Complete sequence, recombinant analysis and binding to laminins and sulphated ligands of the N-terminal domains of laminin α 3B and α 5 chains

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The N-terminal sequences of mouse laminin α 3B and α 5 chains have been completed and demonstrate the presence of a signal peptide followed by a complete laminin N-terminal (LN) module (domain VI). These signal peptides were released after recombinant production of larger fragments comprising domains VI/V (45–65 kDa) from this region yielding properly folded proteins, which were secreted from HEK-293–EBNA cells. Pepsin digestion of these fragments yielded products of 25–35 kDa, which consisted only of domain V. The α VI/V fragments were able to inhibit self-assembly of laminin-1, with those from the α 3B and α 5 chains being more active than those from α 1 and α 2 chains. Domain V fragments, however, showed a reduced activity, indicating the major contribution of the LN module in inhibition. These interactions were confirmed by surface-plasmon-resonance assays demonstrating moderate affinities ($K_d = 0.02$ to $> 6 \mu\text{M}$)

for the binding to laminin-1. This indicated that laminins containing α 3B or α 5 chains should also be able to form non-covalent networks by polymerization. The LN modules also showed heparin binding in affinity chromatography, which was strongest for α 1/ α 2, moderate for α 3B, whereas no binding was observed for α 5. They all bound to heparan sulphate chains of perlecan and to sulphatides, with a lower variability in binding activity. Specific antibodies were raised against α 3BVI/V and α 5VI/V and were shown to stain basement membrane zones in various mouse tissues. These antibodies also allowed the identification of a new laminin assembly form 5B consisting of α 3B, β 3 and γ 2 chains.

Key words: heparin binding, immunohistology, laminin α chains, polymerization, recombinant production.

INTRODUCTION

The laminins represent a large protein family mainly located in basement membranes and are involved in cell matrix and other homo- and hetero-typic protein interactions. They are assembled from α , β and γ chains, and so far 12 different chains (α 1– α 5, β 1– β 3, γ 1– γ 3) have been identified and sequenced, demonstrating a complex modular structure [1,2]. All α chains share a unique C-terminal G domain which consists of five laminin G (LG) modules, but differ considerably in the length of their N-terminal short arms. These short arms consist of rod-like elements on the basis of tandem arrays of laminin-type epidermal growth factor-like (LE) modules [3] and several globular domains, referred to as laminin N-terminal (LN) and L4 modules and domain IVb, which has not been classified to date. The modular structure of the 400 kDa α 1 and α 2 chains is very similar when compared with the larger α 5 chain [4], whereas the α 4 chain is considerably smaller (200 kDa) due to the presence of a short N-terminal domain. The α 3 chain, originally identified as a 200 kDa variant in biosynthetic studies [5] and by sequence analysis [6], is now referred to as the α 3A variant [1,2]. A larger splice variant α 3B was subsequently discovered [6,7] and shown to have a modular structure similar to the α 5 chain [8,9]. Both splice variants are under the control of different promoters [10] and are expressed in various tissues at variable levels, as shown by mRNA protection assays [7,9].

Nevertheless, the N-terminal sequences of α 3B [8] and α 5 [4] chains are still incomplete, and the presence of a signal peptide has not been identified. Both chains showed, however, the presence of an incomplete LN module, which, in the α 1 and α 2 chains, follows the signal peptide. Such LN modules consist of approx. 250 residues and are also found in the N-terminal region of the β 1, β 2, β 3, γ 1 and γ 3 chains, but not in the α 3A and α 4

chains [1,2]. These LN modules are essential for polymerization into large non-covalent networks, as shown initially for laminin-1 (α 1 β 1 γ 1) and laminin-2 (α 2 β 1 γ 1), which crucially depend on the presence of three different LN modules within the proteins [11–13]. This self-assembly is considered to be crucial for the integrity of basement membranes [12], as highlighted by genetic forms of muscular dystrophy containing the deletion of the LN module from the α 2 chain [14]. The α 5 chain is associated in laminins-10/11 with β 1/ β 2 and γ 1 chains [15], raising the interesting possibility that the α 5 LN module may also participate in self-assembly. By using appropriate recombinant fragments it was shown further that the LN modules of the α 1 [16,17] and α 2 [18] chains bind to heparin, heparan sulphate, α 1 β 1 and α 2 β 1 integrins, and are thus substrates for cell adhesion.

With one exception [19], similar properties have not been shown so far for the LN modules of the α 3B and α 5 chains. In the present study we have completed the N-terminal sequencing of mouse laminin α 3B and α 5 chains and identified the signal peptide cleavage sites. This has allowed the production of properly folded and secreted recombinant forms of the LN modules of α 3B and α 5 together with adjacent LE modules. The recombinant proteins were compared with similar fragments from α 1 and α 2 chains in immunological and binding assays for heparin/heparan sulphate and sulphatides and their ability to inhibit laminin-1 polymerization.

EXPERIMENTAL

Sequence analysis and vector construction

A 5'-Full rapid amplification of cDNA ends (RACE) core set (TaKaRa, Kyoto, Japan) was used with RNA from mouse epidermal Pam 212 cells [20] and mouse embryonic endothelial

Abbreviations used: LE, laminin-type epidermal growth factor-like module; LG, laminin G domain module; LN, laminin N-terminal module; RT, reverse transcriptase; RACE, rapid amplification of cDNA ends; RU, resonance units; TBS, Tris-buffered saline.

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cells [21] in order to complete the 5' coding sequence of the $\alpha 3B$ and $\alpha 5$ chains respectively, following the manufacturer's instructions (also see [22,23]). The final cDNAs were amplified by PCR with Vent polymerase and their sequences were determined on a 373A sequencer (Applied Biosystems, Tokyo, Japan) by using BigDye Terminator Cycle Sequencing Ready Reaction Kit (Applied Biosystems). These RNAs were then amplified by using appropriate primers and reverse transcriptase-PCR to obtain cDNA encoding the amino acids 1–468 and 1–480 of $\alpha 3B$ and $\alpha 5$ chains respectively, including their own signal peptides. The sequences were then inserted into the episomal expression vector pCEP-Pu [24] via *NheI/XhoI* restriction sites. A similar strategy was used to obtain an expression vector for the mouse $\alpha 2$ chain (residues 1–513) using RNA from adult heart. A 1.8 kb *XbaI/StuI* cDNA fragment [16] was amplified by PCR to obtain a vector encoding residues 1–509 of the mouse laminin $\alpha 1$ chain including its own signal peptide. All vectors were verified by sequencing and used for transfection of HEK-293–EBNA cells [24] to obtain serum-free conditioned medium for the purification of the recombinant fragments referred to as $\alpha 1VI/V$, $\alpha 2VI/V$, $\alpha 3BVI/V$ and $\alpha 5VI/V$.

Purification of recombinant fragments

Serum-free medium (approx. 1 l) containing $\alpha 1VI/V$ or $\alpha 2VI/V$ was dialysed against 0.05 M Tris/HCl (pH 7.4) containing 0.1 M NaCl and passed over a heparin–Sepharose column (2.5 cm $\times$ 8 cm) equilibrated with the same buffer. The fragments were subsequently eluted with a linear gradient (600 ml) of 0.1–0.6 M NaCl. The initial purification of $\alpha 3BVI/V$ and $\alpha 5VI/V$ from 1 litre of medium was performed on a DEAE-cellulose column equilibrated with 0.05 M Tris/HCl (pH 8.6), and fragments were eluted with a linear gradient (600 ml) of 0–0.5 M NaCl. Fragments $\alpha 3BVI/V$ and $\alpha 5VI/V$ were eluted at 0.15–0.25 M NaCl and 0.2–0.3 M NaCl respectively. Final purification was achieved by molecular-sieve chromatography on a Superose 12 (HR 16/50; Amersham Biosciences, Piscataway, NJ, U.S.A.) column equilibrated with 0.2 M ammonium acetate (pH 6.8). Purification of the $\alpha 1V$ fragment of the mouse laminin $\alpha 1$ chain has been described previously [16].

