Mutation of a basic sequence in the laminin α2LG3 module leads to a lack of proteolytic processing and has different effects on β1 integrin-mediated cell adhesion and α-dystroglycan binding

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Abstract A RRKRRQ sequence unique to the LG3 module of the laminin α2 chain was previously shown to be sensitive to endogenous proteolysis during the recombinant production of the tandem array α2LG1-3. Mutation of RQ surrounding the cleaved peptide bond did not prevent this processing and intracellular degradation. Alanine mutagenesis of three alternate basic residues, however, was shown to prevent the cleavage in α2LG1-3, allowing for the α2LG3 module to be obtained as a folded, globular fragment. The mutation did not change heparin and sulfatide binding or cell adhesion of α2LG1-3 which can be mediated by α3β1 and α6β1 integrins. It did, however, cause a 10-fold reduction in α-dystroglycan binding. The data favor the interpretation that binding epitopes for heparin/sulfatides, β1 integrins and α-dystroglycan occupy different parts of the α2LG1-3 structure.

Key words: Basement membrane; Cell receptor; Proteolysis; Recombinant protein; Site-directed mutagenesis

1. Introduction

Laminins are abundant basement membrane proteins which assemble into heterotrimers (αβγ) and are involved in cellular interactions and in the supramolecular organization of large extracellular structures [1]. They exist as at least 11 different isoforms based on five α chains, α1–α5, which have as a characteristic feature a large globular G domain (about 1000 residues) at their C-terminal end. This G domain consists of a tandem array of five LG modules with moderate sequence identities (20–30%) and similar modules have been identified in several other extracellular and transmembrane proteins [1,2]. The major functions of the LG modules of laminins are considered to be the promotion of cell adhesion through integrins [3] and α-dystroglycan [4] and the binding of heparin, sulfatides and several extracellular matrix ligands [5–8]. This has led to several recombinant studies in mammalian and insect cells in order to analyze structure-function relationships [7–11]. The data demonstrated that LG modules represent autonomous folding units [9,11]. Furthermore, similar binding functions identified for laminin α1 and α2 chains are located on different modules such as α1LG4 and α2LG5 [8].

In previous studies, we have prepared the individual laminin α2 LG modules α2LG1, α2LG2, α2LG4 and α2LG5 as well as the tandem arrays α2LG1-3 and α2LG4-5 by recombinant production in mammalian cells [9]. Functional analyses demonstrated that heparin and sulfatide binding are shared by several but not all LG modules, while binding to α-dystroglycan required tandem arrays [8]. Previous data for α2 chain-containing laminins derived from tissues have also indicated that limited proteolysis takes place and in the recombinant fragment α2LG1-3, the cleavage site could be located to a unique sequence of five basic residues joined to a glutamine in the α2LG3 module [9]. The two fragments of α2LG1-3 remained non-covalently associated. The same cleavage may have occurred in the recombinant production of α2LG3 where it apparently initiated a complete intracellular destruction of the module.

In the present study, we have used site-directed mutagenesis of the protease cleavage region of α2LG3 in order to study requirements for endogenous proteolysis as well as the effects on binding activities. This demonstrated that proteolysis could be abolished by changing the basic nature of this sequence but not by changing the two residues which contribute to the natural cleavage site. Lack of cleavage had no effect on β1 integrin-mediated cell adhesion to α2LG1-3 but distinctly reduced α-dystroglycan binding.

2. Materials and methods

2.1. Vector construction and recombinant protein production

The mouse laminin α2 chain LG1-3 domain construct [9] was used as a template for the construction of vectors encoding mutants of the protease-sensitive site in α2LG3. Two different mutations, 1 (introducing the amino acid changes R2571A, K2573A and R2575A) and 2 (introducing the amino acid changes R2575A and Q2576A), were produced. Site-directed mutagenesis was accomplished by overlap extension PCR with Vent Polymerase. In order to introduce mutation 1, the 5′ mutational primer ACACCACCCGCGAGAGCACGGGCACAAACCACA was used with the α2LG3 3′ primer and the 3′ mutational primer TGTGGTTTGTGCCCGTGCTCTCGCGGGTGGTGT was used together with the 5′ primer for α2LG3 or α2LG1 [9] to produce α2LG3 mutant 1 or α2LG1-3 mutant 1, respectively. The overlapping PCR products were annealed and PCR was used to extend them to their full length. For mutant 2, the same strategy was used. Mutations were introduced into the template using the primers AGAAAACGGGCAGCAACCACACAG and CTGTGTGGTTGCTGCCCGTTTTCT in addition to the terminal primers for α2LG3. PCR products were verified by sequencing and ligated into plasmid pCEP/pu [14] as described [9]. Human embryonic kidney cells 293 which constitutively express the EBNA-1 protein from Epstein-Barr virus (293-EBNA, Invitrogen) were transfected with the constructs. Transfectants were selected with 0.5 μg/ml puromycin (Sigma) and 250 μg/ml G418 (Gibco), washed extensively in phosphate-buffered saline and grown in serum-free DMEM/F12 medium (Gibco) for 2 days after which medium was harvested and new serum-free medium added for another 2 days.

