The N-terminal globular domain of the laminin α1 chain binds to α1β1 and α2β1 integrins and to the heparan sulfate-containing domains of perlecan

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Abstract The N-terminal domains VI plus V (62 kDa) and V alone (43 kDa) of the laminin α1 chain were obtained as recombinant products and shown to be folded into a native form by electron microscopy and immunological assays. Domain VI alone, which corresponds to an LN module, did not represent an autonomously folding unit in mammalian cells, however. Fragment α1VI/V, but not fragment α1V, bound to purified α1β1 and α2β1 integrins, to heparin, and to heparan sulfate-substituted domains I and V of perlecan. This localized the binding activities to the LN module, which contains two basic sequences suitable for heparin interactions.

Key words: Basement membrane; Cell receptor; Heparin binding; Kinetic assay; Recombinant production

1. Introduction

Laminins are ubiquitous basement membrane proteins which form networks or filaments, promote cell adhesion through integrin and dystroglycan receptors and interact with other basement membrane proteins such as nidogen, perlecan and fibulin-1 [1]. Eleven distinct isoforms of laminin have been identified so far, composed of one of each of five different α, three β and two γ chains in a heterotrimeric assembly [2,3]. Electron microscopy of these chain assemblies demonstrated a cross- or T-shaped structure in which the short arms consist of the N-terminal domains of the three chains. In six of the laminin chains (α1, α2, α5, β1, β2, γ1) the most N-terminal region (domains VI/V) consists of a laminin-type N-terminal (LN) module followed by a tandem array of laminin EGF-like (LE) modules [4]. Together they form a globular domain connected to a short rod [5] and have been shown to be crucial elements in the polymerization of certain laminins into large networks [6–8]. It was also indicated that the same region of the laminin α1 chain binds to α1β1 and α2β1 integrins and to heparin [9,10].

In the present study, we have examined the production of laminin α1 chain domains VI/V by recombinant methods that have previously been used to successfully localize nidogen binding to a single LE module in the laminin γ1 chain [11]. This has allowed us to assign integrin, heparin and perlecan binding to the LN module, which corresponds to domain VI. Yet it was also shown that the LN module needed to be connected to LE modules to assure its proper folding.

2. Materials and methods

2.1. Expression vectors and cell transfection

A 1.8 kb *Xba*I/*Stu*I cDNA fragment encoding the entire N-terminal region of the mouse laminin α1 chain [12] was used as a template for amplification by polymerase chain reaction with Vent polymerase (Biolabs) following the manufacturer's instructions. The following synthetic oligonucleotides were used as primers: primer 1, 5′-GCCCCGCTAGCAATGTGCATTTGCTACGGCCAT; primer 2, 5′-GCCCCGCTAGCACAGCAGAGAGGCTTGTTCCC; primer 3, 5′-TCAGTTAGCGGCCGCTCAGCCTCCAACGGAAATGTCTTTT; primer 4, 5′-TCAGTTAGCGGCCGCTCACTCACACTCGTTGCCGGAG; primer 5, 5′-TCAGTTAGCGGCCGCTCAGGAGCAGCCCTCGGGGTTT.

Sequences encoding recombinant laminin fragment α1VI were obtained with the primer pair 1/3, fragment α1VI-1 with the pair 1/4, fragment α1V with the pair 2/4 and fragment α1VI/V with the pair 1/5. These primer combinations introduced a novel *Nhe*I site at the 5′ end and a stop codon and *Not*I site at the 3′ end. The products were restricted with *Nhe*I and *Not*I and, after purification by agarose gel electrophoresis, were inserted in frame with the BM-40 signal peptide [11] in an expression vector restricted with the same enzymes. The episomal expression vectors used were pCEP-sh [13] or pCEP-Pu [14], which contain either the phleomycin or puromycin resistance genes, respectively. The sequences of the constructs were verified by cycle sequencing on a 373A DNA sequencer (Applied Biosystems).

Human embryonic kidney 293 or 293-EBNA cells were transfected using lipofectamine (Gibco) as described by the supplier. After antibiotic selection, cells were grown either in a Tecnomouse hollow fiber system [15] or in a factory of 10 stacked trays (Nunc). Serum-free culture medium was then regularly collected for the purification of recombinant fragments.