Characterization of proteins and analytical methods

Digestion with neutral proteases (trypsin, elastase and chymotrypsin; Serva, Heidelberg, Germany) was performed at an enzyme/substrate ratio of 1:100 for 1–24 h at 37 °C. Pepsin digestion at the same ratio was carried out in 0.1 M glycine/HCl (pH 1.9) for 2 h at 25 °C. Large pepsin-resistant fragments were purified on Superose 12. Edman degradation was performed on 473 and Procise sequencers following the manufacturer's instructions (Applied Biosystems). Lyophilized $\alpha 1VI/V$ (0.1 nmol) was digested with 1 m-unit of pyroglutamate aminopeptidase (TaKaRa) for 2.5 h at 75 °C. Protein digests were separated by standard SDS/PAGE under non-reducing conditions and blotted on to PVDF membranes for sequencing. Electron microscopy was carried out using standard protocols following rotary shadowing of proteins [25]. Protein and hexosamine content was determined on a Biotronik LC 3000 analyser (Maintal, Germany) after hydrolysis (16 h, 110 °C) with 6 M or 3 M HCl respectively.

Inhibition of laminin polymerization

A complex of laminin-1 ($\alpha 1\beta 1\gamma 1$) with nidogen-1 as well as its fragment E4 ($\beta 1VI/V$) were purified from the mouse Engelbreth–Holm–Swarm tumour [26] and dialysed in the cold against Tris-buffered saline [TBS; 0.05 M Tris/HCl (pH 7.4) and 0.15 M

NaCl]. Laminin (0.32 μ M in a final volume of 0.5 ml), after the addition of 1 mM $CaCl_2$, was polymerized by incubation at 35 °C for 4 h [11] followed by centrifugation at 9500 *g* for 15 min at 20 °C. The precipitated material was subsequently dissolved overnight at 4 °C in 0.5 ml TBS containing 1 mM EDTA. Control incubations were performed in the same way in TBS containing 1 mM EDTA to block most of the polymerization. Inhibitors of Ca^{2+} -dependent polymerization were added at concentrations of 0.32 and 1.6 μ M prior to incubation. All tubes were set up in triplicate and each inhibitor was examined three to four times in order to calculate means $\pm$ S.D. The laminin concentrations in the supernatants after polymerization and in the dissolved precipitates were measured by an inhibition RIA specific for laminin fragment $\alpha 1LG4-5$ [27]. None of the inhibitors used in the polymerization inhibited the RIA even when added at 1000-fold molar excess over the reference inhibitor laminin-1.

Affinity chromatography and solid-phase binding assays

Analytical affinity chromatography was carried out on a 1 ml heparin–HiTrap column (Amersham Biosciences) equilibrated with 0.05 M Tris/HCl (pH 7.4) in order to determine the NaCl concentrations required for displacement within ± 0.01 M [27]. Solid-phase binding assays with laminin fragments immobilized on to the plastic wells of microtitre plates were used to measure the binding of the perlecan fragment Vc being substituted with a single heparan sulphate chain [16,28]. The assay with immobilized sulphatides was carried out as described previously [27]. Antisera against perlecan or the laminin α chain fragments were used to detect binding.

Surface-plasmon-resonance assays

Binding assays were performed with BIAcore 1000 equipment (BIAcore AB, Stevenage, Herts., U.K.) as described in detail previously [29]. For the binding to immobilized laminin and laminin fragments, soluble ligands (0.1–5 μ M) were dissolved in TBS containing 1 mM $CaCl_2$ and examined at 25 °C. Kinetic constants were calculated by non-linear fitting of association and dissociation curves (recorded for 8 min) according to the 1:1 model following the manufacturer's instructions (BIAevaluation software version 3.0). All binding curves could be fitted with $\chi^2 < 3$.

Heparin (approx. 5 kDa) was biotinylated with sulphosuccinimido-biotin following the manufacturer's instruction (Pierce, Rockford, IL, U.S.A.). Since commercial streptavidin SA chips (BIAcore AB) had a high non-specific binding of the laminin fragments, we immobilized streptavidin (200 μ g/ml; Sigma, St. Louis, MO, U.S.A.) in 0.1 M sodium acetate buffer (pH 4.0) by carbodiimide coupling to CM5 chips (BIAcore AB) at a flow rate of 5 μ l/min, yielding approx. 10000 resonance units (RU). Biotinylated heparin (4 mg/ml) was then bound to the chip (2 μ l/min) to approx. 300 RU. Binding of soluble laminin fragments (0.02–8 μ M) in TBS (pH 7.4) had an uptake of 30–11000 RU, whereas non-specific binding to streptavidin alone was in the range 4–80 RU. This non-specific binding was subtracted during non-linear fitting of the binding curves with $\chi^2 < 3$.

Immunological assays

Immunization of rabbits, affinity purification of antibodies and ELISA titration were carried out using established protocols. Immunoblotting and indirect immunofluorescence on frozen tissue sections of adult mice were carried out as described previously [30]. Rabbit antibodies against laminin fragments

$\alpha 1$	1				MRGS	GTGAALLVLL	ASVLWVTVRS	24	
$\alpha 2$	1					MPAATAGIL	LLLLLTLEG	19	
$\alpha 3B$	1				M	AVALGRAPRS	LPLLLTLLL	31	
$\alpha 5$	1				MAKRGGQLCA	GSAPGALGPR	SPAPRPLLLL	40	
							LAGLAIVGEA	40	
$\alpha 1$	25		.QQRGLFPAI	LNLATNAHIS	<u>ANATCGEKGP</u>	E	MECKLV....	EHVPGRPVR	69
$\alpha 2$	20	SQTQRQSQA	HQQRGLFPAV	LNLASNALIT	<u>TNATCGEKGP</u>	E	MYCKLV....	EHVPGQPVV	75
$\alpha 3B$	32	...VVGQDHP	MSSRSLHPPY	FNLAQAARIW	ATATCGERDP	..EVSRRPPE	LFCKLVGGPA	AQGS.GHTIQ	95
$\alpha 5$	41	RTPG	GDGFSLHPPY	FNLAEGARIT	ASATCGEEAP	TRSVSRPTED	LYCKLVGGPV	AGGDPNQTIQ	104
$\alpha 1$	70	HAQCRVCDGN	STNPRERHPI	SHAIIDGTNNW	WQSPSIQNGR	EYHWVTVTLD	LQVVFQVAYI	IKAANAPRP	139
$\alpha 2$	76	NPQCRICNQ	SSNPYQRHPI	TNAIDGKNTW	WQSPSIKNGV	EYHYVTITLD	LQVVFQIAYV	IVKAANSRP	145
$\alpha 3B$	96	GQFCDYC..N	SEDSRKAHPA	SHAIIDGSEW	WQSPPLSSGT	QYNQVNLTL	LGQLFHVAYI	LIFANSRP	163
$\alpha 5$	105	GQYCDIC..T	AANSNKAHPV	SNAIDGTERW	WQSPPLSRGL	EYNEVNVTL	LGQVFHVAYV	LIFANSRP	172
$\alpha 1$	140	GNWILERSVD	.GVKFKPWQY	YAVSDTECLT	RYKITPRRGP	PTYRADNEVI	CTSYYSKLVP	LEHGEIHTSL	208
$\alpha 2$	146	GNWILERSLD	.DVEYKWPQY	HAVTDTECLT	LYNIYPRTGP	PSYAKDDEVI	CTSFSYKIHP	LENGEIHISL	214
$\alpha 3B$	164	DLWILERSVD	FGSTYSPWQY	FAHSRRDCVE	QFG...QEAN	MAITQDDQML	CVTEYSRIVP	LENGEIVVSL	230
$\alpha 5$	173	DLWVLERSTD	FGHTYQPWFQ	FASSKRDCL	RFG...PRTL	ERITQDDVVI	CTTEYSRIVP	LENGEIVVSL	239
$\alpha 1$	209	INGRPSADD..	.PSPOLLEFT	SARYIRLRLO	RIRTLNADLM	TLSHRDRLDL	DPIVTRRRYY	SIKDISVGGM	276
$\alpha 2$	215	INGRPSADD..	.PSPELLEFT	SARYIRLRFO	RIRTLNADLM	MFAHKDPREI	DPIVTRRRYY	SVKDISVGGM	282
$\alpha 3B$	231	INGRPGAKKF	AFSDTLREFT	KATNIRLRFL	RTNTLLGHLI	SKAER....	DPTVTRRRYY	SIKDISVGGR	295
$\alpha 5$	240	VNGRPGALNF	SYSPLLRDFT	KATNIRLRFL	RTNTLLGHLM	GKALR....	DPTVTRRRYY	SIKDISIGGR	304

