ligand	$k_d \times 10^3$ (s^{-1})	$k_d \times 10^{-3}$ ($M^{-1}s^{-1}$)	K_d (nM)
α2LG1-3	fibulin-1	0.56	42	13
	fibulin-2	0.40	28	14
	nidogen-1	no binding		
	nidogen-2	1.3	22	59
α2LG4-5	fibulin-1	1.1	7.4	148
	fibulin-2	1.1	9.4	117
	nidogen-1	no binding		
	nidogen-2	1.4	27	51

Assays were performed in neutral buffer (pH 7.2) containing 1 mM $CaCl_2$, with soluble ligands in the concentration range 0.3–1 μM. Values are means of two to four independent determinations.

graphy, indicating that both assays measure relative binding strengths. Laminin-1 fragment E3 (α1LG4-5) has also recently been shown to bind distinctly to the heparan sulfate chains of perlecan domain I (fragment IA; Sasaki *et al*., 1998). Similar studies with α2LG1-3 and α2LG4-5 failed to show this binding, indicating a significant difference between laminin α1 and α2 chains.

A solid-phase assay with immobilized sulfatides was used as a third binding test. The binding was again dose-dependent (Figure 1B) and for most ligands showed a good correlation (within a factor of two) with the concentrations yielding half-maximal binding to heparin (Table I). There were, however, two noticeable exceptions: α2LG4 and α2LG4-5 bound distinctly better to sulfatides than to heparin. Furthermore, ligands with tandem arrays showed a 4- to 6-fold stronger binding than individual LG modules, indicating cooperativity in the interactions.

Binding to various extracellular matrix proteins

Several extracellular matrix proteins, mostly typical basement membrane components, were screened by solid-phase binding assays to identify further ligands. Fibulin-1C, fibulin-2 and nidogen-2 bound particularly well to immobilized α2LG1-3 and α2LG4-5. No or only low binding was observed for collagens I and IV, BM-40 and perlecan, however, while inconsistent results were obtained with nidogen-1 (data not shown).

The kinetic and thermodynamic constants for the most relevant interactions were determined by surface plasmon resonance assay (Table II). This confirmed strong binding (K_d = 13–14 nM) of fibulin-1 and fibulin-2 to α2LG1-3 and a 10-fold lower affinity for α2LG4-5. Nidogen-2 bound both α2 chain ligands to the same extent (K_d = 51–59 nM). No measurable interactions were observed with nidogen-1, indicating that the inconsistent solid-phase assay binding data represent artefacts.

Binding to α-dystroglycan

Since the binding of α-dystroglycan has been localized to fragment E3 (α1LG4-5) of laminin-1, it seemed possible that its strong interaction with α2 chain laminins may occur through identical or similar G domain structures (Gee *et al*., 1993; Yamada *et al*., 1994, 1996; Brancaccio *et al*., 1995; Pall *et al*., 1996). This was examined in solid-phase assays with immobilized α-dystroglycan, using

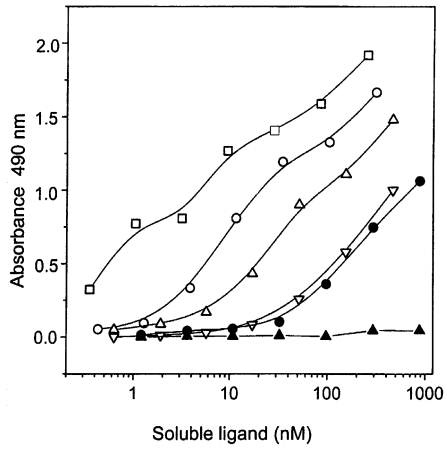

Fig. 2. Solid-phase binding assays of laminin α1 and α2 chain and perlecan fragments to chick muscle α-dystroglycan. Soluble ligands were fragments α2LG1-3 (○), α2LG4-5 (△), α1LG4-5 (▽), perlecan PGV (□), α1LG4 (●) and α1LG5 (▲).