2.2. Protein purification and modification

Culture medium (0.1–0.4 l) containing recombinant fragment α1VI/V was dialyzed against 0.05 M Tris-HCl pH 7.4, 0.1 M NaCl and passed over a heparin-Sepharose column (2.5×15 ml) equilibrated in the same buffer. Bound fractions were eluted with a linear 0.1–0.6 M NaCl (600 ml) gradient, concentrated and further purified on a Sepharose CL-6B column [16]. Medium containing fragment α1V was initially passed over a DEAE cellulose column (2.5×25 cm) which was equilibrated in 0.05 M Tris-HCl pH 8.6. After eluting with a 0–0.5 M NaCl gradient (1000 ml), the relevant fractions were further purified on a Superose 12 HR 16/50 column (Pharmacia) equilibrated in 0.2 M ammonium acetate pH 6.9. Degradation with neutral proteases was performed in 0.05 M Tris-HCl pH 7.4 at 37°C at an enzyme-substrate ratio of 1:100. A 1 ml heparin HiTrap column (Pharmacia) equilibrated in 0.05 M Tris-HCl pH 7.4 and eluted with a linear 0–0 .6M NaCl gradient was used for chromatography of small protein samples (0.2–1 mg).

Laminin-1, several of its proteolytic fragments and the heparan sulfate proteoglycan perlecan were prepared from a mouse tumor [17]. Recombinant perlecan fragments IA [18] and V and Vc [19] were obtained as described. In addition, we prepared a modified fragment I (I-Mut) where heparan sulfate attachment was prevented by Ala mutagenesis of Ser-65, Ser-71 and Ser-76 (T. Sasaki, M. Costell, R. Timpl, in preparation). Affinity-purified α1β1 integrin from human placenta and α2β1 integrin from human platelets were those used previously [9]. Some of these integrins were also kindly supplied by J. Eble, Münster.

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2.3. Binding assays

Solid-phase assays with plastic-immobilized ligands followed a previously used procedure [20]. Binding was detected with specific antisera raised against perlecan fragments IA and V [18,19]. Surface plasmon resonance assays were performed with BIAcore instrumentation (BIAcore AB, Uppsala) using fragments α1VI/V and α1V immobilized by covalent coupling to CM-5 sensor chips [21]. Binding assays with perlecan fragments were carried out in 0.05 M Tris-HCl, 0.15 M NaCl, 0.05% P20 detergent (TBS) and those with integrins in TBS containing 1 mM $MgCl_2$, 1 mM $MnCl_2$ and 0.1 mM $CaCl_2$. The divalent cations were replaced by 4 mM EDTA as a control for specificity [9]. Soluble ligands were used in the concentration range 0.3–1 μM and chips were regenerated by spontaneous dissociation (integrins) or by treatment with saturated NaCl (perlecan). Further details of the procedure and evaluation of the kinetic constants by the 1:1 model have been previously described [21].

2.4. Analytical and other methods

Samples were hydrolyzed with 6 M or 3 M HCl (16 h, 110°C) for the determination of the amino acid or hexosamine contents, respectively, on a LC3000 analyzer (Biotronik). SDS gel electrophoresis followed standard protocols. Edman degradation on Procise and 473 sequencers (Applied Biosystems) followed the manufacturer's instructions. Mixtures of protein fragments were electrophoretically separated and blotted onto Immobilon PSQ membranes (Millipore) for sequencing. Circular dichroism (CD) spectroscopy [22], electron microscopy after rotary shadowing [23] and immunological assays [24] followed standard protocols.