Figure 1 Sequence alignment of the N-terminal region of mouse laminin $\alpha 1$, $\alpha 2$, $\alpha 3B$ and $\alpha 5$ chains

The arrow head denotes the cleavage site of the signal peptides. Cysteine residues are in bold and N-linked glycosylation sites are underlined. The sequences for $\alpha 1$ [32] and $\alpha 2$ [33] have been published and are shown here with some minor corrections. The sequences of $\alpha 3B$ and $\alpha 5$ start with those determined by RACE up to positions 49 ($\alpha 3B$) and 83 ($\alpha 5$) and continue with some corrections to published sequences [4,8]. The novel sequences of $\alpha 3B$ and $\alpha 5$ have been previously deposited in the GenBank® nucleotide sequence database (accession numbers AJ 293592 and AJ 293593 respectively).

$\alpha 3AIIIa$, $\alpha 3LG4-5$, $\beta 3VI/V$, $\gamma 2L4m$ and $\gamma 2LE4-6$ were as used previously [31]. Additional rabbit antibodies were raised against laminin $\gamma 1VI/V$ following the procedure used for $\alpha 1VI/V$ [16]. A rat monoclonal antibody BE1/4 raised against mouse nidogen-1 was kindly supplied by Dr Marie Dziadek (Department of Anatomy and Cell Biology, University of Melbourne, Australia) [32]. Detergent tissue extracts (150 μ l) were immunoprecipitated with 5 μ g of purified antibodies and Protein A-Sepharose [30], and then used for electrophoresis and immunoblotting with antibodies (1 μ g/ml).

RESULTS

Completion of the sequence of mouse laminin $\alpha 3B$ and $\alpha 5$ chains

Since the sequences were incomplete at the N-terminus of the $\alpha 3B$ [8] and $\alpha 5$ [4] chains, a RACE approach was used to identify a potential starting methionine residue. This added 49 residues to the $\alpha 3B$ and 83 residues to the $\alpha 5$ chain sequences (Figure 1). Together with results of Edman degradation of recombinant fragments (see below), this demonstrated that the size of the signal peptide was 31 and 40 residues for the $\alpha 3B$ and $\alpha 5$ chain respectively, in agreement with a long hydrophobic stretch of amino acids within these sequences being typical of signal peptides. The sequences of the LN modules (domain VI) of the mouse laminin $\alpha 1$, $\alpha 2$ and $\alpha 3B$ and $\alpha 5$ chains were also aligned (Figure 1) and showed a strict conservation of six cysteine residues, although separated by some sequence gaps, but not of the potential N-glycosylation sites. The size of these modules varied between 252 and 264 residues. A sequence identity of 72–77% was shown between $\alpha 1/\alpha 2$ and $\alpha 3B/\alpha 5$ chains respectively, and 53–60% identity was observed between both groups.

Recombinant production and properties of N-terminal fragments of laminin $\alpha 1$, $\alpha 2$, $\alpha 3B$ and $\alpha 5$ chains

Previous recombinant studies with the LN module of the $\alpha 1$ chain [16] and of the $\beta 1$ and $\gamma 1$ chains (T. Sasaki, unpublished

Figure 2 SDS/PAGE of purified recombinant N-terminal fragments of laminin α chains and their corresponding pepsin-resistant fragments

The lanes were loaded with $\alpha 1VI/V$ (lane 1), $\alpha 2VI/V$ (lane 2), $\alpha 3BVI/V$ (lane 3) and $\alpha 5VI/V$ (lane 4) prior to (A) and after (B) pepsin degradation, separated by SDS/PAGE under non-reducing conditions, and the gels were stained with Coomassie Blue. The fragments in (B) were also used for Edman degradation and the same sequence was obtained for each band examined in a particular lane, indicating that the mobility differences reflect different levels of N-glycosylation. Molecular-mass markers (in kDa) are shown on the left.

work) have shown that this module does not represent an autonomous folding unit and requires the addition of several adjacent, rod-like LE modules from domain V to achieve efficient

Figure 3 Rotary shadowing electron microscopy of laminin fragments

Electron micrographs are shown for laminin fragments $\alpha 2VI/V$ (A) and $\alpha 5VI/V$ (B). Scale bar represents 100 nm.

production in mammalian cells. We therefore designed expression vectors for the LN modules of $\alpha 3B$ and $\alpha 5$ chains containing their predicted signal peptides, LN modules and three additional LE modules, referred to in the following as fragments $\alpha 3BVI/V$ and $\alpha 5VI/V$. In addition, we prepared similar vectors for $\alpha 1VI/V$ and $\alpha 2VI/V$ containing an additional four LE modules comprising the complete domain V of these chains [33,34]. This allowed the efficient production and secretion (5–20 $\mu\text{g}/\text{ml}$ per day) of all four fragments, which could be readily purified to electrophoretic homogeneity (Figure 2A) by a combination of DEAE-cellulose, heparin and molecular-sieve chromatography. The purity was also confirmed by showing a single N-terminal Val-Val-Gly-Gln-Asp-His-Pro sequence for $\alpha 3BVI/V$ and an Arg-Thr-Pro-Gly-Gly-Asp sequence for $\alpha 5VI/V$; findings in agreement with the presence of a typical signal peptide. A single N-terminal Ser-Gln-Thr-Gln-Arg-Arg sequence was also found for $\alpha 2VI/V$, whereas the N-terminal sequence of $\alpha 1VI/V$ was blocked, suggesting cleavage of a Gln-Gln sequence followed by cyclization. This was verified by pyroglutamate aminopeptidase treatment, which liberated an Arg-Gly-Leu-Phe-Pro-Ala sequence, indicating that release of the signal peptide occurs through cleavage in front of the first or second glutamine residue.

Electron microscopy of $\alpha 2VI/V$ demonstrated a globular domain connected to a short rod (Figure 3A), consistent with its modular structure and the shape of the N-terminal regions of most laminins [1,2]. The same shape was shown previously for $\alpha 1VI/V$ [17]. A comparable globular domain was also demonstrated for $\alpha 3BVI/V$ (results not shown) and $\alpha 5VI/V$ (Figure 3B), whereas the rod was only infrequently visible, presumably because of its short length (approx. 7 nm) [3]. The proper folding and disulphide bond connectivity of the rods was therefore examined by pepsin digestion, since most laminin LE modules are pepsin-resistant, as in the laminin fragment P1 [26]. This was also the case for the fragments examined here, as shown by the persistence of fragments, which could be obtained as single or doublet bands in the range of 25–35 kDa after non-reducing electro-

Figure 4 Inhibition of the polymerization of laminin-1 by various N-terminal fragments from laminin α and $\beta 1$ chains

Inhibitors were added at equimolar concentrations (0.32 μM) or in 5-fold excess (1.6 μM) and their effects determined by inhibition RIA. Results are means $\pm$ S.D.

phoresis (Figure 2B). The same pattern was also seen after electrophoresis under reducing conditions (results not shown). All of these fragments started with a single N-terminal sequence, which was close to the C-terminal end of the LN modules (Figure 1) at positions 271 for $\alpha 1$, 277 for $\alpha 2$, 290 for $\alpha 3B$ and 299 for $\alpha 5$. Taken together, this demonstrated a lack of internal cleavage within disulphide loops, making the pepsin fragments good equivalents for domain V structures to be used in binding studies.