Table III. Solid-phase assay binding of immobilized α-dystroglycan from muscle to LG modules derived from the laminin α2 and α1 chains and from perlecan (PG)

Soluble ligand	([a])	+ heparin[b]	+ NaCl[c]
α2LG1-3	15	25	100
α2LG3M	160	n.d.	n.d.
α2LG4-5	50	60	150
α1LG4-5	200	NB	NB
α1VI/V	150	n.d.	n.d.
PGV	3	3	25
PGVa	40[d]	50	200
PGVb	2000[d]	n.d.	n.d.
PGIII-3	NB	n.d.	n.d.

Assays were performed in physiological buffer in the absence of inhibitors[a] or in the presence of heparin (0.3 mg/ml)[b] or 0.5 M NaCl[c]. Activities are recorded as concentrations (nM) of soluble ligands required for half-maximal binding. NB denotes no significant binding above background up to 500 nM. n.d., not determined. [d]Assay with kidney α-dystroglycan.

various soluble ligands possessing LG modules (Figure 2; Table III). Both α2 chain tandem fragments showed distinct binding profiles, with α2LG1-3 being ~3-fold stronger than α2LG4-5. Fragment α2LG3M had a 10-fold decreased binding activity compared with α2LG1-3 (Table III). However, none of the other fragments with a single LG module (α2LG1, α2LG2, α2LG4, α2LG5) showed any significant binding up to 500 nM (data not shown).

A comparison with the corresponding laminin α1 chain fragments demonstrated binding of α1LG4-5 but not of fragment E8, which contains the LG1-3 modules, confirming previous observations (Gee *et al*., 1993; Brancaccio *et al*., 1995). The binding activity of α1LG4-5 was ~4-fold lower than that of α2LG4-5. Furthermore, the binding activity could be mapped to α1LG4, which was nearly as active as α1LG4-5, while no binding was observed with α1LG5 (Figure 2). In addition, we used an unrelated heparin-binding fragment α1VI/V from the laminin α1 chain and found a comparable interaction as with fragment α1LG4. The most surprising observation, however, was the strong binding of perlecan fragment V, which exceeded that of the most active laminin fragment

(α2LG1-3) by a factor of five (Figure 2; Table III). Two subfragments, Va containing two LG modules and Vb consisting of the most C-terminal LG module (Brown *et al*., 1997), were also examined. This demonstrated a 10-fold (Va) and 1000-fold (Vb) reduction in binding activity (Table III), indicating, as for the α2 chain, the need for several LG modules to achieve a high level of interaction. The interaction with PGV was apparently specific, since a structurally unrelated perlecan fragment, PG III-3, had no binding activity for α-dystroglycan.

We also compared α-dystroglycans obtained from skeletal muscle and kidney and found no difference in the binding to α2LG1-3, α2LG4-5, α1LG4-5 and PGV. Most of the other data were then obtained with the skeletal muscle α-dystroglycan unless otherwise stated.

The binding of α2LG1-3, α2LG4-5 and PGV to α-dystroglycan could be inhibited completely by 10 mM EDTA (not shown), as has previously been shown for the α1LG4-5 structure (Gee *et al*., 1993, 1994; Brancaccio *et al*., 1995; Pall *et al*., 1996; Yamada *et al*., 1996). In addition, we examined the effects of high concentrations of heparin (0.3 mg/ml) or of NaCl (0.5 M) on the binding to α-dystroglycan. This abolished binding of α1LG4-5 but caused only a slight to moderate shift in the binding profile of α2LG4-5. Similarly, α2LG1-3 and perlecan fragments V and Va also showed a low sensitivity to both inhibiting conditions (Table III).

A major question which arose from these observations was whether the binding epitope on α-dystroglycan was the same or at least similar for laminin α1 and α2 chains and perlecan LG modules. This was initially examined in competition assays with the strongest ligand, PGV, used at a fixed low concentration (40 nM) and competitors at equivalent or higher molar ratios. Fifty percent inhibition was achieved with a 20-fold excess of α2LG1-3 and a 300-fold excess of α2LG4-5 (Figure 3A). These differences correlated with the relative strengths in direct binding assays (Table III), but were accentuated by a factor of four to twenty. Fragment α1LG4-5 showed no inhibition up to a 240-fold excess. In a second experiment, α2LG1-3 was used at a low concentration (40 nM) and combined with various competitors (Figure 3B). As expected, fragment PGV inhibited 90% of the binding even at equimolar concentrations. However, a 100- to 200-fold excess of fragments α2LG4-5, α1LG4-5 and α1VI/V was needed to cause 30–50% inhibition. These data again correlated with those of the direct binding assay and, since they were carried out with the competitors in solution, define a hierarchy of relative affinities for α-dystroglycan extending over more than three orders of magnitude.

