Structural analysis and proteolytic processing of recombinant G domain of mouse laminin α2 chain

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Abstract **Four individual LG modules from the C-terminus of the laminin α2 chain (LG1, LG2, LG4 and LG5) and combinations of these modules were prepared as recombinant products from transfected mammalian cells. This demonstrated that LG modules represent autonomously folding protein domains. Successful production depended on proper alignment of module borders and required a sequence correction at the C-terminus which added an extra cysteine. The LG modules were glycosylated and shown by electron microscopy to have a globular shape, indicating proper folding. Evidence is provided for the splicing of a 12 bp exon in LG2, although this did not impair folding. Proteolytic cleavage at the C-terminus of a basic sequence was observed close to the N-terminus of LG3. A similar processing also occurs in tissue-derived laminin-2 and -4 which contain the α2 chain.**

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1. Introduction

Laminins are major basement membrane proteins for which at least 11 isoforms exist. This diversity is mainly due to five different α chains (α1–α5) of 200–400 kDa which combine with smaller numbers of β and γ chains into disulfide-bonded heterotrimers [1]. A common hallmark of the laminin α chain structure is the presence of five laminin-type G domain (LG) modules arranged at the C-terminus in a tandem array. LG modules consist of 180–200 residues and have been identified in various extracellular proteins and receptors, although the sequence identity is generally low (20–30%). As well as being found in laminins, they also occur in the basement membrane proteoglycans perlecan [2] and agrin [3,4] and in less related proteins such as hormone-binding proteins [5], neurexin receptors [6] and proteins involved in *Drosophila* differentiation [7], indicating that they are involved in important functions. In laminins, modules LG1–LG5 form a large globular domain at the distal end of the long arm which appears to consist of subdomains when visualized by negative staining [8]. It has not yet been established whether individual LG modules are autonomously folding units or whether they need to be incorporated into larger structures for proper folding.

Potential functions of LG modules have been most extensively studied using laminin-1 (chain composition α1β1γ1). The laminin-1 LG modules bind to heparin, sulfatides, perlecan and fibulin-1 [9], as well as cellular receptors including α6β1, α7β1 and some other integrins [10], and α-dystroglycan [11]. Laminin-2 (α2β1γ1) and laminin-4 (α2β2γ1) are the iso-

forms most similar to laminin-1, and also show a broad tissue expression [12,13] but only a partially overlapping deposition at certain anatomical sites [14,15]. These α2 chain-containing laminins are particularly prominent in skeletal muscle and are directly or indirectly involved in several inherited or transgenic muscular dystrophies [1,16–18]. Despite such specific features, laminin-2 and laminin-4 are similar to laminin-1 in that they self-assemble into networks [19], mediate cell adhesion via β1 integrins, have a moderate affinity for heparin [20] and promote neurite outgrowth [21]. It therefore appears very likely that several of these activities are mediated by the LG modules of the laminin α2 chain.

Laminin-2 and -4 can be purified from tissues [20,22] but because of their large size and low yields they are not appropriate for structural and functional mapping studies. Recombinant production methods, as shown for the LG modules of the laminin α1 chain [23], seem to be more suitable. In the present study we have used established technologies to prepare individual LG modules and some larger fragments of the mouse laminin α2 chain in mammalian cells in order to ensure their proper folding and post-translational processing. The initial aim was to study module borders and folding properties and to examine proteolytic cleavage as indicated from tissue studies [20,24], but their successful production has also set the stage for binding studies.