The LN modules were largely destroyed by pepsin. However, they showed a variable degree of resistance to neutral proteases, including the release of several fragments of 31–39 kDa starting close to the N-terminus. This confirmed the proper folding of these modules into globular structures as shown by electron microscopy. The various $\alpha VI/V$ fragments contained 3–11 residues of glucosamine, consistent with the partial occupation of 1–3 Asn-Xaa-Thr/Ser acceptor sites present in their sequence. Accordingly, bands of faster electrophoretic mobility were generated by treatment with N-glycosidase F in the presence of SDS (results not shown).

Inhibition of laminin polymerization

A major function of LN modules is apparently the promotion of self-assembly of laminins into non-covalent quasi-hexagonal networks in a Ca^{2+} -dependent fashion, as shown initially for laminin-1 (composition $\alpha 1\beta 1\gamma 1$) [12] and subsequently for $\alpha 2$ chain-containing laminins [13]. On the basis of previously established parameters of this polymerization [11–13], we have now developed a quantitative inhibition RIA to study the interference by the α chain fragments described in the present study. The assay was designed to allow the polymerization of 30–38% of laminin-1 (subsequently set to 100%), which was reduced to only a few per cent in the presence of EDTA (Figure 4). The inhibitors were then added in stoichiometric amounts (0.32 μM) or in 5-fold molar excess (1.6 μM) prior to starting polymerization. Low activity for $\alpha 1VI/V$ and $\alpha 2VI/V$ at 0.32 μM was observed, which exceeded 50% inhibition at 1.6 μM . $\alpha 3BVI/V$ and $\alpha 5VI/V$ fragments, however, were distinctly more

Table 1 Surface-plasmon-resonance assay of the binding of N-terminal fragments to laminin-1 and themselves k_{diss} , dissociation constant; k_{ass} , association constant.

Immobilized ligands	Soluble ligands	$k_{\text{diss}} \times 10^3$ (s^{-1})	$k_{\text{ass}} \times 10^{-3}$ ($\text{M}^{-1} \cdot \text{s}^{-1}$)	K_{d} (μM)
Laminin-1	$\alpha 1\text{VI}/\text{V}$	0.5	2.4	0.21
	$\alpha 2\text{VI}/\text{V}^*$	—	—	> 10
	$\alpha 3\text{BVI}/\text{V}$	0.18	7.5	0.024
	$\alpha 5\text{VI}/\text{V}$	0.32	4.6	0.069
	$\alpha 3\text{BV}\dagger$	—	—	> 1
$\alpha 3\text{BVI}/\text{V}$	$\alpha 1\text{VI}/\text{V}$	1.1	2.0	0.55
	$\alpha 3\text{BVI}/\text{V}$	12.5	4.2	3.0
	$\alpha 5\text{VI}/\text{V}$	9.0	3.6	2.5
$\gamma 1\text{VI}/\text{V}$	$\alpha 1\text{VI}/\text{V}$	13.0	2.1	6.2
	$\alpha 3\text{BVI}/\text{V}$	2.3	8.0	0.29
	$\alpha 5\text{VI}/\text{V}$	2.7	0.6	4.5

* no binding;

 $\dagger$ low uptake of RU.

active and, at $1.6 \mu\text{M}$, had effects that approached the level of inhibition induced by EDTA (Figure 4). As a further positive control we used the laminin E4 fragment corresponding to $\beta 1\text{VI}/\text{V}$, which has been used in previous inhibition studies [11], and found activity approximately identical with that of $\alpha 1\text{VI}/\text{V}$. The recombinant $\alpha 1\text{V}$ fragment [16] as well as the corresponding pepsin-resistant fragment $\alpha 5\text{V}$ caused only little inhibition at $1.6 \mu\text{M}$. Some higher activities were, however, found for the $\alpha 2\text{V}$ and $\alpha 3\text{BV}$ fragments. This clearly indicated that the LN modules of the $\alpha 1$, $\alpha 2$, $\alpha 3\text{B}$ and $\alpha 5$ chain fragments make major contributions to the inhibitory effects observed.

In addition, surface-plasmon-resonance assays were used to examine the binding potential of the N-terminal fragments of laminin-1 and recombinant fragments after their immobilization on the sensor chips (Table 1). This demonstrated a distinct binding of $\alpha 3\text{BVI}/\text{V}$ ($K_{\text{d}} = 0.024 \mu\text{M}$) to laminin, whereas 3–10-fold lower affinities were observed for $\alpha 5\text{VI}/\text{V}$ and $\alpha 1\text{VI}/\text{V}$. No binding was detected for $\alpha 2\text{VI}/\text{V}$ up to the highest concentration used ($4 \mu\text{M}$), indicating a K_{d} value higher than $10 \mu\text{M}$. Some weaker interactions were also detected between laminin fragments containing LN modules with immobilized $\alpha 3\text{BVI}/\text{V}$, which bound moderately to $\alpha 1\text{VI}/\text{V}$ ($K_{\text{d}} = 0.55 \mu\text{M}$) and also to $\alpha 3\text{BVI}/\text{V}$ and $\alpha 5\text{VI}/\text{V}$, with 5-fold lower affinity. A different pattern was, however, observed with immobilized $\gamma 1\text{VI}/\text{V}$ from the laminin $\gamma 1$ chain, which bound $\alpha 3\text{BVI}/\text{V}$ distinctly better than the homologous $\alpha 1$ and $\alpha 5$ chain fragments (Table 1). The pepsin-resistant fragment $\alpha 3\text{BV}$, however, showed a low uptake of RU, indicating a relatively high K_{d} value compared with that of $\alpha 3\text{BVI}/\text{V}$ (Table 1) despite a distinct activity in the inhibition assay (Figure 4).

Binding to heparin/heparan sulphate and sulphatides

Another major binding activity of LN modules from the $\alpha 1$ and $\alpha 2$ chains includes the binding to heparin and heparan sulphate [16–18]. These properties were examined by heparin affinity chromatography and demonstrate a strong binding of $\alpha 1\text{VI}/\text{V}$ and $\alpha 2\text{VI}/\text{V}$, which required 0.24–0.27 M NaCl for displacement. Binding of $\alpha 3\text{BVI}/\text{V}$ was apparently weaker (displacement at 0.14 M NaCl), whereas no binding was observed for $\alpha 5\text{VI}/\text{V}$ (Table 2). This binding activity could be attributed exclusively to the LN modules, since no binding was observed for the pepsin-resistant fragments $\alpha 1\text{V}$, $\alpha 2\text{V}$, $\alpha 3\text{BV}$ and $\alpha 5\text{V}$.

Table 2 Binding of N-terminal fragments of laminin α chains to heparin, heparan sulphate and sulphatides

Heparin binding was determined by affinity chromatography in 0.05 M Tris/HCl (pH 7.4) and binding strength denoted by NaCl concentrations required for displacement. Solid-phase assays with immobilized sulphatides and heparan sulphate-substituted perlecan fragment Vc were used to determine the concentrations for half-maximal binding (nM). No binding was observed with non-substituted perlecan fragment V ($> 1000 \text{ nM}$).