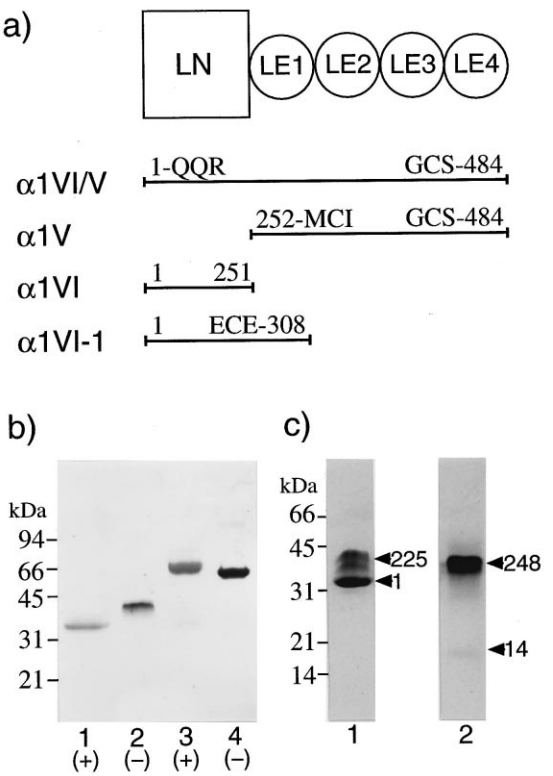

Fig. 1. Modular structure of the N-terminal domain VI/V of the laminin α1 chain (a) and electrophoresis of purified recombinant fragments (b) and of an elastase digest of fragment α1VI/V (c). The structure in a shows the N-terminal laminin-type module (LN) and laminin-type epidermal growth factor-like (LE) modules with the recombinant fragments identified underneath by their terminal sequences and position numbers [12]. Each vector also encoded an N-terminal APLA sequence derived from BM-40 [11]. Electrophoresis in b shows fragments α1V (lanes 1, 2) and α1VI/V (lanes 3, 4) used in reduced (+) and non-reduced (−) form. c: Elastase digests of fragment α1VI/V which either bind to heparin (lane 1) or lack this property (lane 2) were examined under non-reducing conditions. Numbers beside the bands denote starting positions of the major N-terminal sequence as determined after electroblotting. Calibration of both electrophoretic runs is indicated in kDa in the left margins.

3. Results

The N-terminal region of the laminin α1 chain, which corresponds to domains VI and V [12] (positions 1–484), consists of one LN module and a tandem array of four LE modules (Fig. 1a). Expression vectors designed to express the entire structure (α1VI/V) or the individual domains α1VI and α1V were used to transfect mammalian 293 cells. Electrophoresis of serum-free culture medium demonstrated strong bands of the expected size for fragments α1VI/V and α1V, but α1VI was not detected either by protein staining or immunoblotting. A larger vector, α1VI-1, encoding the LN and an adjacent LE module (Fig. 1a), was therefore constructed, but this only gave rise to low production and secretion of a 35 kDa product, as identified by immunoblotting. This suggested that the LN module does not represent an autonomously folding protein domain and requires additional structures for stabilization.

The high production and secretion of fragments α1VI/V and α1V (40–180 μg/ml, as determined by radioimmunoassay) facilitated their efficient purification from serum-free culture medium. Fragment α1VI/V bound quantitatively to a heparin-Sepharose column and, after molecular sieve chromatography, appeared as a 62 kDa band when examined by electrophoresis under non-reducing conditions (Fig. 1b). DEAE cellulose and molecular sieve chromatography were sufficient to obtain the 43 kDa fragment α1V in a pure form. Electrophoresis under reducing conditions (Fig. 1b) caused a slightly slower migration for fragment α1VI/V, indicating the opening of internal disulfide bridges. In contrast, fragment α1V migrated slightly faster after reduction, as has previously been observed for a similar tandem array of LE modules from the laminin γ1 chain [11]. Edman degradation of each fragment demonstrated a single N-terminal sequence which started with

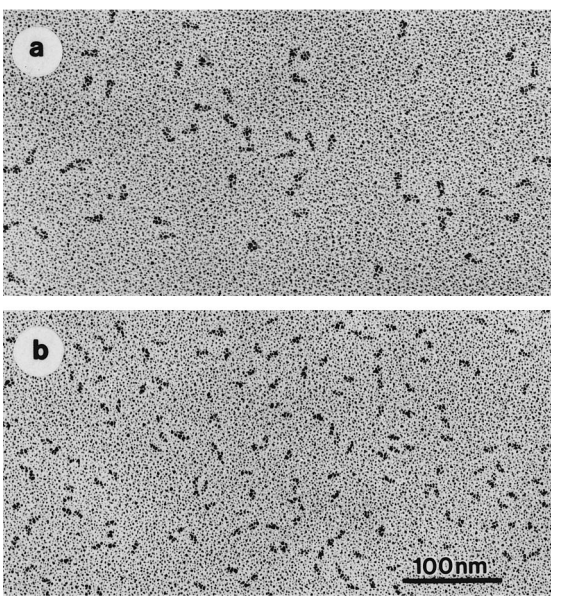

Fig. 2. Electron micrographs of rotary shadowed recombinant fragments α1VI/V (a) and α1V (b). The bar indicates the magnification for both parts of the figure.