2. Materials and methods

2.1. Vector construction and recombinant protein production

Mouse laminin α2 chain cDNA clone pmm 16 [13] was used as a template to amplify the sequence encoding the five LG modules individually or in combination (Table 1) by polymerase chain reaction (PCR) with Vent polymerase (New England Biolabs) following the manufacturer's instructions. The 5′ end primers used were GTCAGC-TAGCGGGAGGTGACTGTGTTC (LG1, LG1–3), GTCAGCTA-GCCAGCCCACAAGTCGAAGAT (LG2), GTCAGCTAGCTGTG-GAGCTTGCCGCTGT (LG3), GTCAGCTAGCGTCGGTGCCC-ACCCCTGC (LG4, LG4–5) and GTCAGCTAGCTGCGAATGCA-GAGAGTGGG (LG5.1, LG5.2). The 3′ end primers used were GT-CACTCGAGCTAGCTGACAGTACATCCCTTAC (LG1), GTCA-CTCGAGCTACTCCACAAAACCAGGCTTG (LG2), GTCACTC-GAGCTACGACTGAGGCTGGACAATA (LG3, LG1–3), GTCAC-TCGAGCTACGCAAAGCATGTCCCAA (LG4), GTCACTCGAG-TCAGTTCCAGGGCCTTGGC (LG5.1) and GTCACTCGAGT-TAGGTAGTCGGGCATGATAC (LG5.2, LG4–5). In addition to the coding sequences, these primers introduced a stop codon and single *Nhe*I and *Xho*I restriction sites in order to allow in-frame insertion of the cDNA distal to the BM-40 signal peptide sequence [25] present in the episomal expression vector pCEP-Pu [26]. The PCR products were initially ligated into plasmid pUC18 (Pharmacia) and sequences were verified on an 373A automated sequencer (Applied Biosystems). Since several sequence differences were found the original cDNA was also sequenced using the same primers. The PCR products were cut out by *Nhe*I and *Xho*I digestion and ligated into plasmid pCEP-Pu.

Human embryonic kidney cells 293 which constitutively express the

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EBNA-1 protein from Epstein-Barr virus (293-EBNA, Invitrogen) were transfected with the constructs [27] and transfectants were selected with 0.5 μg/ml puromycin (Sigma) and 250 μg/ml G418 (Gibco). Transfected cells were washed extensively with phosphate-buffered saline and grown in serum-free DMEM/F12 medium (Gibco) for 2 days after which medium was harvested and replaced by new serum-free medium for another 2 days.

2.2. Purification of proteins

Conditioned serum-free medium (1 l) was dialyzed against 50 mM Tris-HCl, pH 8.6, containing 2 mM EDTA, 0.5 mM phenylmethylsulfonyl fluoride (Serva) and 0.5 mM *N*-ethylmaleimide (Merck). Recombinant proteins were isolated on a DEAE-cellulose column (2×30 cm) equilibrated in the same buffer and eluted with a linear NaCl gradient (0–0.4 M, 500 ml). They were then purified on a Superose 12 column (HR 16/50, Pharmacia) equilibrated in 0.2 M ammonium acetate, pH 6.8, lyophilized and dissolved in 0.2 M NH_4HCO_3. Chromatography on heparin-Sepharose in 50 mM Tris-HCl pH 7.4 and elution with a salt gradient (0–0.5 M NaCl) was used for additional purification. Reverse phase chromatography in 0.1% trifluoroacetic acid was performed on C4 and C18 columns in order to purify the LG3 fragment and its CNBr peptides. Laminin-2 and laminin-4 were obtained from human placenta [20].

2.3. Analytical and miscellaneous methods

Protein and hexosamine concentrations were determined on a Biotronik LC 3000 amino acid analyzer after hydrolyzing samples with 6 M and 3 M HCl, respectively (110°C, 16 h). Amino acid sequences were determined by Edman degradation on an Applied Biosystems model 473 sequencer, following the manufacturer's instructions. Electrophoresis in SDS-polyacrylamide gradient gels followed established protocols. Electron microscopy of rotary shadowed protein samples was performed by a standard procedure [28]. MALDI mass spectra were obtained with a Bruker Reflex III time-of-flight mass spectrometer using 2,5-dihydroxybenzoic acid as matrix as previously described [29]. Total RNA was isolated as described [30] and fractionated by agarose gel electrophoresis. The fractionated RNA was transferred to Hybond N[+] membranes (Amersham) and hybridized to ^{32}P-oligolabeled cDNA clones encoding LG4–5 or LG1–3 in Church buffer at 65°C. The filters were subsequently washed in 0.02 M sodium phosphate solutions containing 5% SDS followed by 1% SDS for 2×1 h at 65°C before autoradiography. Immunization of rabbits with recombinant proteins (2×0.2 mg in complete Freund's adjuvant) and ELISA titration followed established procedures. Immunoblotting was carried out as described previously [31].