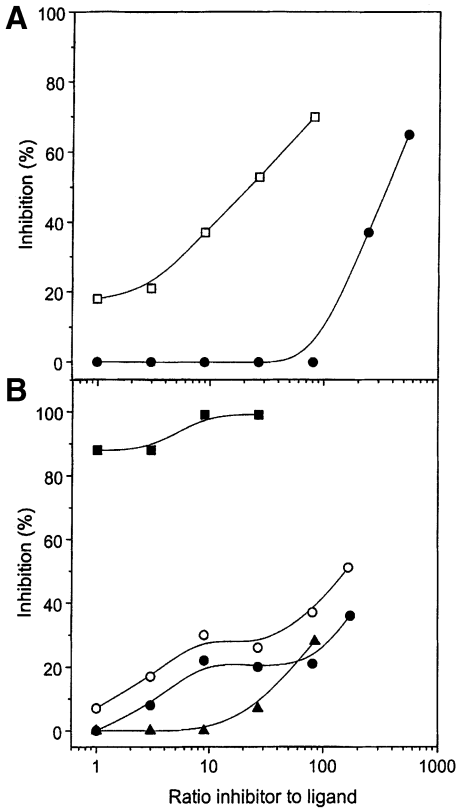

Fig. 3. Competition between perlecan and laminin α chain fragments for binding to α-dystroglycan. (**A**) A fixed amount of fragment PGV (40 nM) was mixed with increasing concentrations of fragments α2LG1-3 (□) or α2LG4-5 (●) prior to incubation with immobilized kidney α-dystroglycan. Detection of binding was with an antiserum against PGV which did not cross-react with the laminin fragments. (**B**) A fixed amount of fragment α2LG1-3 (40 nM) was mixed with increasing concentrations of fragments PGV (■), α1LG4-5 (○), α2LG4-5 (●) and α1VI/V (▲), and analyzed with muscle α-dystroglycan. Detection of binding was with an antiserum-specific for α2LG1-3.

Localization of the heparin-binding epitope of α2LG5 by site-directed mutagenesis

Heparin-binding epitopes in a variety of proteins often include regions containing clusters of Arg and/or Lys residues which are not necessarily contiguous in the sequence (Lander, 1994). The participation of Lys and Arg residues in the heparin-binding epitope of α2LG5 was initially examined by specific modification of their side chains. Acetylation of lysine completely abolished binding both in the affinity chromatography and solid-phase binding assays. Blocking of arginine by phenylglyoxal did not change the affinity chromatography profile but strongly reduced binding in the solid-phase assay. Two Lys-rich regions exist in the mouse laminin α2LG5 sequence (Bernier *et al*., 1994; Talts *et al*., 1998) and were selected for mutation in the mutants K1 (KKIK; position 3027–3030) and K2 (KLTKGTGK; position 3088–3095). Expression vectors were produced containing mutations to convert the Lys codons to Ala. Both vectors produced mRNA levels comparable to the wild-type (not shown, but see Talts *et al*., 1998) but only mutant K2 could be obtained as a protein product and purified. This indicated that the mutations introduced in K1 interfered with the proper folding of the α2LG5 module.

The ELISA titration profiles of an antiserum against α2LG4-5 were similar for mutant K2 and α2LG5, indicating a comparable folding. Yet the mutant no longer bound to the heparin affinity column and showed a strongly reduced binding to heparin and sulfatides in the solid-phase assays (Figure 4).