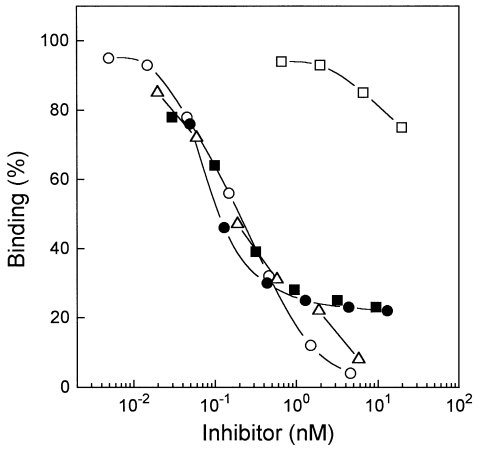

Fig. 3. Radioimmuno-inhibition assay for epitopes present in the N-terminal domain of the laminin α1 chain. The assay consisted of ^{125}I-labeled fragment α1VI/V (1 ng) and a fixed concentration of a rabbit antiserum against the same fragment. Inhibitors used were recombinant fragments α1VI/V (○) and α1V (●), laminin-1 (△) and its fragments E1XNd (■) and E4 (△). Similar profiles were obtained with an antiserum against laminin-1 (not shown).

APLA derived from the vector and then continued with the authentic α1 chain sequence shown in Fig. 1a. Fragment α1VI/V was substituted with 13.5 residues of glucosamine and 2 residues of galactosamine, which were reduced to 5.5 and 1 residues, respectively, in fragment α1V.

The CD spectra of both fragments were similar to those of other fragments containing LE models [22], indicating the presence of α helical and β strand conformation (data not shown). Rotary shadowing electron microscopy of fragment α1V revealed short rods of about 10 nm length (Fig. 2b). Similar rods connected to a small globular domain were observed for fragment α1VI/V (Fig. 2a). A rabbit antiserum raised against fragment α1VI/V showed a high titer in radioimmunoassays ($1:3\times10^4$), as did several antisera against laminin-1 from a mouse tumor. A sensitive radioimmuno-inhibition assay demonstrated almost identical inhibition profiles for fragment α1VI/V and laminin-1 with $IC_{50} = 0.2$ nM (Fig. 3). Recombinant fragment α1V and the laminin-1 short arm fragment E1XNd were also comparable inhibitors, but inhibition was incomplete, with 20–25% residual binding. This indicated antibody binding to epitopes on both the LE and LN modules. A related fragment E4 from the N-terminus of the laminin β1 chain (domain VI/V) was, however, only a poor inhibitor (Fig. 3). The antibodies against fragment α1VI/V were purified by affinity chromatography and found to react strongly with basement membrane zones in immunofluorescence on several frozen mouse tissue sections (data not shown).

Fragment α1VI/V was shown to have a high affinity for heparin and could be eluted from an analytical HiTrap column in a sharp peak at 0.3 M NaCl. The same elution profile was obtained for laminin fragment E3 from the C-terminal end of the laminin α1 chain. Fragment α1V, however, as well as recombinant laminin fragments β1VI/V and γ1VI/V (T. Sasaki, R. Timpl, unpublished), did not bind to the heparin column at low ionic strength. Several neutral proteases were found to cleave recombinant α1VI/V into distinct fragments which were used for further mapping. A short digestion (1 h) with pancreatic elastase produced two major bands of ~40 kDa which both started at position 225 within the LN module. A further band of 34 kDa started with the original N-terminus (Fig. 1c). All of these bands showed an undiminished binding to heparin. A more prolonged digestion (24 h) abolished heparin binding. The latter digest contained a major 38 kDa band which started at positions 248/251 just in front of domain V and a faint 16 kDa band that had lost 13–18 residues from the N-terminus of the LN module (Fig. 1c). Since this 16 kDa component is considerably smaller than its parental 34 kDa band, a substantial cleavage must also have occurred at the C-terminus.

