Structural and functional analysis of the globular domain IVa of the laminin $\alpha 1$ chain and its impact on an adjacent RGD site

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The globular domain IVa (about 250 residues) of the laminin $\alpha 1$ chain was obtained in recombinant form from mammalian cell clones. It was prepared either with ($\alpha 1$ IVa-R) or without ($\alpha 1$ IVa) an adjacent cell-adhesive RGD site which seems to be masked in laminin-1. The recombinant products could be visualized as globular structures by rotary shadowing, were resistant to trypsin and shared immunological epitopes with laminin-1, indicating folding into a native structure. Sequence analysis of pepsin fragments demonstrated the insertion of the globular domain into an epidermal growth factor-like scaffold which is characteristic of the extracellular laminin domain IV (L4) module. Only

little immunological cross-reaction was found, however, with other L4 modules from perlecan and different laminin isoforms. Fragment $\alpha 1$ IVa-R, but not fragment $\alpha 1$ IVa, bound to $\alpha V\beta 3$ integrin, although to a distinctly lower level than a laminin fragment where the RGD site is fully exposed. The fragments also had no or only little cell attachment activity. This confirmed previous predictions that the globular domain $\alpha 1$ IVa masks the RGD site in laminin-1. Domain $\alpha 1$ IVa showed, in addition, a weak binding activity for the basement-membrane protein fibulin-1.

INTRODUCTION

Laminins represent a distinct family of extracellular matrix proteins with seven different isoforms identified so far. They are heterotrimeric proteins composed of distinct α , β and γ chains and have a cross- or T-shaped structure formed from several rod-like and globular domains [1–3]. Most of the globular domains of the short arms correspond to one of two different motifs, the 200-residue laminin N-terminal (domain VI) (LN) module and the 250-residue laminin domain IV (L4) module (see [4] for details of nomenclature). The LN modules are located at the distal ends of the short arms and are the binding structures involved in the polymerization of laminins into large networks [5]. The L4 modules are found in central positions in the short arms and have so far only been identified in the $\alpha 1$, $\alpha 2$ and $\gamma 1$ chains of laminin as well as in the proteoglycan perlecan. They are considered to consist of a large globular insert between Cys-3 and Cys-4 of an epidermal growth factor-like (LE) module, both structures together forming the entire module [1,6–8]. Structural evidence for this arrangement was recently provided by a recombinant perlecan fragment III-3, which consisted of one L4 module and three additional LE modules [9]. So far no functions have been identified for the L4 modules of laminin and perlecan.

The L4 modules of laminin-1 (chain composition $\alpha 1\beta 1\gamma 1$) correspond to its three globular domain IV sequences, as shown by sequence analysis and electron microscopy [1,10]. Pepsin digestion destroys these globules but leaves the adjacent LE modules, which form the rod-like elements of the short arms, intact [7]. The resulting large fragment, P1, was shown to strongly mediate cell adhesion through RGD-dependent $\beta 1$ and $\beta 3$ integrins [6,11]. A single RGD was identified in the mouse laminin $\alpha 1$ chain sequence and localized to the disulphide-bonded loop d of an LE module just adjacent to the L4 module of domain IVa [10]. This led to the hypothesis that the large L4 module masks the RGD site in mouse laminin-1, and a possible

mechanism for its exposure was postulated to involve unknown proteolytic pathways probably acting during tissue repair [6]. However, the change of the RGD sequence into the non-adhesive RAD or DAK sequence in the human laminin $\alpha 1$ and mouse $\alpha 2$ chains [12,13] also indicated that such a mechanism would be isoform and species specific.

In the present study, two structural questions were addressed by the recombinant production of domain IVa of the mouse laminin $\alpha 1$ chain in mammalian cell clones. We used an expression vector corresponding to just the L4 module in order to find out whether the module comprises an autonomous protein folding unit, a matter which could not be concluded from our previous study with a larger perlecan fragment [9]. We also used two different vectors which included or lacked the adjacent RGD loop for examining the masking hypothesis. The data clearly supported the view that L4 modules are not dependent on other structures for proper folding, as well as being consistent with the masking hypothesis.