2.2. Purification and sources of proteins

Conditioned serum-free medium of α2LG mutants was dialyzed against 50 mM Tris-HCl, pH 7.4, containing phenylmethylsulfonyl fluoride (Serva) and *N*-ethylmaleimide (Merck). Recombinant proteins were isolated on a heparin affinity column (2×30 cm) equili-

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brated in the same buffer. Proteins were eluted with a linear NaCl gradient (0–0.6 M, 500 ml). After purification on a Superose-12 column (HR 16/50, Pharmacia) equilibrated in 0.2 M ammonium acetate, pH 6.8, they were lyophilized and dissolved in 0.2 M NH_4HCO_3. Other α2LG fragments were those previously described [9]. Chicken kidney α-dystroglycan was kindly supplied by A. Brancaccio [15,16]. Heparin coupled to bovine serum albumin and bovine brain sulfatides were from a commercial source (Sigma).

2.3. Analytical and binding assays

Protein samples were hydrolyzed with 6 M HCl (16 h, 110°C) in order to determine their concentrations on a Biotronik LC3000 amino acid analyzer. N-terminal sequencing and rotary shadowing electron microscopy followed established protocols. Solid phase binding assays with immobilized heparin-albumin, sulfatides and α-dystroglycan and analytical heparin affinity chromatography were according to previously used methods [8]. Cell adhesion assays based on crystal violet staining and colorimetry [17] were carried out with cell lines previously shown to adhere to α2 chain-containing laminins [13] but also included C2C12 muscle cells provided by U. Mayer. Inhibitors were added to the cells prior to seeding them onto the substrates. Inhibitory monoclonal antibodies to human integrin subunits were those used previously [13].

3. Results

The α2LG3 module of the mouse and human laminin α2 chain [18,19] contains a unique basic sequence RRKRRQ (position 2571–2576) which was shown to be completely cleaved at the RQ site by an unknown protease in the recombinant production of the tandem array α2LG1-3 in mammalian cells [9]. This cleavage was apparently also responsible for the failure to obtain α2LG3 in a recombinant form. This cleavage region was now studied by site-directed mutagenesis (Fig. 1A) in order to replace three basic residues by alanines (mutant 1) or to change the cleavage site to an Ala-Ala sequence (mutant 2). The mutations were introduced into expression vectors for α2LG1-3 and/or α2LG3 and all gave rise to high levels of specific mRNA in transfected human EBNA-293 cells (Fig. 1B). Analysis of serum-free culture medium by SDS-gel electrophoresis was used to identify production of the recombinant proteins as indicated in Fig. 1A. This is illustrated for α2LG3 and its mutants and showed only for mutant 1 the expected band of 34 kDa (Fig. 1C, lane 3) when compared to medium of non-transfected cells (Fig. 1C, lane 1). Medium of α2LG3 mutant 2 showed instead two weaker extra bands of 30 and 26 kDa (Fig. 1C, lane 4). These two weak bands both started with an AATTQAYY sequence, demonstrating cleavage of the novel Arg-Ala bond in mutant 2. This is then very likely followed by extensive degradation of the recombinant product.

Mutant 1 variants of α2LG1-3 and α2LG3 could be efficiently purified by heparin and molecular sieve chromatography, as shown by electrophoresis (Fig. 1D). They started with a single APLAGGD (α2LG1-3, position 2147) or APLAVE-