Discussion

Laminins that share the α2 chain are particularly prominent in basement membranes of striated and smooth muscles, of peripheral nerves and placenta (Engvall *et al*., 1990;

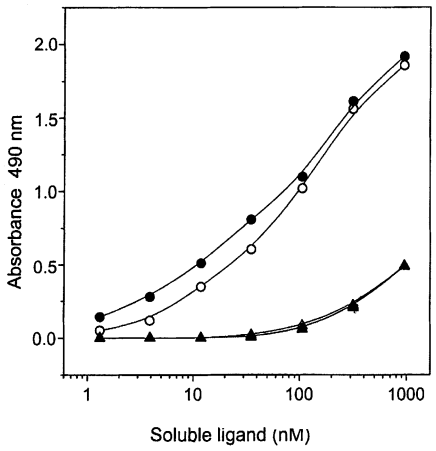

Fig. 4. Change in solid-phase assay binding of fragment α2LG5 and mutant K2 mutation to heparin–albumin (open symbols) and sulfatides (closed symbols). Soluble ligands were fragment α2LG5 (○, ●) and mutant K2 (△, ▲). The latter two curves were superimposable.

Miner *et al*., 1997). Natural mutations in the α2 chains and their absence in transgenic mice (Xu *et al*., 1994; Helbing-Leclerc *et al*., 1995; Miyagoe *et al*., 1997) cause severe forms of muscular dystrophy, suggesting interference with important cell–matrix interactions. This led us to examine the potential functions of the G domain of the laminin α2 chain and in the present study we demonstrate a distinct binding of this domain to heparin, sulfatides and α-dystroglycan. The same binding properties have been previously identified for the α1LG4-5 modules of the laminin α1 chain (Ott *et al*., 1982; Taraboletti *et al*., 1990; Gee *et al*., 1993) but, as shown here, frequently differ in binding strength and/or localization to a particular LG module. A further biological property exclusively associated with fragment α2LG1-3 includes firm cell attachment and spreading, which is still under examination (J.F.Talts and R.Timpl, unpublished).

A strong heparin affinity indicates the potential to bind matrix or membrane-bound heparan sulfate proteoglycans through clusters of basic amino acids (Lander, 1994). At least two heparin-binding sites of comparable strength exist on fragments α2LG1-3 and α2LG4-5 and in the latter case could be exclusively mapped to the α2LG5 module. A comparison with the homologous structure α1LG4-5 demonstrated that in the case of the α1 chain, the α1LG4 but not the α1LG5 module makes the major contribution to heparin binding. This suggests that during evolutionary diversification of laminin α chains, gain or loss of heparin binding may have depended on a few crucial amino acid substitutions. Alanine mutagenesis of a single lysine cluster close to the C-terminal end of α2LG5 (mutant K2) abolished heparin binding, while another mutation (mutant K1) impaired protein folding. These data emphasize the importance of the K2 region for heparin binding, but do not exclude the participation of additional basic regions, probably non-contiguous in sequence, in this interaction. In fact, recent and more comprehensive mutagenesis data for α1LG4 demonstrated that two different non-contiguous basic regions are required for efficient heparin-binding (Z.Andac, T.Sasaki, K.Mann, A.Brancaccio and R.Timpl, submitted). This precise mapping could not be achieved for α2LG1-3 since the two active modules (α2LG1, α2LG3M) showed a 4- to 8-fold decreased binding activity indicating that they cooperatively enhance the binding of α2LG1-3.

Several extracellular matrix proteins have been shown to bind sulfatides, which was interpreted to indicate a role in promoting cell adhesion (Roberts, 1987) and, as in the case of heparin, may depend on sulfate groups. For laminin-1, it was also shown that sulfatides enhance polymerization into networks in the close vicinity of artificial lipid bilayers (Kalb and Engel, 1991). Here we show that sulfatide binding is also shared by α2 chain-containing laminins, mediated through four different modules of the G domain. With one exception, the binding strength correlated with that of heparin-binding, indicating that these two ligands may share binding epitopes. This was further supported by the loss of binding to both of these ligands observed for mutant K2. The potential biological importance of sulfatide binding was underscored by recent observations that α2 chain-containing laminins also polymerize into networks (Cheng *et al*., 1997), and the genetic loss of this ability causes a special form of muscular dystrophy (Xu *et al*., 1994; Timpl, 1996a).