EXPERIMENTAL

Materials

Laminin-1 complexed to nidogen, collagen IV and the laminin-1 fragments E1XNd, P1X and P1 [14,15] were prepared from the mouse Engelbreth–Holm–Swarm tumour. A mixture of laminin-2 and laminin-4 was obtained from human placenta [16]. Vitronectin [17] and fibronectin (Behringwerke) from human plasma were purified by heparin-affinity chromatography. Mouse fibulin-1 variants C and D [18], mouse perlecan domains III-1, III-2 and III-3 [9,19] and human BM-40 [20] were prepared in recombinant form. The integrins $\alpha 1\beta 1$, $\alpha 2\beta 1$, $\alpha V\beta 3$ and $\alpha IIb\beta 3$ were obtained from human platelets or placenta [21]. Pepsin (Boehringer Mannheim) and trypsin (Worthington) were of commercial origin. Human embryonic kidney 293 cells were from American Type Culture Collection. All other cell lines used have been described previously [6].

Abbreviations used: LE module, laminin epidermal growth factor-like module; LN module, laminin N-terminal (domain VI) module; L4 module, laminin domain IV module.

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Methods of recombinant production

A 4.0 kb cDNA clone (pA01/0.3) encoding domain IVa of the mouse laminin $\alpha 1$ chain [10] was used as a template for amplification by PCR according to the supplier's protocol (New England Biolabs). Two 5' primers were used, both of which introduced an *NheI* restriction site, primer 1: GTCAGCTAG-CCAGTAAGTGCCAAGCC and primer 2: GTCAGCTAG-CAGCCCCCTGCTTCTGC. A 3' primer 3: GTCAGCGCCG-CCTCGAGCTAGGGCACACAAGGAGC contained an in-frame stop codon and an *XhoI* site. Primers 1 and 3 were used to obtain a PCR product of 841 bp representing fragment $\alpha 1$ IVa-R (positions 1113–1385) and primers 2 and 3 for generating the 787 bp fragment $\alpha 1$ IVa (positions 1131–1385). The amplified cDNAs were purified by gel electrophoresis, cut with *NheI* and *XhoI* and subcloned in vector pCRII (Invitrogen). The inserts were released with *NheI/XhoI* and ligated in-frame to the BM-40 signal peptide cDNA within the eukaryotic expression vector pCis [22], as previously described [23]. The correct structure of the inserts was verified by restriction mapping and complete sequencing. These vectors were used for cotransfection of 293 cells with plasmid pSV_{pac} [20]. Stably transfected clones were then selected with puromycin and the clones which produced the recombinant fragments identified by SDS gel electrophoresis.

Protein purification and fragmentation

The recombinant laminin fragments were purified from serum-free culture medium (about 1 litre) initially on DEAE-cellulose, which was equilibrated in 0.05 M Tris/HCl, pH 8.6, and eluted with an NaCl gradient [9]. A lyophilized pool of the fragments was then used for final purification on a Superose 12 (HR 16/50) column equilibrated in 0.2 M ammonium acetate, pH 6.8. Proteolytic digests were prepared at an enzyme/substrate ratio of 1:100 with either pepsin in 0.1 M glycine/HCl, pH 1.9 (2 h, 25 °C), or with trypsin in 0.2 M NH₄HCO₃ (4 h, 37 °C). The lyophilized pepsin digest was then passed over Superose 12 and pools containing small peptides were further purified by reverse-phase HPLC [24].

Binding assays

Solid-phase assays with plastic-immobilized and soluble protein ligands followed previously used protocols [25]. Binding of the soluble ligands was detected by specific antibodies. A similar assay, but with a buffer which promotes receptor interactions [0.05 M Tris/HCl (pH 7.4)/0.15 M NaCl/1 mM MnCl₂/1 mM MgCl₂/1 mM CaCl₂/0.04 % Tween 20] was used in the binding study with immobilized integrins [21]. The protocol for cell adhesion studies using immobilized substrates and cells suspended in Dulbecco's modified Eagle's medium has been described [26]. Adherent cells were quantified colorimetrically after staining with Crystal Violet.