3. Results

The alignment of LG modules to determine module borders in non-interrupted arrays such as found in laminins meets with some difficulties due to variable module size and only moderate sequence identities [5,7]. Our choice for the laminin α2 chain is shown schematically in Fig. 1A and was based on two short conserved sequences and two cysteines close to the C-terminus which were rather invariant in all five laminin α chains. It also made use of the information that a single cys-

teine close to the N-terminus of the LG4 module is essential for the proper folding of the same module in the α1 chain (Z. Andac and R. Timpl, unpublished). The starting points of the slightly overlapping primers for PCR amplification (Table 1) were chosen accordingly. A comprehensive sequence analysis of the amplified products as well as of the parental cDNA clone revealed several differences to the previously published cDNA sequence of the mouse laminin α2 chain [13]. The most striking one was a 25 amino acid residue sequence including a 14 residue extension at the C-terminus (Fig. 1B), which added an extra cysteine and made the sequence more similar to that of the mouse laminin α1 chain [32]. The correctness of the novel sequence was demonstrated by recombinant analysis (see below). The other major change was the deletion of the sequence SMKA in module LG2 (positions 2476–2479 in the mouse). This sequence was identified in both the mouse and human laminin α2 chain [12,13] and, as discussed later, probably represents a splice variation. More minor differences include 17 single amino acid replacements based on single bp changes, 14 of which made the mouse sequence identical to the human sequence (see legend to Fig. 1B).

The successful expression of recombinant products was analyzed by electrophoresis of serum-free culture medium obtained from transfected cells. Strong bands of about the expected size were seen for the individual fragments LG1, LG2 and LG4 and the larger combinations LG1–3 and LG4–5. For LG5 it was important to use the primer 5.2 corresponding to the novel C-terminus as a construct with the more upstream primer 5.1 (Fig. 1B) was ineffective in recombinant protein production. In addition, fragment LG3 could not be obtained using the chosen borders (Table 1), even though LG3 is expressed in the larger fragment LG1–3, as discussed later. In order to exclude inefficient transfection as a cause of low protein production, we carried out Northern blotting of transfected cells with appropriate probes (Fig. 1C). This showed similar mRNA levels for LG3 and LG5 transfections, the latter with primer 5.1, when compared to the level of LG5 mRNA obtained with primer 5.2. This clearly demonstrated that factors other than mRNA levels contribute to low protein production.

The recombinant fragments were purified by a combination of ion exchange and molecular sieve chromatography, as well as heparin affinity chromatography where appropriate (fragments LG1–3, LG4–5 and LG5). The yields of purified products were 0.6–1.8 mg/l and their purity was examined by electrophoresis (Fig. 2). Fragments LG1, LG2, LG5 and LG4–5 showed a single major band which started at the expected N-terminal sequence as shown by Edman degradation (Table 1).

Table 1
Sequence borders and structural properties of recombinant LG modules of the mouse laminin α2 chain

Fragment[a]	Sequence positions	Molecular mass (kDa)		N-terminal sequence[d]
		calculated[b]	observed[c]	
LG1	2147–2327	19.9	29	GGDC
LG2	2327–2534	22.7	35	SPQV
LG4	2729–2932	22.2	37, 33	SVPXPA
LG5	2932–3118	20.0	26	ANAE
LG1–3	2147–2729	64.4	84, 60, 26	GGD
LG4–5	2729–3118	42.1	53	SVP

[a]The LG3 module (2534–2729) could not be obtained as a recombinant product.
[b]From amino acid sequence.
[c]From electrophoretic mobility.
[d]APLA derived from vector has been omitted.