The α2 chain-containing laminins were also shown to be strong ligands for α-dystroglycan (Yamada *et al*., 1994, 1996; Pall *et al*., 1996). This transmembrane receptor is known to provide a crucial linkage between various ligands of the extracellular matrix and cytoskeletal components (Henry and Campbell, 1996). A major binding site was located to the α1LG4-5 modules (fragment E3) but not the α1LG1-3 modules (fragment E8) of laminin-1 (Gee *et al*., 1993; Smalheiser *et al*., 1993; Brancaccio *et al*., 1995). Yet as shown here, the constellation is different for the laminin α2 chain, in which both α2LG1-3 and α2LG4-5 were able to bind, but with α2LG1-3 being the stronger ligand, again indicating genetic diversification. Except for a relatively weak affinity of α2LG3M, none of the four individual recombinant α2LG modules showed any binding activity for α-dystroglycan, suggesting that at least two modules are required for the binding epitope. This is different to the laminin α1 chain, where the binding activity could be mapped to the α1LG4 module and showed a partial overlap with the heparin-binding epitope (Z.Andac, T.Sasaki, K.Mann, A.Brancaccio, R.Deutzmann and R.Timpl, submitted). A novel observation was that the recombinant heparin binding fragment α1VI/V from the N-terminus of the laminin α1 chain (Ettner *et al*., 1998) was also a ligand for α-dystroglycan, with a binding activity comparable to that of fragment α1LG4-5 (Table III). This indicates that certain other heparin binding structures may be able to bind to α-dystroglycan. This may not be a general rule, since α-dystroglycan binding to laminin α1 chains but not α2 chains can be inhibited by heparin and high salt concentrations (Gee *et al*., 1993; Yamada *et al*., 1994; Brancaccio *et al*., 1995; Pall *et al*., 1996; McDearmon *et al*., 1998), as was confirmed in our study with the recombinant G domain fragments.

The C-terminal domain V of the major basement membrane proteoglycan perlecan also contains three LG modules (Timpl, 1993) which bind to heparin (Brown *et al*., 1997), but these have not previously been examined for α-dystroglycan binding. Here we show that the corresponding recombinant fragment PGV was the strongest α-dystroglycan ligand of all those tested. Binding requires the modules LG1 and/or LG2 present in

fragment Va while module LG3 (fragment Vb) had only low activity. As for the laminin α2 chain, binding was sensitive to EDTA but not to heparin. Together, the data indicate that perlecan is a stronger α-dystroglycan ligand than the laminin α1 and α2 chains. Yet the data of the competition assays (Figure 3) would be compatible with the assumption that α-dystroglycan binds to these diverse ligands through the same or a set of overlapping epitopes. Given the broad occurrence of perlecan in tissues (Timpl, 1993), it could in fact be a more important ligand for α-dystroglycan than laminin α1 and α2 chains. Elimination of the dystroglycan gene by homologous recombination was shown to cause early embryonic lethality in mice, probably due to an abnormal development of Reichert's membrane (Williamson *et al*., 1997). In this context, it is of interest that Reichert's membrane stains heavily for perlecan and laminin α1 chain but is negative for the laminin α2 chain (J.F.Talts, unpublished), suggesting that the failure of cellular contacts to perlecan and perhaps to the weaker binding α1 chain are involved in the mutant phenotype. A further place of interactions could be neuromuscular junctions where perlecan and α-dystroglycan co-localize (Peng *et al*., 1998).

The proteoglycan agrin was identified as another major α-dystroglycan ligand (Gee *et al*., 1994; Gesemann *et al*., 1996; Yamada *et al*., 1996). Agrin was originally characterized as a protein responsible for the clustering of acetylcholine receptors in neuromuscular junctions and, like perlecan, possesses three LG modules at its C-terminus (McMahan *et al*., 1992; Patthy and Nikolics, 1993). These LG modules were shown to be involved in both biological activities to a variable extent, while heparin binding to the agrin LG2 module depended on a special splice variation (Gesemann *et al*., 1996; Hopf and Hoch, 1996). This again emphasizes that the binding epitopes for α-dystroglycan and heparin are not necessarily the same.