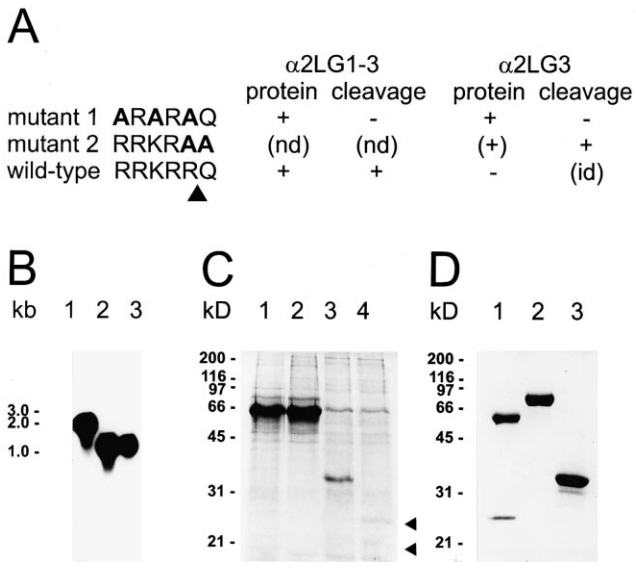

Fig. 1. Mutations in the protease-sensitive region of the laminin α2LG3 module and their effects on recombinant production. A: The mutations were introduced in a basic hexapeptide region (position 2571–2576 of the mouse laminin α2 chain) and are shown in bold. An arrowhead marks the cleavage site in the wild-type sequence. Both mutations were introduced in episomal expression vectors encoding the tandem array α2LG1-3 and mutation 1 also in the α2LG3 module alone. Their effects on protein production and protease cleavage are schematically outlined. Probable intracellular degradation is denoted by (id). One of the potential vectors was not done (nd). The data for the wild-type are from [9]. B: Northern blots show the efficiency of 293-EBNA cell transfections with vectors for α2LG1-3 mutant 1 (lane 1), α2LG3 mutant 1 (lane 2) and α2LG3 mutant 2 (lane 3). C: SDS-gel electrophoresis of serum-free medium from non-transfected EBNA-293 cells (lane 1) and cells transfected with α2LG3 (lane 2) or its mutants 1 (lane 3) and 2 (lane 4). Only lane 3 showed a characteristic 34 kDa band. Two weak bands in lane 4 (marked by arrowheads) were shown to be fragments of α2LG3. D: SDS-gel electrophoresis of purified recombinant wild-type α2LG1-3 (lane 1), α2LG1-3 mutant 1 (lane 2) and α2LG3 mutant 1 (lane 3).

LA (α2LG3, position 2533) sequence, with APLA being derived from the signal peptide cleavage region of the expression vectors [14]. Rotary shadowing electron microscopy of both mutants demonstrated a rather uniform population of globular particles which, as expected, differed in size (Fig. 2). This strongly indicated that the mutation has not interfered with proper folding of the proteins.

The α2LG1-3 and α2LG3 mutants 1 were also compared with the processed α2LG1-3 (see Fig. 1D) in various binding assays (Table 1). In analytical heparin affinity chromatography, the mutants eluted from the column at a lower ionic strength (0.2 M NaCl) than α2LG1-3 (0.36 M NaCl). In solid

Table 1
Effect of mutations in the protease-sensitive region of α2LG3 on binding to immobilized ligands

Soluble ligands	Heparin		Sulfatides (nM)[c]	α-Dystroglycan (nM)[c]
	(M NaCl)[a]	(nM)[b]		
α2LG1-3	0.36	50	60	25
α2LG1-3 mutant 1	0.20	110	110	150
α2LG3 mutant 1	0.19	80	80	300

[a]Heparin affinity chromatography in 0.05 M Tris-HCl, pH 7.4, showing the NaCl concentration required for displacement.
[b]Solid phase assays with immobilized heparin-albumin conjugate recording the concentrations required for half-maximal binding.
[c]Half-maximal binding to immobilized sulfatides and α-dystroglycan.

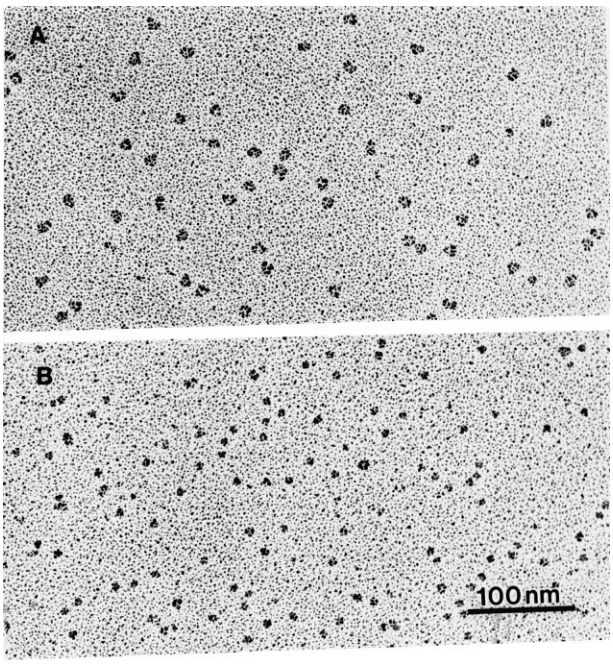

Fig. 2. Electron micrographs of rotary-shadowed α2LG1-3 mutant 1 (A) and α2LG3 mutant 1 (B). Note the rather uniform globular structures which differ in size. Bar: 100 nm.

phase binding assays carried out at physiological ionic strength, however, only marginal differences were observed in the binding of the three components to immobilized heparin-albumin conjugate and to sulfatides. A clear 6–12-fold reduction in binding affinity for α-dystroglycan was observed for both mutants.