A

B

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                            ←—5.1—     ←—5.2—
Mα2c 3088 KLTKGTGKPLEVNFAKALELRGVQPVSCPTT*
Mα1  3029 RLSRGS-QVQSLDLSRAFDLQGVFPHSCPGPEP*
Mα2  3088 KLTKGTANRWRLILPRPWN*
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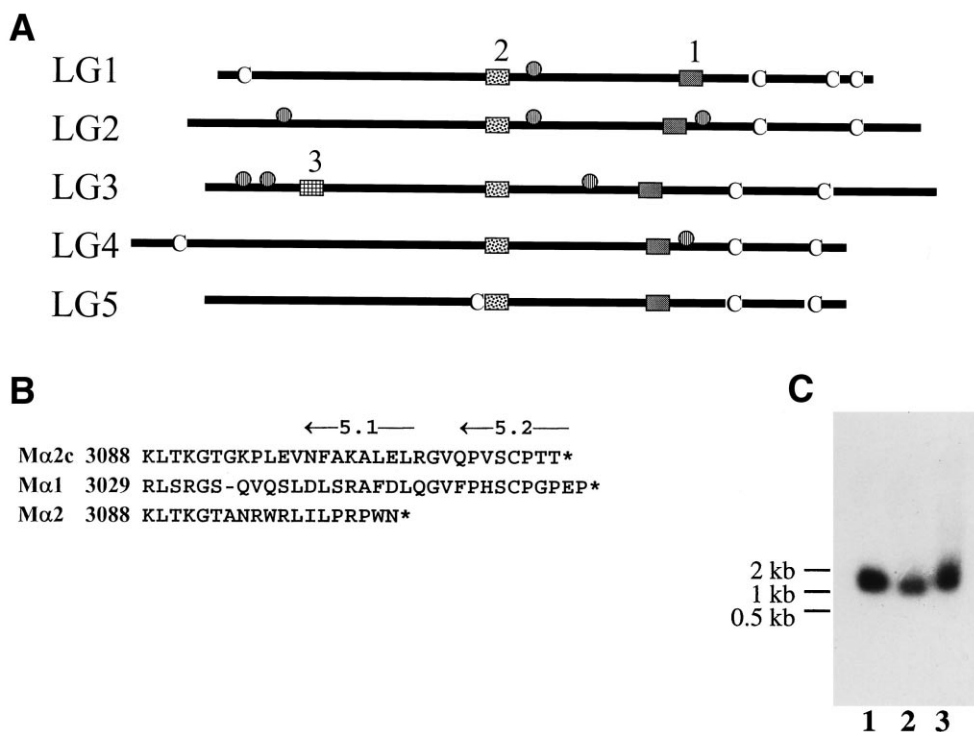
C

Fig. 1. Outline of critical parameters for the efficient recombinant production of LG modules of the mouse laminin α2 chain. A: Schematic alignment of the LG modules used to define their borders. The alignment is based on two rather conserved sequences (boxed and numbered on top) typically represented by FVGGLP (1) and DGXWHX (2). A further box (No. 3) in LG3 indicates a RRKRR sequence involved in proteolytic processing. C denotes positions of cysteines and circles potential N-glycosylation sites. B: Correction of the amino acid sequence at the C-terminus of the mouse LG5 module. The corrected sequence (Mα2c) is caused by insertion of a G between nt positions 9333 and 9334 of mouse α2 cDNA and is compared with the mouse laminin α1 chain (Mα1) and the previous [13] mouse α2 chain sequence (Mα2). Lines on top (5.1, 5.2) denote positions of oligonucleotide primers used in PCR (see Section 2). Numbers refer to N-terminal sequence positions and ignore the loss of a small exon in LG2 (see text). Further single amino acid changes which were identical to the human α2 chain sequence [12] were: in LG1, I2204 to W and GF2214/2215 to EY; in LG2, N2523Y; in LG3, I2642M; in LG4, V2773A, G2802A, G2810A, F2820Y, S2829D and G2878V; and in LG5, G2945A, I2953K and Y3022H. Three more changes (V2739A, R2831S, I2976V) did not correspond to the human sequence. All these changes were also present in the parental cDNA and have now been deposited to the GenBank (U12147). C: Examples of Northern blots showing the efficiency of 293-EBNA cell transfections. Lanes were loaded with 10 μg total RNA after transfection with LG5 using either primer 5.2 (lane 1) or 5.1 (lane 2) or after transfection with LG3 (lane 3). The size of the mRNA (about 1.2 kb) was as expected. Only the cells shown in lane 1 showed efficient protein production.