Another function of the laminin α2 chain G domain could be the binding to extracellular matrix ligands rather than to cellular receptors (Brown *et al*., 1994). The heparan sulfate-containing perlecan fragment PGIA failed to show such binding, in contrast to its distinct binding to the α1LG4-5 structure (Sasaki *et al*., 1998). The screening of further ligands demonstrated binding of fragments α2LG1-3 and α2LG4-5 to fibulin-1, fibulin-2 and nidogen-2, however, with moderate affinities (Table II). The laminin α2 chain G domain therefore has the potential to participate in the supramolecular assembly of the extracellular matrix. Binding of fibulin-1 has also been demonstrated for the laminin α1LG4-5 structure (Pan *et al*., 1993b). These binding ligands have been localized to several basement membrane zones including vessel walls (Pan *et al*., 1993a; Kohfeldt *et al*., 1998), in agreement with a similar localization of laminin α2 chains (Engvall *et al*., 1990; Miner *et al*., 1997). A more precise localization at the electron microscopic level and the distinction between different binding epitopes by site-directed mutagenesis will now be required to understand the complex binding repertoire of the G domain of the laminin α2 chain.

Materials and methods

Sources of proteins, antibodies and other ligands

Purified recombinant mouse laminin α2 chain fragments α2LG1-3, α2LG4-5, α2LG1, α2LG2, α2LG4 and α2LG5 (Talts *et al*., 1998) and perlecan fragments IA (Costell *et al*., 1997), III-3 (Schulze *et al*., 1995), and V, Va and Vb (Brown *et al*., 1997) have been described previously. The N-terminal fragment α1VI/V of the laminin α1 chain was prepared in recombinant form (Ettner *et al*., 1998). Laminin-1 fragments E3 and E8, perlecan and collagen IV were obtained from the mouse Engelbreth-Holm-Swarm tumor (Timpl *et al*., 1987). Neutral salt-soluble collagen I from rat skin was prepared as described previously (Stoltz *et al*., 1972). Mouse fibulin-1C (Sasaki *et al*., 1995) and fibulin-2 (Pan *et al*., 1993a), mouse nidogen-1 (Fox *et al*., 1991), human nidogen-2 (Kohfeldt *et al*., 1998) and human BM-40 (Nischt *et al*., 1991) were prepared by recombinant procedures. Chicken skeletal muscle and kidney extracts were used for the purification of α-dystroglycan, following a combination of two previously described procedures (Brancaccio *et al*., 1995; Gesemann *et al*., 1998). Heparin coupled to bovine serum albumin (BSA) and bovine brain sulfatides were from a commercial source (Sigma). The rabbit antisera against α2LG1-3 and α2LG4-5 have been characterized previously (Talts *et al*., 1998). Side-chain modifications of Lys and Arg followed standard procedures (Fraenkel-Conrat, 1957; Takahashi, 1968).

Recombinant production of laminin α1 chain LG modules

A cDNA clone encoding the C-terminal part of the mouse laminin α1 chain (Deutzmann *et al*., 1988) was used to construct expression vectors for the α1LG4 (positions 2666–2871) and α1LG5 (positions 2877–3060) modules. These sequences were amplified by polymerase chain reaction (PCR) with vent polymerase (New-England Biolabs) following the manufacturer's instructions and using the primer combinations GCCCCGCTAGCTCTGCACAGAGAACACGGGG plus TCAGTTGCGGCCGCTTAATAGCACCTGTCCACAGC for α1LG4 and GCCCCGCTAGCTGGAACTTTCTTTGGAAGGAAG plus TCAGTTGCGGCCGCTTAGGGCTCAGGCCCGGG for α1LG5. The purified fragments were then ligated in-frame with the BM-40 signal peptide in the episomal expression vector pCEP-Pu and used to transfect human kidney 293-EBNA cells (Kohfeldt *et al*., 1997). The recombinant proteins were purified from serum-free culture medium either by heparin affinity chromatography in the case of α1LG4 or by DEAE cellulose chromatography in the case of α1LG5, following previous experimental protocols (Brown *et al*., 1997). Fragment α1LG4 eluted from the heparin column as a 36 kDa electrophoretic band of >95% purity. Fragment α1LG5 was found in the DEAE flow-through fraction at pH 8.6, and, after addition of 0.15 M NaCl, was concentrated by ultrafiltration. This fragment appeared as a 27 kDa band.