Human placental laminin-2 and -4, which contain the α2 chain, were previously shown to promote strong adhesion of HBL-100, HT1080 and Rugli cells mediated through β1 integrins [13]. These and some other cell lines were now used to analyze the adhesive properties of various recombinant LG modules of the mouse laminin α2 chain. As shown for human HBL-100 cells, fragments α2LG1-3 and α2LG1-3 mutant 1 showed nearly identical dose-response profiles to laminin-2/-4 (Fig. 3) and adhesion was followed by distinct cell spreading. No or only low adhesion was observed to all other fragments, including α2LG4-5 and individual LG modules. Similar activities were found for rat RN22 and Rugli tumor cells and for the muscle cell line C2C12. Fragment α2LG1-3 was, however, only a poor substrate for human HT1080 cells. HBL-100 cell adhesion to α2LG1-3 was also examined in the presence of blocking monoclonal antibodies against human integrin subunits (Fig. 4). Nearly complete inhibition was observed with anti-β1 antibodies but only partial inhibition with antibodies against α3 and α6 subunits. Furthermore, a synthetic GRGDS peptide was non-inhibitory up to 0.4 mM for the adhesion of Rugli, RN22 and C2C12 cells to α2LG1-3. No inhibition was observed for heparin (1 mg/ml) in assays with HBL-100, RN22 and Rugli cells.

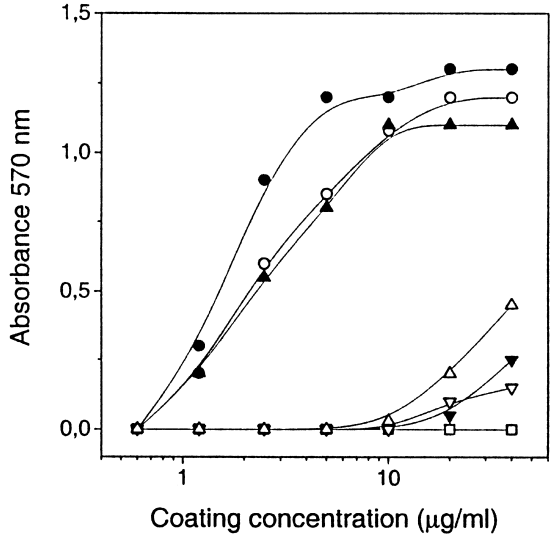

Fig. 3. Adhesion profiles of human HBL-100 cells to α2 chain-containing laminins and recombinant fragments of the G domain. Substrates used were a mixture of laminin-2 and -4 (●) and fragments α2LG1-3 (○), α2LG1-3 mutant 1 (▲), α2LG4-5 (△), α2LG1 (▼), α2LG2 (△) and α2LG3 mutant 1 (□).

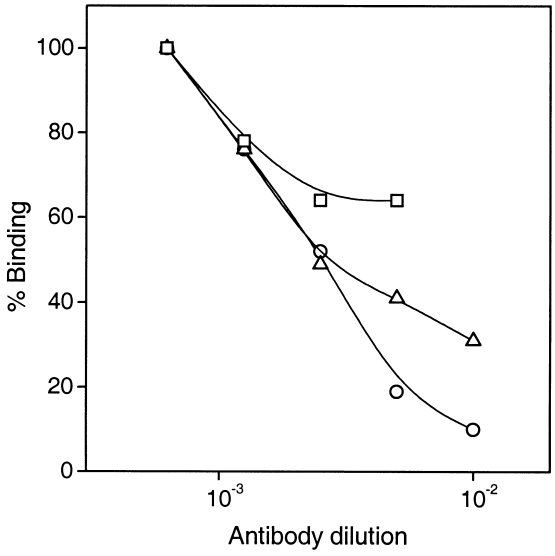

Fig. 4. Inhibition of adhesion of HBL-100 cells to integrin subunits β1 (○), α3 (△) and α6 (□) by monoclonal antibodies. They were used as hybridoma media at the dilutions indicated.