Fragment	Heparin (M NaCl)	Heparan sulphate (nM)	Sulphatides (nM)
$\alpha 1\text{VI}/\text{V}$	0.27	45	40
$\alpha 2\text{VI}/\text{V}$	0.24	200	20
$\alpha 3\text{BVI}/\text{V}$	0.14	250	50
$\alpha 5\text{VI}/\text{V}$	0	250	70

Surface-plasmon-resonance assays using biotinylated heparin immobilized on streptavidin chips were used as a second method to compare the affinity of binding, following correction for non-specific binding to the streptavidin carrier. Table 3 shows that $\alpha 1\text{VI}/\text{V}$ had the highest binding affinity for heparin ($K_{\text{d}} = 13 \text{ nM}$), whereas the other $\alpha \text{VI}/\text{V}$ fragments had 4–70-fold lower affinities, consistent with their strength of binding in affinity chromatography (Table 2). This also indicated that $\alpha 5\text{VI}/\text{V}$ can

Table 3 Surface-plasmon-resonance assay of the binding of N-terminal fragments to biotinylated heparin immobilized on streptavidin chips

Curves were corrected for non-specific binding to streptavidin chips in the range of 10% ($\alpha 1\text{VI}/\text{V}$, $\alpha 2\text{VI}/\text{V}$, $\alpha 1\text{LG4-5}$) to 30% ($\alpha 3\text{BVI}/\text{V}$, $\alpha 5\text{VI}/\text{V}$). Pepsin-resistant fragments $\alpha 1\text{V}$, $\alpha 2\text{V}$, $\alpha 3\text{BV}$ and $\alpha 5\text{V}$ showed the same low binding to heparin–streptavidin and streptavidin alone, indicating a $K_{\text{d}} > 5 \mu\text{M}$. k_{diss} , dissociation constant; k_{ass} , association constant.

Soluble ligand	$k_{\text{diss}} \times 10^3$ (s^{-1})	$k_{\text{ass}} \times 10^{-3}$ ($\text{M}^{-1} \cdot \text{s}^{-1}$)	K_{d} (nM)
$\alpha 1\text{VI}/\text{V}$	0.29	23.0	13 ± 2
$\alpha 2\text{VI}/\text{V}$	0.19	3.8	53 ± 6
$\alpha 3\text{BVI}/\text{V}$	0.90	5.0	185 ± 45
$\alpha 5\text{VI}/\text{V}$	1.90	2.2	863 ± 90
$\alpha 1\text{LG4-5}$	0.22	109.0	2 ± 1

Figure 5 Binding of recombinant laminin VI/V fragments to immobilized sulphatides in solid-phase assays

Soluble ligands were $\alpha 1\text{VI}/\text{V}$ (∇), $\alpha 2\text{VI}/\text{V}$ (Δ), $\alpha 3\text{BVI}/\text{V}$ ($\square$), $\alpha 5\text{VI}/\text{V}$ ($\circ$) and the pepsin-resistant domains V of $\alpha 3\text{B}$ ($\blacksquare$) and $\alpha 5$ ($\bullet$). No binding was also observed for $\alpha 1\text{V}$ and $\alpha 2\text{V}$.

Figure 6 Titration of antisera by ELISA

ELISA titration of rabbit antisera against fragments $\alpha 3\text{BVI/V}$ ($\circ$, $\triangle$) and $\alpha 5\text{VI/V}$ ($\bullet$, $\blacktriangle$). The antigens used were $\alpha 3\text{BVI/V}$ ($\circ$, $\bullet$) and $\alpha 5\text{VI/V}$ ($\triangle$, $\blacktriangle$). No binding for $\alpha 1\text{VI/V}$ and $\alpha 2\text{VI/V}$ was observed with both antisera.

bind to heparin at physiological ionic concentrations, although with low affinity. The strongest affinity, however, was observed for the C-terminal fragment $\alpha 1\text{LG4-5}$ ($K_d = 2 \text{ nM}$), indicating that it harbours the major heparin-binding epitope of the laminin $\alpha 1$ chain [27].

Figure 7 Immunofluorescence localization of laminin $\alpha 3$ chain on mouse tissues

Affinity-purified rabbit antibodies against fragments $\alpha 3\text{BVI/V}$ and $\alpha 3\text{AIIIa}$ were used to stain neonatal skin (1), adult oesophagus (2) and embryonic day-18.5 lung (3). Scale bar represents $50 \mu\text{m}$.

Figure 8 Double immunofluorescence staining of $\alpha 5\text{VI/V}$ and nidogen-1

Sections of neonatal mouse skin (1), adult mouse kidney (2) and adult skeletal muscle (3) were stained with rabbit anti- $\alpha 5\text{VI/V}$ sera and a rat monoclonal anti-(nidogen-1) antibody. Scale bar represents $50 \mu\text{m}$.

A perlecan fragment substituted with a single heparan sulphate chain [28] and sulphatides were used as further ligands in solid-phase binding assays. The results in Table 2 and Figure 5 show a distinct binding of all four α chain fragments, with only moderate differences in their relative activities. Pepsin treatment of $\alpha 3\text{BVI/V}$ and $\alpha 5\text{VI/V}$ was again found to destroy the binding activity (Figure 5, and results not shown).

Immunological analysis of tissues

Rabbit antisera were prepared against recombinant $\alpha 3\text{BVI/V}$ and $\alpha 5\text{VI/V}$ and, as determined by ELISA, had high titres against the antigens used for immunization, and were only marginally cross-reactive with fragments VI/V obtained from the other α chains (Figure 6). These antibodies were purified by affinity chromatography and used for immunofluorescence staining of various adult mouse tissues. Antisera against $\alpha 3\text{BVI/V}$ were compared with antibodies raised against recombinant $\alpha 3\text{AIIIa}$ (Figure 7), a domain shared by the short and long form of the laminin $\alpha 3$ chain [7]. Both showed a distinct staining in skin, particularly the basement membrane zones underneath the epidermis and around hair follicles. The antibodies also stained the basement membrane of the squamous-stratified epithelium of the oesophagus, and reacted more weakly with the underlying smooth muscle of the lamina muscularis mucosae as well as with capillaries. A distinct staining pattern was also observed in lung, including, in particular, alveolar and bronchial basement membranes and capillaries. Taken together, these data, and those

Figure 9 Immunoblotting evidence for a new assembly form laminin-5B

Immunoblotting was performed with anti- α 3BVI/V antibodies after SDS/PAGE under reducing conditions of immunoprecipitates obtained from mouse lung extracts with antibodies against various recombinant laminin fragments. The antibodies used for immunoprecipitation were: anti- α 3BVI/V (lane 1), anti- α 3AIIa (lane 2), anti- α 3LG4-5 (lane 3), anti- γ 2L4m (lane 4), anti- γ 2LE4-6 (lane 5), anti- β 3VI/V (lane 6), anti- γ 1VI/V (lane 7) and normal rabbit IgG (lane 8). The run was calibrated with laminin α 1 chain (α 1, 400 kDa) and myosin (200 kDa) and used for the estimation of apparent molecular masses of the blotted bands.

from several other tissues (results not shown), indicate a broad tissue distribution of the α 3BVI/V structure.

Antibodies against α 5VI/V were used in double staining with a monoclonal antibody against nidogen-1, which is a ubiquitous basement-membrane protein [32]. A distinct co-localization of both proteins was demonstrated in glomerular and tubular basement membranes of the mouse kidney and around capillaries (Figure 8). A similar co-localization was observed for basement membranes at the dermal-epidermal junctions and around hair follicles of skin. Nidogen-1, however, was also detected in restricted dermal regions and around capillaries (Figure 8). In skeletal muscle, staining for α 5VI/V was restricted to capillaries and some synapses, where it co-localized with nidogen-1. The laminin α 5 chain was, however, not detected along the muscle endomysium, which was strongly stained for nidogen-1.