Preparation of laminin α2 chain mutants

The mouse laminin α2 chain LG5 domain construct (Talts *et al*., 1998) was used as a template for the construction of vectors encoding mutants K1 and K2. Site-directed mutagenesis was accomplished by overlap extension PCR with Vent polymerase. To produce mutant K1, a 5′ primer GTCACTGCCGCGGCGATCGCAAACCGTCTT, which introduced the mutation Lys to Ala at amino acid positions 3027, 3028 and 3030 (Bernier *et al*., 1994), was used with the α2LG5 3′ primer GTCACTCGAGTTAGGTAGTCGGGCATGATAC; and a α2LG5 5′ primer GTCAGCTAGCTGCGAATGCAGAGAGTGGG was used with the 3′ primer AAGACGGTTTGCGATCGCCGCGGCAGTGAC introducing the same mutations. These two overlapping PCR products were then annealed and PCR was used to extend them to the full-length of α2LG5. Mutant K2, with Lys to Ala mutations at positions 3088, 3091 and 3095, was constructed using the same strategy. The mutational primers were CGATCTCTGGCGCTCACCGCAGGCACTGGCGCACCGCTGGAG at the 5′ end, and CTCCAGGGCTGCGCCAGTGCCTGCGGTGAGCGCCAGAGAGAG at the 3′ end. Mutations R2571A, K2573A, R2575A were introduced into α2LG3 by the primers ACACCACCCGCGAGAGCACGGGCACAAACCACA and TGTGGTTTGTGCCCGTGCTCTCGCGGGTGGTGT using in addition the terminal primers for α2LG3 (Talts *et al*., 1998) for mutating the protease-sensitive site in the vector α2LG3M. All PCR products were ligated into plasmid pUC18 (Pharmacia) and sequences were verified by DNA sequencing. They were then ligated into the pCEP-Pu vector and used for transfection (see above). The mutants K2 and α2LG3M were then purified from serum-free culture medium by a combination of DEAE cellulose or heparin affinity and molecular sieve chromatography (Talts *et al*., 1998).

Protein ligand binding assays

Solid-phase assays were carried out with various proteins (5 μg/ml) coated onto the plastic surface of microtiter wells at 4°C following a previously used procedure (Aumailley *et al*., 1989) with some modifica-

tions. Wells were then blocked at room temperature (2 h) with 0.05 M Tris–HCl pH 7.4, 0.15 M NaCl (TBS), 1% BSA, 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 an absorbance at 490 nm of 1.5–2.0 in regular ELISA. After a further wash, the 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), 0.001% H_2O_2. Heparin binding was assayed by coating with 10 μg/ml heparin–BSA (Sigma) and incubating for 3 h with soluble ligands. The latter variation was also used for α-dystroglycan binding in TBS–BSA buffer containing 1 mM $CaCl_2$ and 1 mM $MgCl_2$. Some binding assays were also carried out in buffer containing heparin (0.3 mg/ml) or 0.5 M NaCl. Coating with sulfatides dissolved in methanol (0.2 mg/ml; 50 μl) was achieved by drying at room temperature overnight.

A 1 ml heparin HiTrap column (Pharmacia) equilibrated in 50 mM Tris–HCl pH 7.4 was used for affinity chromatography. After loading the protein samples (0.2–0.3 mg), the column was eluted at a flow rate of 0.5 ml/min with a 0–0.6 M NaCl gradient (30 ml). Eluted proteins were monitored at 280 nm and by SDS–PAGE of individual fractions.

Surface plasmon resonance assays were performed with BIAcore instrumentation (BIAcore AB Uppsala) using fragments α2LG1-3 or α2LG4-5 covalently coupled by carbodiimide to CM-5 sensor chips (Maurer *et al*., 1995). Binding assays were performed in neutral buffer (TBS) containing 1 mM $CaCl_2$ and taking precautions to avoid mass transport problems (Göhring *et al*., 1998). Dissociation and association rate constants were calculated according to the 1:1 model following the manufacturer's instructions (BIAevaluation software version 3.0).

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