Their molecular masses, as estimated from their electrophoretic migration, were, however, distinctly higher than those calculated from the sequence (Table 1) which, apart from LG5, may in part be attributed to N-glycosylation. Fragment LG4 was produced as a double band (37 and 33 kDa), both of which started with the same N-terminus. They could be partially separated from each other and from some aggregated material by molecular sieve chromatography. Fragment LG1–3 consistently appeared as a 60 kDa fragment starting with the expected N-terminal sequence and a 26 kDa fragment with an apparently blocked N-terminus. An uncleaved variant (about 84 kDa) was only barely visible. Electron microscopy of individual LG modules showed uniformly small globular structures as illustrated for LG2 and LG4 (Fig. 3A,B). As expected, a somewhat bigger size was observed for fragments LG4–5 (Fig. 3C) and LG1–3 (Fig. 3D). This indicates close contacts between adjacent LG modules which cannot be resolved after rotary shadowing.

Hexosamine analyses demonstrated negligible galactosamine levels for individual LG modules (0.3–1.1 residues) but 4.3 residues for LG1–3 indicating some O-glycosylation of the LG3 module. N-Glycosylation was somewhat higher (2–5 residues in LG1, LG2, LG4 and LG4–5 and 14 residues in LG1–3). Yet these numbers are low when compared to

potential N-glycosylation sites (Fig. 1A), indicating either modification by mannose-rich oligosaccharides or incomplete substitution.

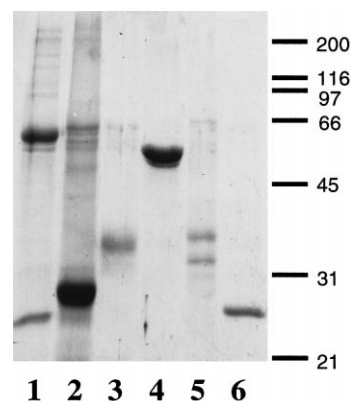The existence of two electrophoretic bands in fragment

Fig. 2. Electrophoretic comparison of purified recombinant fragments from the G domain of the laminin α2 chain. These fragments were LG1–3 (lane 1), LG1 (lane 2), LG2 (lane 3), LG4–5 (lane 4), LG4 (lane 5) and LG5 obtained with primer 5.2 (lane 6). Samples were reduced and the gel was calibrated with reduced markers, denoted in kDa.