Heparin binding of laminin-1 domains has been considered to correspond to interactions with various heparan sulfate proteoglycans. A previous study with perlecan, however, demonstrated that only fragment E3 and no other proteolytic laminin fragment had binding activity [25]. Using solid-phase assays, we could now demonstrate that perlecan also binds to fragment α1VI/V with a binding profile distinct from that of fragment E3 (Fig. 4a). Heparan sulfate attachment to perlecan

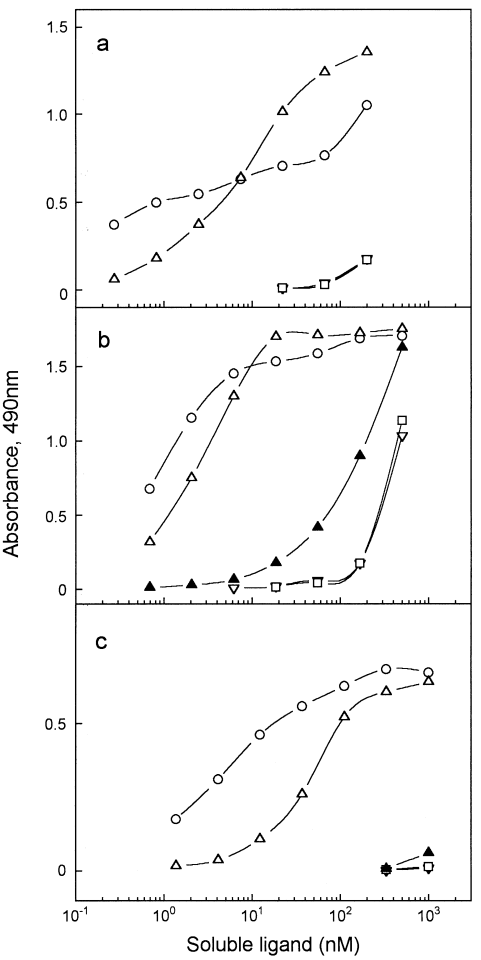

Fig. 4. Binding of perlecan and its heparan sulfate-containing fragments to laminin-1 fragments in solid-phase assays. Soluble ligands were perlecan (a) and the fragments Vc (b) and IA (c). Immobilized ligands were the laminin fragments E3 (○), α1VI/V (△) and α1V (▽) and, as a background control, serum albumin (□). In all assays, binding to serum albumin and α1V was nearly superimposable. Further controls included the non-substituted perlecan fragments V (▲) in (b) and I-Mut (▲) in (c) using immobilized laminin fragment α1VI/V as ligand. Detection of binding was with antibodies against perlecan (a) and its fragments V (b) and IA (c).

Table 1
Binding of immobilized laminin fragment α1VI/V to integrin and perlecan ligands in surface plasmon resonance assays

Soluble ligands	$k_{diss} \times 10^3$ (s^{-1})	$k_{ass} \times 10^{-3}$ (M^{-1} s^{-1})	K_d (nM)
α1β1 integrin	0.36	40	9 ± 6
α2β1 integrin	1.4	31	45 ± 30
perlecan fragment IA	0.57	106	5.3 ± 0.8
perlecan fragment I-Mut		no binding	
perlecan fragment Vc	0.25	33	7.5 ± 1.2
perlecan fragment V		no binding	
perlecan fragment II		no binding	
perlecan fragment III-2		no binding	

Average values of 3–4 independent determinations with S.D. values given for K_d.

occurs through its domains I and V, which are located at opposite ends of the protein core [18,19]. Corresponding recombinant perlecan fragments IA and Vc were both shown to bind to laminin fragment α1VI/V with an affinity comparable to (Vc, Fig. 4b) or about 10-fold lower (IA, Fig. 4c) than fragment E3. Neither perlecan nor its two heparan sulfate-containing fragments showed significant binding to fragment α1V. Furthermore, perlecan fragments V (Fig. 4b) and I-Mut (Fig. 4c), which lack heparan sulfate chains, showed a strongly reduced binding. Together, the data demonstrate that binding occurs between the LN module and the heparan sulfate chains of perlecan. These data were confirmed in a surface plasmon resonance assay, which demonstrated similar affinities for the interaction between immobilized fragment α1VI/V and perlecan fragments IA and Vc (Table 1). Here again, no or only questionable binding was observed with fragment α1V (data not shown).

Previous studies with purified α1β1 and α2β1 integrins suggested that they should bind to the N-terminal region of the laminin α1 chain [9]. This could now be directly demonstrated in surface plasmon resonance assays with immobilized fragment α1VI/V, which showed a five-fold difference in its affinity for the two integrins (Table 1). These interactions were abolished by EDTA and did not occur with fragment α1V, which underscored the specificity of the binding as well as the involvement of the LN module.