Analytical, immunological and other methods

Amino acid compositions and protein concentrations were determined on an LC 3000 analyser (Biotronik) after hydrolysis (6 M HCl, 16 h, 110 °C). Edman degradation was performed in 470A and 473A sequencers following the manufacturer's instructions (Applied Biosystems). Electrophoresis in SDS/polyacrylamide gels and calibration of the runs with globular proteins followed standard protocols. Established procedures were used for immunization, ELISA and radioimmunoassays [27]. Rotary shadowing was used to visualize protein shapes by electron microscopy [28].

RESULTS

Recombinant expression of laminin domain $\alpha 1$ IVa

The central globular domain $\alpha 1$ IVa of the mouse laminin $\alpha 1$ chain short arm is considered to consist of a 200-residue globular portion inserted into an LE module [1], as shown schematically in Figure 1. This structure is also referred to as the L4 module [4]. Two expression vectors were designed, one encoding exactly the L4 module (fragment $\alpha 1$ IVa, $\alpha 1$ chain positions 1131–1385) and the second fragment $\alpha 1$ IVa-R containing in addition an 18-residue disulphide-bonded loop with a cell-adhesive RGD sequence (Figure 1). These vectors also contained the BM-40 signal peptide [23], which added an N-terminal APLA sequence to the secreted products. Stably transfected human kidney 293 cell clones could be obtained with both vectors and expressed a novel 0.8 kb mRNA as shown by Northern blot hybridization. A distinct electrophoretic protein band of the expected size could be detected in the culture medium of the clones, demonstrating efficient folding and secretion of the protein products. The secretion rates of the recombinant proteins were about 1–3 $\mu\text{g} \cdot 24 \text{ h}^{-1} \cdot \text{ml}^{-1}$.

The recombinant fragments were purified from serum-free culture medium on DEAE-cellulose where they eluted at 0.14–0.18 M NaCl in the gradient. Subsequent molecular-sieve chromatography on Superose 12 yielded products of sufficient purity for further analysis. SDS gel electrophoresis of fragment $\alpha 1$ IVa-R demonstrated a single 33 kDa band with a purity of > 95 %. Fragment IVa had a slightly higher mobility. Both fragments were of somewhat lower electrophoretic mobility after reduction, indicating the formation of internal disulphide bonds (Figure 2, lanes 1–4). Edman degradation of the major bands demonstrated the expected N-terminal sequences for fragment $\alpha 1$ IVa-R (APLASKXQAG) and fragment $\alpha 1$ IVa (APLAPXFXFGL).

Protease stability and conformation

Trypsin treatment of fragments $\alpha 1$ IVa-R (Figure 2, lanes 6 and 7) and IVa (results not shown) did not alter their electrophoretic mobility, indicating that both recombinant products were in a

Figure 1 Schematic structure of recombinant laminin domain $\alpha 1$ IVa and a larger variant containing an RGD loop ($\alpha 1$ IVa-R)

The sequence and position numbers of cysteines are those of the mouse laminin $\alpha 1$ chain [10]. The large globular portion of the L4 module corresponds to positions 1143–1344 which is embedded between Cys-3 and Cys-4 of an LE scaffold. Bars at the bottom identify the size of the two recombinant fragments. Both contain in addition an N-terminal APLA sequence derived from the signal peptide region of BM-40 [23].

Figure 2 SDS gel electrophoresis of recombinant laminin fragments and of some proteolytic digests

Lanes were loaded with fragment $\alpha 1IVa-R$ (lanes 1 and 2), fragment $\alpha 1IVa$ (lanes 3 and 4), a pepsin digest of $\alpha 1IVa-R$ (lane 5) and a trypsin digest of $\alpha 1IVa-R$ (lanes 6 and 7). Samples were either non-reduced (—) or reduced with dithiothreitol (+). Positions of non-reduced marker proteins are denoted in kDa on the left of the Figure.

Table 1 N-Terminal sequences of peptides obtained by pepsin digestion of fragment $\alpha 1IVa-R$

The triple sequence of Pep-1 was identified without prior separation of the individual constituents. * Indicates the additional presence of peptides one N-terminal residue shorter; X, indicates unidentified residues which were in most cases Cys.