LG1–3 in about stoichiometric proportions suggested endogenous proteolysis, probably close to the N-terminus of the LG3 module, which contains a unique basic sequence (Fig. 1A). This was of special interest since the $\alpha 2$ chain of tissue-derived laminin-2 and -4 was previously shown to consist of 300 and 80 kDa components [20]. The two bands of LG1–3 could not be separated from each other on a Superose 12 column, indicating non-covalent association. Separation was achieved at acidic pH by reverse phase chromatography, allowing the 26 kDa band to be obtained in pure form. Mass spectroscopy of this component showed a major signal of 19.7 kDa, which was smaller than the calculated mass for the entire LG3 module (22.1 kDa). Cleavage of the truncated fragment LG3 by CNBr should give rise to four fragments [13] (including I2642M, Fig. 1) with different molecular masses (in parentheses with position numbers): CB1 from the N-terminus of unpredicted size, CB2 (4348.5 Da; 2603–2642), CB3 (5348.2 Da; 2643–2691) and the C-terminal CB4 (4801.5 Da; 2692–2735). Edman degradation of the whole digest identified the sequences of CB2–CB4 but no additional N-terminal sequences. Mass spectroscopy demonstrated major signals of 4348 and 4802 Da, matching CB2 and CB4, while CB3 could not be detected, presumably because it was glycosylated. A third major signal of 3020 Da was seen, however, and this matched the calculated mass of 3020.5 of a more N-terminal sequence (2576–2602; starting with QTT), assuming the N-
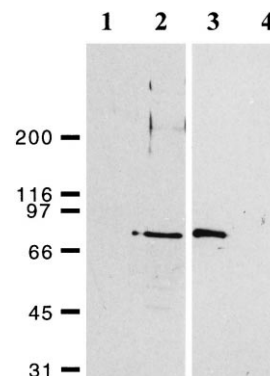
Fig. 4. Immunoblotting of laminins with antisera raised against the G domain of the mouse laminin $\alpha 2$ chain. The antisera were against fragments LG1–3 (lanes 1, 2) and LG4–5 (lanes 3, 4). A mixture of human placenta laminin-2 and -4 (lanes 2, 3) and of mouse laminin-1 (lanes 1, 4) was used at 0.5 μg/lane. Samples were reduced and the electrophoresis was calibrated with reduced marker proteins indicated in kDa in the left margin.

terminal Q has been converted to pyroglutamic acid and contains homoserine lactone at the C-terminus. This peptide CB1 had, as expected, a blocked N-terminus and started just adjacent to the cluster of basic amino acid residues. The calculated mass of the truncated fragment LG3 was therefore 17.6 kDa, indicating that the 2 kDa larger mass actually found (see above) is due to post-translational modifications.

For the further characterization of tissue forms of the laminin $\alpha 2$ chain, rabbit antisera were generated against mouse fragments LG1–3 and LG4–5 and both showed a high ELISA titer ($1:10^5$) for the antigen used in immunization. The cross-reaction between the two fragments and with corresponding $\alpha 1$ chain fragments of mouse laminin-1 (E3, E8) was at least 30-fold lower. However, both antisera showed a distinct cross-reaction with human laminin-2 and -4 which contain the $\alpha 2$ chain. Immunoblotting of these human laminins demonstrated staining of an 80 kDa band by the antiserum against LG4–5. The same staining was observed with the antiserum against LG1–3, but in addition a weaker reaction with a band of about 250–300 kDa was seen (Fig. 4). The same 80 kDa band could be also detected in detergent extracts of mouse muscle (data not shown). No reaction was observed with the laminin-1 $\alpha 1$ chain, underscoring the specificity of these antibodies (Fig. 4).

4. Discussion

The successful recombinant production of four LG modules in mammalian cells demonstrates that they represent autonomously folding units within the laminin $\alpha 2$ chain G domain. It is likely that this is the case for most if not all of the LG modules in the other four laminin α chains, since they are similar in length and module borders. The accurate selection of borders is crucial, as shown here by the need to correct the C-terminal end of the LG5 module. Module LG3 was not obtained in detectable amounts, which could reflect its vulnerability to proteolysis, a process which may be more extensive if the module is not protected by its non-covalent association with other LG modules as in fragment LG1–3. All LG modules showed a globular shape by electron microscopy, indicative of proper folding. In addition, they were modified mainly
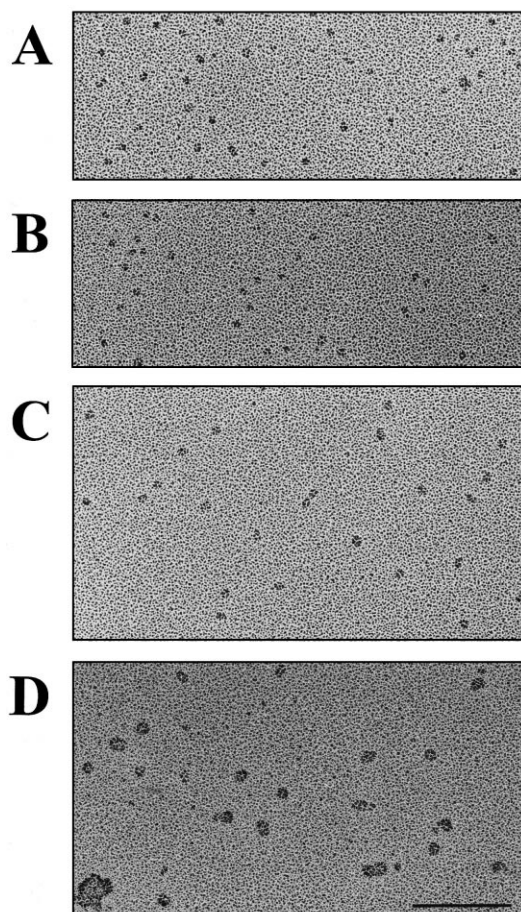