4. Discussion

In the present study we used an established recombinant expression system in mammalian cells to obtain the N-terminal domain VI/V of the laminin α1 chain (module structure $LNLE_4$) and domain V alone (LE_4) in high yields and purity. Both recombinant fragments were folded into their native structure as shown by CD spectroscopy, electron microscopy, immunological assays and partial resistance to proteolysis. They were also modified mainly by *N*-glycosylation, consistent with four or two potential acceptor sites which are present in the α1VI/V and α1V segments, respectively [12]. Fragment α1V formed 10 nm long rods, which is in agreement with the crystal structure of three LE modules of the laminin γ1 chain [26]. Fragment α1VI/V showed an additional globular domain, consistent with previous interpretations of the laminin structure [5,6]. Yet we failed to produce this globular domain VI alone, indicating that the LN module has no autonomous folding properties and gets rapidly degraded, as found for other recombinant deletion fragments [27]. This was a surprising observation, since single LE [11], and globular L4 [28] and LG [29] modules of laminins can be readily obtained by the same recombinant procedures. In recent experiments, however, we also failed to prepare LN modules of the laminin β1 and γ1 chains (T. Sasaki and R. Timpl, unpublished) and thus imperfect folding could be a general feature. Apparently, more than one single adjacent LE module seems to be required to initiate folding and/or stabilization of LN modules.

Despite the failure to obtain the LN module for binding studies, a comparison of the activities of fragments α1VI/V and α1V localized heparin, perlecan and integrin binding to the LN module. Heparin binding had a salt sensitivity (0.3 M NaCl) clearly above physiological levels and comparable to that of fragment E3, which was previously considered to represent the major binding site of laminin-1 for heparin and perlecan [17,25,30]. A somewhat weaker heparin affinity (0.18 M NaCl) was also observed for a larger related α1 chain fragment (α1VI-IVb) and attributed to the LN module [10]. Here we also showed that the two perlecan fragments IA and Vc, when substituted with heparan sulfate chains [18,19], bound with comparable affinities (K_d = 5–7 nM) to fragment α1VI/V at physiological salt concentration. These perlecan binding domains, as well as the corresponding laminin α1 chain binding epitopes, are located at opposite ends of the proteins. This raises the possibility that they could form linear copolymers which may contribute to basement membrane structure. A brief elastase digestion of fragment α1VI/V apparently cleaved a single peptide bond at position 224/225 in the LN module but did not influence heparin binding of either fragment. The regions flanking the cleavage site, RYIRLRLQRIR (positions 205–215) and RDLRDLDPIVTRRYYYSIK (positions 227–245), are rich in basic amino acid residues and their loss upon more extensive digestion abolished heparin binding. This indicated that both regions, together or individually, contribute to one or two binding epitopes.

Another major activity of the LN module is involvement in cell adhesion, as indicated from the binding of purified α1β1 and α2β1 integrins to laminin fragment E1XNd [9] and, for α1β1 integrin, from cell adhesion and antibody inhibition studies [10]. Here we showed distinct divalent cation-dependent binding of both integrins to fragment α1VI/V, with a five-fold difference in affinity, while no binding occurred to fragment α1V. For α2β1 integrin, the same affinity was found in previous studies with laminin-1 and fragment E1XNd [9], demonstrating that the recombinant fragment possesses a fully active binding epitope. The α1β1 and α2β1 integrins were originally identified as major receptors for several collagen types [31,32] but they have also been shown to bind to

laminin-1, although with lower efficiency [9]. As shown for α1β1 integrin binding to collagen IV, the collagen epitope requires a single Arg and two Asp residues to be fixed in triple-helical conformation for proper binding [33]. The complementary epitope on the integrins has been localized to the A domain [34,35]. Based on the X-ray structure of the integrin α2 chain A domain [36], it was speculated that a shallow groove formed by the cation-binding MIDAS motif would allow a collagen triple-helix to dock perfectly. Yet it is not clear and hard to predict whether this binding epitope would also fit a structure on the laminin α1 chain LN module. In this context, however, it is of interest that the K_d values measured for the binding of the two A domains to collagen I [35] are of the same order of magnitude and show the same difference between the two integrins as determined here for the laminin fragment α1VI/V.

The present recombinant study has set the stage for examining the various binding properties of the laminin α1 chain LN module, including its potential involvement in self-assembly [6–8], by site-directed mutagenesis. The high production rates will also make crystallization feasible, providing support for epitope mapping.

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