Peptide	Sequence*	Position
Pep-1	APLASKXQAGTF	N-Terminus
	ALRGDNPQGKSP	1121
	LLELVXPPGTA*	1341
3/2	MDAPAPENGVRQDY*	1255
3/4	EVGRKAV*	1324
3/6	IKASYGQ	1305
3/8	HVVSQSN	1163

folded state. However, pepsin digestion at low pH caused considerable cleavage of fragment $\alpha 1IVa-R$ and only a single fragment, Pep-1, of about 7 kDa could be detected by electrophoresis (Figure 2, lane 5). This fragment disappeared after reduction, indicating that it consisted of several disulphide-bonded peptides. This was confirmed by purification of Pep-1, which appeared in the first peak of a Superose 12 run and accounted for about 15% of the total digest. Edman degradation demonstrated approximately equal proportions of the N-terminal sequence and two more sequences starting within the RGD loop and shortly before the fourth Cys within the L4 module (Table 1, Figure 1). Further examination of several smaller peptic peptides, which were purified by reverse-phase HPLC, demonstrated their exclusive origin from the large insert between Cys-3 and Cys-4 (Table 1). Together, the data confirm the continuous disulphide connection within the L4 module (Figure 1), as indicated previously for perlecan [9], and in addition, a separate disulphide connection in the extra RGD loop.

After rotary shadowing, fragments $\alpha 1IVa-R$ and $\alpha 1IVa$ appeared as small globular structures (results not shown), consistent with previous predictions [1]. CD spectroscopy of fragment $\alpha 1IVa-R$ yielded an ellipticity profile very similar to that of the L4 module-containing perlecan fragment III-3 [9] (R. Battistutta and B. Schulze, unpublished work), indicating a 55% content of α -helix and β structure.

Figure 3 ELISA titration of an antiserum against fragment $\alpha 1IVa-R$ with various laminin and perlecan antigens

The coated antigens were fragments $\alpha 1IVa-R$ (■) and $\alpha 1IVa$ (□), mouse laminin-1-nidogen complex (●), a mixture of human laminin-2 and laminin-4 (○) and the recombinant perlecan fragments PGIII-1 (◆), PGIII-2 (△) and PGIII-3 (▲).

Figure 4 Comparison of recombinant laminin fragments with laminin and authentic laminin fragments by radioimmuno-inhibition assay

The test system consisted of ^{125}I -labelled fragment $\alpha 1IVa-R$ and an antiserum against fragment E1XNd. Inhibitors of the reaction were recombinant fragments $\alpha 1IVa-R$ (○) and $\alpha 1IVa$ (□), laminin-1-nidogen complex (◆) and laminin-1 fragments E1XNd (◇), P1X (▲) and P1 (△).

Immunochemical analysis

A rabbit antiserum was raised against fragment $\alpha 1IVa-R$ and used in ELISA titration for a comparative analysis with related antigens (Figure 3). This demonstrated almost identical binding curves for fragments $\alpha 1IVa-R$ and $\alpha 1IVa$ and for laminin-1, indicating that they share most of the epitopes. Laminin-2 and laminin-4, which possess an $\alpha 2$ instead of an $\alpha 1$ chain [16], and the recombinant L4 module-containing perlecan fragments III-1, III-2 and III-3 showed no or only little binding activity. Antisera against laminin-1 or its fragment E1XNd also cross-reacted with fragment $\alpha 1IVa-R$ but with lower titres than with the antigens used for immunization, reflecting their larger epitope diversity (results not shown). These cross-reactions were used for a further comparison by radioimmuno-inhibition assay (Figure 4). Recombinant fragments $\alpha 1IVa-R$ and $\alpha 1IVa$, laminin-1 and the short

Figure 5 Binding of laminin fragments and other ligands to immobilized $\alpha V\beta 3$ (A) and $\alpha IIb\beta 3$ (B) integrins in a solid-phase assay

Soluble ligands were vitronectin (○), fibrinogen (●) and the laminin-1 fragments P1 (□), $\alpha 1IVa$ -R (◇) and $\alpha 1IVa$ (△). Binding was detected with specific antisera against the soluble ligands.