Evidence for a new assembly form laminin-5B

The α 5 chain has been shown previously to associate with γ 1 and β 1 or β 2 chains to form laminins-10 and -11 [8]. To date, similar information is not available for the α 3B chain. We therefore used a lung detergent extract for immunoprecipitation with chain-specific anti-laminin antibodies, followed by immunoblotting with anti- α 3BVI/V sera (Figure 9). Two bands of 325 and 260 kDa were observed, which were specifically immunoprecipitated by antibodies against α 3BVI/V, α 3IIa, two γ 2 chain fragments and β 3 chain. These bands were not detected by using antibodies against the γ 1 chain or normal IgG (Figure 9, lanes 7 and 8). However, only the larger band was immunoprecipitated by antibodies against α 3LG4-5, which corresponds to the C-terminal end shared by the α 3B and α 3A chains [7]. This strongly indicated the chain composition α 3B β 3 γ 2 for the laminin present in the lung extract. This interpretation was further confirmed by immunoblotting for the β 3 and γ 2 chains, which revealed bands of 140 and 155 kDa respectively, corresponding to the non-processed forms [5,31] of these chains (results not shown).

DISCUSSION

The α 3B [7-9] and α 5 [4] chains are the largest laminin α chains identified in the mouse to date. Their N-terminal sequences were completed in the present study and the nature and release of the signal peptides verified by recombinant production. For the α 3B chain, this extended the sequence to 3333 residues, including a 31-residue signal peptide and rectified a previously incorrect prediction of the signal peptide cleavage site [9]. The addition of

83 more residues to the N-terminus of α 5 included a 40-residue signal peptide. Taken together with a 26-residue extension at the C-terminus followed by a stop codon found in an expressed sequence tag bank clone (AR 096946; H. Wizemann and R. Timpl, unpublished results), this completed the mouse α 5 chain sequence (3719 residues). It has also enabled the recombinant production of their N-terminal LN modules (domain VI) together with additional LE modules in order to allow proper folding and secretion. A major aim of the present study was, however, to examine the role of these modules for their potential to promote laminin self-assembly [12] and the binding to sulphated ligands, which could be modulators of such interactions [35,36].

A general concept for the supramolecular organization of basement membranes includes the formation of two independent networks of collagen IV and laminins, which are connected and stabilized by further interactions with nidogens, perlecan and some other proteins [1,12,37]. Such polymerization was initially shown for laminin-1 (α 1 β 1 γ 1) from the Engelbreth-Holm-Swarm tumour, which depended on a minimal concentration (0.1–0.2 μ M), moderate temperature and Ca^{2+} , and could be reversed by EDTA and cooling, demonstrating a non-covalent assembly process [38,39]. At high concentrations (> 1 μ M), this process leads to the formation of a quasi-hexagonal network, similar to those also found *in situ*, and involves the N-terminal globular domains of all three laminin-1 chains, as shown in inhibition and aggregation studies with proteolytic fragments [11,40–42]. It was shown further that α 2 chain-containing laminins-2 and -4 can both self-assemble and co-polymerize with laminin-1. Laminin-5 (α 3A β 3 γ 2) and -6 (α 3A β 1 γ 1), however, failed to form aggregates and to co-polymerize with laminin-1 [13]. Laminins containing the α 3B chain have not been properly identified to date, and no suitable source of laminins containing the α 5 chain has been available to provide amounts sufficient for polymerization studies.

Therefore we have used α 3BVI/V and α 5VI/V fragments in comparison with α 1VI/V and α 2VI/V to evaluate their activity in laminin-1 polymerization inhibition assays, similar to those used previously [11,41,42] as a measure of their potential for self-assembly. The present results demonstrate a high activity of α 3BVI/V and α 5VI/V, with 50–65% inhibition of laminin-1 polymerization being achieved at equimolar concentrations of laminin-1. However, a 5-fold molar excess was required for α 1VI/V, α 2VI/V and β 1VI/V to produce comparable effects, in agreement with previous data for similar α 1 and β 1 chain fragments [11,13,17,41]. However, the affinities of such interactions have been difficult to evaluate and a $K_d = 1.4 \mu\text{M}$ was estimated from equilibrium gel-filtration for the binding of β 1VI/V to a laminin fragment containing the LN modules of the α 1 and γ 1 chains [11]. In the present study, we have now used surface-plasmon-resonance assays to examine the kinetic parameters of such postulated hetero- and homo-typic interactions between LN modules. This showed that the K_d values for the binding to laminin-1 were in the range of 0.024–0.21 μM for α 3BVI/V, α 5VI/V and α 1VI/V, with a good correlation with their potency for polymerization inhibition. However, no binding activity could be detected for α 2VI/V ($K_d > 10 \mu\text{M}$) and α 3BV, a finding which cannot be explained at present. It may indicate the need of an activation step favoured by the longer incubation time and higher temperature in the inhibition assay. Binding was also demonstrated between individual N-terminal fragments with approx. 10-fold lower affinities. This suggests that their stronger binding to immobilized laminin may include up to two different LN modules, as postulated by the three-arm interaction model of Yurchenco and Cheng [11]. A total of nine LN modules have been identified so far in the known laminin isoforms [1,2]. On the

basis of our present results, it now seems feasible to examine their potential for self-assembly by using surface-plasmon-resonance assays to reveal new possibilities for laminin self-interactions.

The formation of quasi-hexagonal lattices upon laminin self-assembly has been primarily shown by electron microscopy and implicated the formation of ternary complexes, probably between different LN modules derived from three laminin molecules [11,12,40,42]. Whether additional LE modules from the adjacent rod-like domain V also play a role has not been examined to date. The inhibition data with the pepsin-resistant domain V fragments indicated little contribution to the binding of $\alpha 1$ and $\alpha 5$ chain fragments, but a higher activity for the $\alpha 2$ and $\alpha 3B$ chain fragments. In the latter case, the activity was actually equivalent to that of $\alpha 1VI/V$, $\alpha 2VI/V$ and $\beta 1VI/V$, indicating a distinct residual binding activity. This raises the interesting possibility that related N-terminal LE modules of the $\alpha 3A$, $\alpha 4$ and $\gamma 2$ chains, which lack LN modules, have similar binding activities. However, taken together, our present results confirm a major role of LN modules in this process, and now add laminins containing the $\alpha 5$ chain [8,15] in particular, and probably the $\alpha 3B$ chain, to the list of potential candidates for self-assembly. It is likely that the $\alpha 3B$ and $\alpha 5$ chains would also have the ability to co-polymerize with other laminin isoforms, as shown for laminins-1, -2 and -4, which contain either $\alpha 1$ or $\alpha 2$ chains.

Several other modulators of laminin-1 polymerization have been identified and include sulphatides, which enhance aggregation on phospholipid bilayers [35], and heparin, but not heparan sulphate chains, which cause compaction of laminin networks assembled *in vitro* [36]. These three ligands showed some variable binding patterns for the four α chain fragments, and the LN modules were identified as being exclusively responsible. Fragments $\alpha 1VI/V$, $\alpha 2VI/V$, $\alpha 3BVI/V$ and $\alpha 5VI/V$ showed comparable binding with sulphatides in solid-phase assays. This property had not been examined before and could serve to increase laminin concentrations in the vicinity of cells enabling efficient interactions with integrin and α -dystroglycan receptors. Affinity chromatography demonstrated a distinct binding of $\alpha 1VI/V$ and $\alpha 2VI/V$ to heparin at levels above physiological salt concentrations, as shown previously [16–18], and a more moderate binding of $\alpha 3BVI/V$. However, $\alpha 5VI/V$ did not bind to heparin in this assay. This is in contrast with another report [19] that showed strong binding ($K_d = 6$ nM) to biotinylated heparin in surface-plasmon-resonance assays. We have therefore repeated these kinetic assays and found a good correlation with the activities determined by affinity chromatography and only a low K_d (approx. $1 \mu M$) for $\alpha 5VI/V$. The discrepancy between both sets of data cannot be explained, but may be due in part to non-specific binding of such laminin fragments to the streptavidin carrier not observed before [19].

All four fragments showed some affinity for heparan sulphate on a perlecan fragment, indicating that the LN modules have the ability to integrate the major proteoglycan into basement membranes. Binding of all three sulphated ligands has been shown previously to be shared by several globular LG modules derived from the C-terminal ends of laminin $\alpha 1$ and $\alpha 2$ chains [27,43,44]. Furthermore, site-directed mutagenesis of LG modules demonstrated a crucial role of some basic residues for binding, and a considerable overlap between the binding epitopes for heparin and sulphatides [27,44]. Whether this is also the case for the LN modules can now be examined by mutagenesis of the fragments described in the present study. A restriction of heparin binding to a few basic residues of the $\alpha 5LN$ module has been reported recently [19].