Fig. 3. Electron microscopy after rotary shadowing of recombinant LG fragments. A: LG2, B: LG4, C: LG4–5 and D: LG1–3. The magnification bar (100 nm) is representative for all sections.

by *N*-glycosylation, in accordance with the presence of potential acceptor sites.

Sequencing of the parental cDNA clone pmm 16 as well as of a partial PCR product revealed the deletion of an SMKA sequence in the LG2 module not found before for the cDNA sequence of human and mouse laminin α2 chain [12,13]. This deletion corresponds exactly to the 12 bp exon 52 of the human α2 chain gene [33], suggesting that this loss has occurred by mRNA splicing. This deletion does not seem to interfere with efficient folding of the corresponding recombinant fragment. Of particular interest, however, is that similar short deletions occur in two of the LG modules of agrin and are correlated with a loss of acetylcholine receptor clustering activity [4,34].

Recombinant fragment LG1–3 consistently showed an almost complete conversion into 60 and 26 kDa fragments by unknown proteases of the transfected mammalian cells. Mass spectroscopy has now been used to unequivocally localize the cleavage site to a unique sequence region RRKRRQT (positions 2571–2577) which is conserved in the human α2 chain [12] but not found in any other laminin α chain. Cleavage of the R–Q peptide bond is accompanied by cyclization of the Q, which then resists Edman degradation. The preceding sequence is similar to the basic (B) residue sequence motif BXBB, which represents a typical cleavage site for furin-like proteases that are involved in the conversion of precursor forms of various extracellular proteins [35] and is, for example, apparently cleaved in collagen XVI in order to release a globular domain [36]. The formation of a cyclic N-terminal Q is also characteristic of several procollagens and occurs after cleavage of the aminopropeptide by a specific protease [37] as a step preceding efficient fibril formation. The proteases involved in the conversion of the G domain of the laminin α2 chain still remain to be identified. A similar proteolytic cleavage has been reported for the laminin α3 chain and may occur between modules LG3 and LG4 [38]. This indicates that modification of the G domain exists in several laminin isoforms even though it may not involve the same cleavage sites.

The cleavage data obtained with the recombinant LG domains predicts the existence of a fragment consisting of LG4, LG5 and a large part of LG3 in α2 chain-containing laminins with a minimal molecular mass of 73 kDa based on electrophoresis (Table 1) and mass spectroscopy. This is in good agreement with the identification of an 80 kDa component found in purified laminins-2 and -4 [20], which in the present study reacted with antisera against LG4–5 and LG1–3. The latter antiserum also reacted with a 250–300 kDa component which should consist of the remaining part of the α2 chain including the LG1 and LG2 modules. Comparable results were reported for antibodies raised against the C-terminal part of the G domain of the α2 chain and synthetic peptides derived from the LG2 module [24]. Together, the data indicate that the cleavage identified here for LG1–3 also occurs in tissue-derived laminins. This cleavage, however, does not cause the dissociation of the 80 kDa component from laminin [20]. Whether this has functional consequences remains to be studied in further recombinant experiments.

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