Figure 6 Adhesion of A375 melanoma cells to laminin-1 fragments

The fragments used for coating were P1 (○) and the recombinant products $\alpha 1IVa$ -R (△) and $\alpha 1IVa$ (□).

arm fragment E1XNd completely inhibited the reaction between ^{125}I -labelled fragment $\alpha 1IVa$ -R and anti-E1XNd antiserum at similar molar concentrations, indicating preservation of native laminin-1 epitopes. These epitopes are, however, destroyed on the smaller pepsin-derived short arm laminin-1 fragments P1 and P1X. Immunofluorescence studies with the antiserum against fragment $\alpha 1IVa$ -R demonstrated a distinct basement-membrane staining of kidney, skin and muscle tissue sections [19]. Together the data demonstrate extensive epitope sharing between laminin-1 and the recombinant fragments.

Integrin-binding and cell-adhesive activities

The single RGD sequence present in fragment $\alpha 1IVa$ -R is the only one so far identified in mouse laminins and was previously shown to require exposure by pepsin digestion to be recognized in cell-adhesion assays by RGD-dependent integrins [6,11,21]. In solid-phase assays, fragment $\alpha 1IVa$ -R bound to immobilized $\alpha V\beta 3$ and $\alpha IIb\beta 3$ integrins, although 50- to 100-fold less than the laminin pepsin fragment P1, and no binding was observed with fragment $\alpha 1IVa$ (Figure 5). All of these interactions were weaker than those with the most active ligands, vitronectin ($\alpha V\beta 3$) and fibrinogen ($\alpha IIb\beta 3$). Binding was abolished by EDTA, demonstrating the typical cation dependence of specific integrin reactions. No binding was observed between fragments $\alpha 1IVa$ -R and $\alpha 1IVa$ and the integrins $\alpha 1\beta 1$ and $\alpha 2\beta 1$, which are capable

Figure 7 Binding of soluble fibulin-1C to immobilized laminin-1 fragments in a solid-phase assay

The fragments used were E1XNd (▲), $\alpha 1IVa$ -R (○) and $\alpha 1IVa$ (□) and binding was detected with an antiserum against fibulin-1C. Similar results were obtained with fibulin-1D.

of binding to laminin-1 fragment E1XNd (results not shown). These data demonstrated that the RGD site in fragment $\alpha 1IVa$ -R is still partially masked but less so than in laminin-1, which does not bind $\alpha V\beta 3$ integrin at all [21].

Several cell lines which were previously shown to adhere in an RGD-sensitive way to laminin fragment P1 [6] were now used to examine the activity of fragments $\alpha 1IVa$ -R and $\alpha 1IVa$. Only the melanoma A375 cells (Figure 6) and Schwannoma RN22 cells showed adhesion to $\alpha 1IVa$ -R, but the dose-response profiles reached plateau levels that were only 30–40% of those achieved with fragment P1. Several other cell lines (HBL-100, HT 1080, B16F10, Rugli, NR6, SclI) showed no binding to fragment $\alpha 1IVa$ -R. Fragment $\alpha 1IVa$ was an inactive substrate for all the cell lines tested.

Binding to extracellular ligands

Several extracellular ligands which are potential candidates for laminin binding were tested in solid-phase assays for their interaction with fragments $\alpha 1IVa$ -R and $\alpha 1IVa$. Only fibulin-1 showed distinct binding to both recombinant fragments as well as to laminin fragment E1XNd (Figure 7). The somewhat higher activity of fragment $\alpha 1IVa$ compared with $\alpha 1IVa$ -R and E1XNd could be reproduced in several tests. Furthermore, no difference in binding could be detected between the two splice variants C and D of fibulin-1. These reactions were abolished by addition of EDTA. Several other ligands (collagen IV, BM-40, fibronectin, vitronectin) showed no or only little binding to both recombinant laminin fragments.