The recombinant laminin fragments described in the present study were also useful in obtaining α chain-specific rabbit

antibodies for use in tissue analyses. Antibodies specific for the $\alpha 3B$ variant have not been available before and were shown to stain very intensively various basement membranes and capillaries. These patterns were indistinguishable from those obtained with antibodies against a central domain IIIa, which can not distinguish between the $\alpha 3B$ and $\alpha 3A$ splice variants. These findings are consistent with mRNA data [9], demonstrating the predominant or equivalent expression of $\alpha 3B$ transcripts when compared with $\alpha 3A$ in a large variety of human tissues. A similar staining of various basement membranes and capillaries was also observed with the antibodies against $\alpha 5VI/V$. The data obtained largely confirm those described previously with a monoclonal antibody and antibodies against $\alpha 5$ fusion proteins [8,45–47], highlighting the versatility of antibodies against recombinant and properly folded fragments to analyse laminin isoforms in biological samples.

The strong staining of lung for the $\alpha 3B$ splice variant also led us to examine its assembly form by using a combination of immunoprecipitation and immunoblotting. From these studies, the composition $\alpha 3B/\beta 3\gamma 2$ was clearly indicated (Figure 9). We propose to refer to this novel protein as laminin-5B, in order to distinguish it from the previously identified laminin-5, with the chain composition $\alpha 3A/\beta 3\gamma 2$ [5,48]. This laminin-5B was proteolytically modified by the release of the C-terminal LG4–5 modules, as observed for laminin-5 [48], whereas no processing could be detected for the $\gamma 2$ chain using antibodies against $\gamma 2L4m$ and $\gamma 2LE4–6$, which allow processed and non-processed forms to be distinguished [31]. The laminin $\alpha 3A$ chain is also known to assemble with $\beta 1\gamma 1$ and $\beta 2\gamma 1$ chains into laminin-6 and laminin-7 [48,49], but similar variants of $\alpha 3B$ could not be detected in the lung extract by immunoprecipitation with $\gamma 1$ -specific antibodies. As shown in the present study and indicated from previous findings [11–13], they may, however, be present in other tissues and should be ideal candidates for polymerization.

We are grateful to Mischa Reiter, Christa Wendt and Vera van Delden for expert technical assistance. The study was supported by grants of the Deutsche Forschungsgemeinschaft (project Ti 95/8-1) and European Community project QLK3-CT2000-00084.

REFERENCES

- 1 Colognato, H. and Yurchenco, P. D. (2000) Form and function: the laminin family of heterotrimers. *Dev. Dyn.* **218**, 213–234
- 2 Sasaki, T. and Timpl, R. (1999) Laminins. In *Guidebook to the Extracellular Matrix, Anchor and Adhesion Proteins* (Kreis, T. and Vale, R., eds.), pp. 434–443. Sambrook and Tootz, Oxford
- 3 Stetefeld, J., Mayer, U., Timpl, R. and Huber, R. (1996) Crystal structure of three consecutive laminin-type epidermal growth factor-like (LE) modules of laminin $\gamma 1$ chain harboring the nidogen binding site. *J. Mol. Biol.* **257**, 644–657
- 4 Miner, J. H., Lewis, R. M. and Sanes, J. R. (1995) Molecular cloning of a novel laminin chain, $\alpha 5$, and widespread expression in adult mouse tissues. *J. Biol. Chem.* **270**, 28523–28526
- 5 Marinkovich, M. P., Lunstrum, G. P. and Burgeson, R. E. (1992) The anchoring filament protein kalinin is synthesized and secreted as a high molecular weight precursor. *J. Biol. Chem.* **267**, 17900–17906
- 6 Ryan, M. C., Tizard, R., Van Devanter, D. R. and Carter, W. G. (1994) Cloning of the LamA3 gene encoding the $\alpha 3$ chain of the adhesive ligand epiligrin. Expression in wound repair. *J. Biol. Chem.* **269**, 22779–22787
- 7 Galliano, M.-F., Aberdam, D., Aguzzi, A., Ortonne, J. P. and Meneguzzi, G. (1995) Cloning and complete primary structure of the mouse laminin $\alpha 3$ chain. Distinct expression pattern of the laminin $\alpha 3A$ and $\alpha 3B$ chain isoforms. *J. Biol. Chem.* **270**, 21820–21826
- 8 Miner, J. H., Patton, B. L., Lentz, S. I., Gilbert, D. J., Snider, W. D., Jenkins, N. A., Copeland, N. G. and Sanes, J. R. (1997) The laminin α chains: expression, developmental transitions, and chromosomal locations of $\alpha 1$ – $\alpha 5$, identification of heterotrimeric laminins 8–11 and cloning of a novel $\alpha 3$ isoform. *J. Cell Biol.* **137**, 685–701