4. Discussion

Among the 11 laminin isoforms identified so far, only a few of them are known to undergo limited proteolysis during or after assembly and secretion. Laminin-5 (chain composition α3β3γ2) is cleaved at several sites to allow for integration into anchoring filaments [20] and cleavage apparently occurs at a single site in the α2 chain of laminin-2 and -4 [12,13]. The latter was localized to an Arg-Gln bond prefixed by a stretch of basic residues and caused the cyclization of the liberated Gln. This indicated that this processing involved either furin-type proteases requiring basic motifs or a procollagen peptidase acting on Gln [9]. The mutational data shown here demonstrated that an uninterrupted basic sequence determines cleavage and not the presence of a C-terminal Gln which can be replaced by Ala. The minimum basic sequence for furin cleavage is BXBB [21] and further studies will be needed to show whether this is sufficient for α2LG3 conversion. The

crystal structure of the α2LG5 module has recently been determined and showed a compact β sandwich [22]. Homology modeling of α2LG3 onto this structure located the basic sequence to an extended loop between β strands D and E and is therefore well-exposed for cleavage. Mutation 1 also facilitated production of recombinant fragments α2LG1-3 and α2LG3 in a non-processed form and these were used to study the role of proteolytic cleavage and/or the basic sequence in various binding assays.

Mutation 1 had no effect on heparin/sulfatide binding in solid phase assays but lowered the salt concentration required to elute α2LG1-3 from heparin. Fragment α2LG1-3 has another heparin binding site on the α2LG1 module (which elutes at 0.14 M NaCl) [8] and it could well be that the mutation interfered with a cooperativity between LG1 and LG3 binding sites when present in a tandem array. The major binding site of the laminin α1 chain for heparin and sulfatides has been localized to the α1LG4 module and, as shown by mutations, both binding epitopes are nearly identical. The cellular receptor α-dystroglycan also binds to this epitope plus some other residues [11] and this is in agreement with the efficient inhibition of α-dystroglycan binding to α1LG4-5 by heparin. The latter inhibition was not observed for α-dystroglycan binding to recombinant α2LG1-3 and α2LG4-5, indicating that the binding epitopes on the α2 chain are substantially different from those on the α1 chain [8]. This interpretation is now further supported by a 10-fold decrease in binding affinity of α-dystroglycan by introducing the mutation 1 into α2LG3. This affinity is as low as that described for α1LG4 [8,11] and may be caused by a lack of proteolytic activation or directly by interference with the binding epitope through individual mutations.

Salt-extracted laminin-2 and -4 were previously shown to mediate strong cell adhesion via β1 integrins [13]. Our data indicate that adhesion of at least some cells is due to the α2LG1-3 structure. In the case of HBL-100 cells, the interaction is mediated through α3β1 and α6β1 integrins, as indicated by antibody inhibition. These two integrins are well-established laminin receptors [3] and have also been implicated in cell binding to placental laminin preparations obtained by limited pepsin digestion [23,24], which very likely contain substantial amounts of α2 chain laminins. Yet, adhesion of HT1080 cells to laminin-2 and -4 could not be inhibited by antibodies to α3 and α6 integrins [13], in agreement with their low adhesion to α2LG1-3. These cells were recently shown to bind to an N-terminal structure of the α2 chain through α1β1 and α2β1 integrins [25]. Another function ascribed to the C-terminal G domain of the laminin α2 chain is the neural targeting of *Mycobacterium leprae*, where it mediates through its LG modules binding to β4 integrins [10]. This implicates a second binding site within the α2LG1-5 structure for bacterial receptors. The β1 integrin binding site on α2LG1-3 could not be mapped to individual LG modules, but should not include heparin binding epitopes, as shown in inhibition assays.

We have also provided some initial evidence that the binding epitopes for β1 integrins and α-dystroglycan should be different, since mutation 1 modulates the latter activity but not cell adhesion. Both cellular receptors are important for early embryonic development, as shown in transgenic mice which lack the β1 integrin [26,27] or α-dystroglycan [28]. Furthermore, a lack of laminin α2 chain causes severe forms of muscular dystrophy [29,30]. A possible molecular explanation for the receptor deficiencies was also analyzed in homozygous (−/−) embryonic stem cells which demonstrated a crucial role for β1 integrins [31] and α-dystroglycan [32] in the correct formation of basement membranes either in teratomas or embryoid bodies. Based on these observations, we speculate on a distinct interplay of laminin α2LG modules with both receptor-types [33] with these interactions possibly also being modulated by limited proteolysis. Further mutational analysis and a precise measurement of binding affinities will be needed to further substantiate such possibilities.

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