DISCUSSION

The successful production of large quantities of domain IVa of the laminin $\alpha 1$ chain clearly demonstrated that the postulated L4 module [1,4,6,7] indeed represents an autonomously folding protein module. This module is considered to consist of a 50-residue disulphide-bonded scaffold similar to LE modules, with a large globular insert between Cys-3 and Cys-4. We have recently obtained perlecan domain III-3 in recombinant form, but since its L4 module was joined to three further LE modules [9] it was not clear whether a single LE disulphide scaffold is sufficient for folding or whether tandem arrays are required. Pepsin digestion of both the laminin and perlecan fragment demonstrated a continuous disulphide bond connection in the scaffold, as shown for LE modules (see below). The globular

inserts possess a distinct content of secondary structural elements. A native structure, probably corresponding to that of laminin domain $\alpha 1$ IVa, was also indicated by electron microscopy, trypsin resistance and extensive sharing of immunological epitopes.

The immunochemical characterization of domain IVa demonstrated, as expected, complete cross-reaction with laminin-1, which possesses the corresponding $\alpha 1$ chain. No cross-reaction was observed with laminin-2 and laminin-4, in which the $\alpha 1$ chain is replaced by the $\alpha 2$ chain [16], and this can be explained by the limited sequence identity (43%) of their domain IVa structures [13]. A similarly low cross-reactivity and sequence identity was also found for the three L4 modules present in perlecan domain III. This will make the antibodies characterized here useful reagents for the specific detection of laminin $\alpha 1$ chains in biological samples. The antibodies also demonstrated the presence of domain IVa epitopes on the laminin elastase fragment E1XNd but not on the related pepsin fragments P1 and P1X. The latter observation agreed with the failure to detect, except for one Pep-1 sequence (LLE..., Table 1), other typical domain IVa sequences in the pepsin fragments [7]. It also correlated with the unmasking of an RGD cell-adhesive site in fragment P1, which was not detectable in laminin-1 or its fragment E1XNd [6].

The RGD site of mouse laminin $\alpha 1$ chain is located in a disulphide-bonded loop of an incomplete LE module bordering the L4 module on its N-terminal side [10]. Our studies demonstrated that the RGD site in the recombinant fragment $\alpha 1$ IVa-R is largely inaccessible or of low affinity, consistent with the hypothesis that the L4 module is responsible for masking [6]. If integrin recognition of this RGD site has any biological relevance, which is so far unknown, unmasking by pericellular proteolysis seems to be a likely mechanism. Such unmasking was demonstrated *in vitro* for a cryptic RGD of thrombin and caused its binding to endothelial cells [29,30]. Recombinant fragment $\alpha 1$ IVa-R may be a suitable substrate for a similar approach. In addition, laminin fragment E1XNd was also shown to possess, probably on its $\alpha 1$ chain, non-cryptic binding sites for $\alpha 1\beta 1$ and $\alpha 2\beta 1$ integrins [21], which are also collagen receptors and are independent of RGD. Our study failed to demonstrate domain IVa as their binding site, in agreement with another recombinant study indicating $\alpha 1\beta 1$ integrin binding to the terminal LN module of the $\alpha 1$ chain [31].

A distinct binding was, however, observed between fragment E1XNd and its domain IVa and the calcium-binding basement-membrane protein fibulin-1 in solid-phase assays. The strength of binding was comparable to that of BM-40 and collagen IV for which a K_d of 3 μ M has recently been determined [32]. Fibulin-1 was previously shown to interact with various laminin isoforms and the data implicated either the $\alpha 1$ chain C-terminal domain or the $\beta 2$ chain as binding domains [16,18,33]. The relative contributions of each of the three binding sites and their significance for basement-membrane assembly still remain to be established.

This and a previous study [9] provided structural evidence for the unique nature of the L4 module and indicated a similar disulphide bond pattern to that predicted for the LE modules. A connection as shown in Figure 1 was in fact recently demonstrated by X-ray crystallography of several recombinant LE modules of the laminin $\gamma 1$ chain (J. Stetefeld, U. Mayer, R. Timpl and R. Huber, unpublished work). Similar structural approaches have

now become feasible for the related L4 module based on the recombinant methods described here.

We wish to thank Dr. M. Pfaff for help and advice in the integrin-binding studies, Dr. C. Gorman for providing the expression vector and Mrs. Mischa Reiter and Mrs. Hanna Wiedemann for excellent technical assistance. The study was supported by the Deutsche Forschungsgemeinschaft (SFB266) and by the Human Capital and Mobility Programme of the European Community (contract No. CHRX-CT93-0246).

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