- 9 Doliana, R., Bellina, I., Bucciotti, F., Mongiat, M., Perris, R. and Colombatti, A. (1997) The human $\alpha 3b$ is a 'full-sized' laminin chain variant with a more widespread tissue expression than the truncated $\alpha 3a$. *FEBS Lett.* **417**, 65–70
- 10 Ferrigno, O., Virrolle, T., Galliano, M.-F., Chauvin, N., Ortonne, J. P., Meneguzzi, G. and Aberdam, D. (1997) Murine laminin $\alpha 3A$ and $\alpha 3B$ isoform chains are generated by usage of two promoters and alternative splicing. *J. Biol. Chem.* **272**, 20502–20507
- 11 Yurchenco, P. D. and Cheng, Y.-S. (1993) Self assembly and calcium-binding sites in laminin. A three-arm interaction model. *J. Biol. Chem.* **268**, 17286–17299
- 12 Yurchenco, P. D. (1994) Assembly of laminin and type IV collagen into basement membrane networks. In *Extracellular Matrix Assembly and Structure* (Yurchenco, P. D., Birk, D. E. and Mecham, R. P., eds.), pp. 351–388, Academic Press, San Diego
- 13 Cheng, Y.-S., Champlaud, M.-F., Burgeson, R. E., Marinkovich, M. P. and Yurchenco, P. D. (1997) Self-assembly of laminin isoforms. *J. Biol. Chem.* **272**, 31525–31532
- 14 Xu, H., Wu, X.-R., Wewer, U. M. and Engvall, E. (1994) Murine muscular dystrophy caused by a mutation in the laminin $\alpha 2$ (Lama2) gene. *Nat. Genet.* **8**, 297–301
- 15 Kikkawa, Y., Sanzen, N. and Sekiguchi, K. (1998) Isolation and characterization of laminin-10/11 secreted by human lung carcinoma cells. Laminin-10/11 mediates cell adhesion through integrin $\alpha 3\beta 1$. *J. Biol. Chem.* **273**, 15854–15859
- 16 Ettner, N., Göhring, W., Sasaki, T., Mann, K. and Timpl, R. (1998) The N-terminal globular domain of the laminin $\alpha 1$ chain binds to $\alpha 1\beta 1$ and $\alpha 2\beta 1$ integrins and the heparan sulfate-containing domains of perlecan. *FEBS Lett.* **430**, 217–221
- 17 Colognato-Pyke, H., O'Rear, J. J., Yamada, Y., Carbonetto, S., Cheng, Y.-S. and Yurchenco, P. D. (1995) Mapping of network-forming, heparin-binding, and $\alpha 1\beta 1$ integrin recognition sites within the α -chain short arm of laminin-1. *J. Biol. Chem.* **270**, 9398–9406
- 18 Colognato, H., MacGarrick, M., O'Rear, J. J. and Yurchenco, P. D. (1997) The laminin $\alpha 2$ -chain short arm mediates cell adhesion through both the $\alpha 1\beta 1$ and $\alpha 2\beta 1$ integrins. *J. Biol. Chem.* **272**, 29330–29336
- 19 Nielsen, P. K. and Yamada, Y. (2001) Identification of cell-binding sites on the laminin $\alpha 5$ N-terminal domain by site-directed mutagenesis. *J. Biol. Chem.* **276**, 10906–10912
- 20 Utani, A., Nomizu, M. and Yamada, Y. (1997) Fibulin-2 binds to the short arms of laminin-5 and laminin-1 via conserved amino acid sequences. *J. Biol. Chem.* **272**, 2814–2820
- 21 Hatzopoulos, A. K., Folkman, J., Vasile, E., Eiselen, G. K. and Rosenberg, R. D. (1998) Isolation and characterization of endothelial progenitor cells from mouse embryos. *Development* (Cambridge, U.K.) **125**, 1457–1468
- 22 Frohman, M. A., Dush, M. K. and Martin, G. R. (1988) Rapid production of full-length cDNAs from rare transcripts: amplification using a single gene-specific oligonucleotide primer. *Proc. Natl. Acad. Sci. U.S.A.* **85**, 8998–9002
- 23 Maruyama, I. N., Rakow, T. L. and Maruyama, H. I. (1995) cRACE: a simple method for identification of the 5' end of mRNAs. *Nucleic Acids Res.* **23**, 3796–3797
- 24 Kohfeldt, E., Maurer, P., Vannahme, C. and Timpl, R. (1997) Properties of the extracellular calcium binding module of the proteoglycan testican. *FEBS Lett.* **414**, 557–561
- 25 Engel, J. (1994) Electron microscopy of extracellular matrix components. *Methods Enzymol.* **245**, 469–488
- 26 Timpl, R., Paulsson, M., Dziadek, M. and Fujiwara, S. (1987) Basement membranes. *Methods Enzymol.* **145**, 363–391
- 27 Andac, Z., Sasaki, T., Mann, K., Brancaccio, A., Deutzmann, R. and Timpl, R. (1999) Analysis of heparin, α -dystroglycan and sulfatide binding to the G domain of laminin $\alpha 1$ chain by site-directed mutagenesis. *J. Mol. Biol.* **287**, 253–264
- 28 Friedrich, M. V. K., Göhring, W., Mörgelin, M., Brancaccio, A., David, G. and Timpl, R. (1999) Structural basis of glycosaminoglycan modification and of heterotypic interactions of perlecan domain V. *J. Mol. Biol.* **294**, 259–270
- 29 Göhring, W., Sasaki, T., Heldin, C.-H. and Timpl, R. (1998) Mapping of the binding of platelet-derived growth factor to distinct domains of the basement membrane proteins BM-40 and perlecan and distinction from the BM-40 collagen-binding epitope. *Eur. J. Biochem.* **255**, 60–66
- 30 Sasaki, T., Wiedemann, H., Matzner, M., Chu, M.-L. and Timpl, R. (1996) Expression of fibulin-2 by fibroblasts and deposition with fibronectin into a fibrillar matrix. *J. Cell Sci.* **109**, 2895–2904
- 31 Sasaki, T., Göhring, W., Mann, K., Brakebusch, C., Yamada, Y., Fässler, R. and Timpl, R. (2001) Short arm region of laminin-5 $\gamma 2$ chain: structure, mechanism of processing and binding to heparin and proteins. *J. Mol. Biol.* **314**, 751–763
- 32 Dziadek, M., Clements, R., Mitrangas, K., Reiter, H. and Fowler, K. (1988) Analysis of degradation of the basement membrane protein nidogen, using a specific monoclonal antibody. *Eur. J. Biochem.* **172**, 219–225
- 33 Sasaki, M., Kleinman, H. K., Huber, H., Deutzmann, R. and Yamada, Y. (1988) Laminin, a multidomain protein. The A chain has a unique globular domain and homology with the basement membrane proteoglycan and the laminin B chains. *J. Biol. Chem.* **263**, 16536–16544
- 34 Bernier, S. M., Utani, A., Sugiyama, S., Doi, T., Polistina, C. and Yamada, Y. (1994) Cloning and expression of laminin $\alpha 2$ chain (M-chain) in the mouse. *Matrix Biol.* **14**, 447–455
- 35 Kalb, E. and Engel, J. (1991) Binding and calcium-induced aggregation of laminin onto lipid bilayers. *J. Biol. Chem.* **266**, 19047–19052
- 36 Yurchenco, P. D., Cheng, Y. S. and Schittny, J. C. (1990) Heparin modulation of laminin polymerization. *J. Biol. Chem.* **265**, 3981–3991
- 37 Timpl, R. and Brown, J. C. (1996) Supramolecular assembly of basement membranes. *BioEssays* **18**, 123–132
- 38 Yurchenco, P. D., Tsilibary, E. C., Charonis, A. S. and Furthmayr, H. (1985) Laminin polymerization *in vitro*. Evidence for a two step assembly with domain specificity. *J. Biol. Chem.* **260**, 7636–7644
- 39 Paulsson, M. (1988) The role of Ca^{2+} binding in the self-aggregation of laminin-nidogen complexes. *J. Biol. Chem.* **263**, 5425–5430
- 40 Bruch, M., Landwehr, R. and Engel, J. (1989) Dissection of laminin by cathepsin G into its long-arm and short-arm structures and localization of regions involved in calcium dependent stabilization and self association. *Eur. J. Biochem.* **185**, 271–279
- 41 Schittny, J. C. and Yurchenco, P. D. (1990) Terminal short arm domains of basement membrane laminin are critical for its self-assembly. *J. Cell Biol.* **110**, 825–832
- 42 Yurchenco, P. D., Cheng, Y.-S. and Colognato, H. (1992) Laminin forms an independent network in basement membranes. *J. Cell Biol.* **117**, 1119–1133
- 43 Battaglia, C., Mayer, U., Aumailley, M. and Timpl, R. (1992) Basement membrane heparan sulfate proteoglycan binds to laminin by its heparan sulfate chains and to nidogen by sites in the protein core. *Eur. J. Biochem.* **208**, 359–366
- 44 Talts, J. F., Andac, Z., Göhring, W., Brancaccio, A. and Timpl, R. (1999) Binding of the G domains of laminin $\alpha 1$ and $\alpha 2$ chains and perlecan to heparin, sulfatides, α -dystroglycan and several extracellular matrix proteins. *EMBO J.* **18**, 863–870
- 45 Patton, B. L., Miner, J. H., Chiu, A. Y. and Sanes, J. R. (1997) Distribution and function of laminins in the neuromuscular system of developing, adult and mutant mice. *J. Cell Biol.* **139**, 1507–1521
- 46 Tiger, C. F., Champlaud, M.-F., Pedrosa-Domellof, F., Thornell, L.-E., Ekblom, P. and Gullberg, D. (1997) Presence of laminin $\alpha 5$ chain and lack of laminin $\alpha 1$ chain during human muscle development and in muscular dystrophies. *J. Biol. Chem.* **272**, 28590–28595
- 47 Sorokin, L., Pausch, F., Durbec, M. and Ekblom, M. (1997) Differential expression of five laminin α chains in developing and adult mouse kidney. *Dev. Dyn.* **210**, 446–462
- 48 Burgeson, R. E. (1996) Laminins in epidermal structures. In *The Laminins* (Ekblom, P. and Timpl, R., eds.), pp. 65–96, Harwood Academic Publisher, Amsterdam
- 49 Champlaud, M.-F., Lunstrum, G. P., Rousselle, P., Nishiyama, T., Keene, D. R. and Burgeson, R. E. (1996) Human amnion contains a novel laminin variant, laminin 6, covalently associates with laminin 5 to promote stable epithelial-stromal attachment. *J. Cell Biol.* **132**, 1189–1198