Low nidogen affinity of laminin-5 can be attributed to two serine residues in EGF-like motif γ2III4

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Received 2 March 1995

Abstract **High affinity nidogen binding of laminin-1 (chain composition α1β1γ1) has been previously mapped to a single EGF-like motif γ1III4 of its γ1 chain. Two more isoforms, laminin-5 (α3β3γ2) and laminin-7 (α3β2γ1), show low and high binding activity, respectively, indicating that the γ2 chain is of low affinity. This was confirmed by recombinant production of the homologous EGF-like motif γ2III4 of the γ2 chain, which has a 100,000-fold lower binding activity than γ1III4. The crucial heptapeptide binding sequence Asn-Ile-Asp-Pro-Asn-Ala-Val of γ1III4 is modified in γ2III4 by replacing both the central Asn and Val by Ser. Changing these replacements to Asn and Val by site-directed mutagenesis enhanced the activity of γ2III4 to a level which was only 5-fold lower than that of γ1III4. Despite their high sequence identity (77%) motifs γ1III4 and γ2III4 were also shown to differ considerably in immunological epitopes. This indicates distinctly different functions for laminins which differ in the γ chain isoform.**

Key words: Basement membrane; Laminin γ2 chain; Nidogen binding; Site-directed mutagenesis

1. Introduction

The 150 kDa glycoprotein nidogen seems to play a central role in basement membrane assembly due to its binding potential for several laminins, collagen IV, perlecan and the fibulins [1, 2]. The ability of nidogen to form ternary or higher-order complexes with these ligands has led to the concept that it mediates the connection between networks formed separately by laminins and collagen IV and thus stabilizes the supramolecular organization of such extracellular matrices [3, 4]. This was recently underscored in studies with antibodies which block the nidogen binding site of laminin [5] and retard kidney tubulogenesis and lung branching when added to embryonic organ cultures [6]. Nidogen is therefore one of the mediators in these developmental processes, which depend on mesenchymal–epithelial interactions [7]. It is also now becoming increasingly clear that in many of these developmental and repair processes nidogen is contributed by the mesenchyme while certain laminin isoforms originate from the epithelium [8]. The place of their first interaction is then the extracellular space, which explains the inhibitory effects of antibodies.

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Abbreviations: PCR, polymerase chain reaction; IC_{50}, inhibitory capacity, 50%.

The nidogen binding site of laminin-1 has been mapped in proteolytic and recombinant studies to a single EGF-like motif, γ1III4, in the short arm of its γ1 chain [5, 9]. Such motifs consist of 50–60 residues and are predicted to form four disulfide-bonded loops (a to d) based on their homology to EGF and cysteine patterns [10]. Subsequent studies demonstrated that two non-contiguous regions in loops a and c of γ1III4 contribute to high affinity binding (K_d = 0.5nM) in a cooperative manner [11]. The contact region in loop a has been restricted to a heptapeptide sequence while that of loop c is not yet completely mapped. Studies with synthetic peptides and site-directed mutants also showed that only a few residues are crucial for binding and predicted that the laminin γ2 chain should have only a low binding activity.

The laminins represent a large protein family consisting of various α, β, γ chain heterotrimers with seven different isoforms identified so far [2, 12, 13]. Laminin-1, laminin-2 and laminin-4 (α1β1γ1; α2β1γ1; α2β2γ1) share the γ1 chain and high affinity nidogen binding [3, 14, 15]. Laminin-5, previously referred to as kalinin/nicein, however, has the chain composition α3β3γ2 and all chains show truncated short arm structures [16, 17]. It is found primarily in anchoring filaments of squameous epithelium where it is connected to laminin-6 (α3β1γ1) by disulfide bonds [18, 19]. Sequence analysis of the γ2 chain demonstrated an EGF-like motif γ2III4 which showed 77% identity to the nidogen binding motif γ1III4 [20, 21]. In the present study we have analyzed nidogen binding of laminin-5 and laminin-7 (α3β2γ1) and of recombinant EGF-like repeats possibly involved in interactions. This demonstrated that only two residues in γ2III4 of laminin-5 are responsible for low affinity.

2. Experimental

2.1. Sources of proteins

Human laminin-5 was purified by antibody affinity chromatography from SCC25 culture medium [16]. Laminin-7 was obtained from human amnion (M.F. Champliaud, G.P. Lunstrum, M.P. Marinkovich, P. Rouselle, T. Nishiyama, D.R. Keene, R.E. Burgeson, in preparation). Recombinant mouse nidogen and laminin-1 fragment P1 were those used previously [3]. Recombinant motif γ1III4 was purified from the culture medium of stably transfected 293 cell clones as described [5]. The same chromatographic procedures were used to purify γ2III4 and the γ2III4 mutant. SDS-polyacrylamide gel electrophoresis on a 16% gel made from a stock solution of 40% acrylamide, 1.3% bis-acrylamide followed standard protocols.

2.2. Expression vectors and cell transfections

The cDNA clone Kal66c, corresponding to a partial sequence of human laminin γ2 chain [21], was used to generate fragments for expression vectors by polymerase chain reaction (PCR) with Vent polym-

SSDI 0014-5793(95)00438-6

erase (New England Biolabs) according to the supplier's instruction. The following oligonucleotide primers, some with underlined mutations, were synthesized: primer 1,5′-GATCCCTAGCGCCCTGTCAATGCAACA; primer 2,5′-GATCCTCGAGCTAAGCTCGACACTTGTCTG; primer 3,5′-CAACAACAATGTGGATCCCAATGCCGTTGGGAATTGTGACCGGC; primer 4,5′-TGGGATCCACATTGTTG; primer 5,5′-GTAACCATTATAAGCTGC; and primer 6,5′-CCTTTCTCTCCACAGGTG.

Primers 1 and 2 were used to construct the vector encoding the γ2III4 motif (position 516–572 of γ2) and also introduced a 5′ *Nhe*I site and a 3′ stop codon and *Xho*I site for connecting the construct to the BM-40 signal peptide as previously described [5]. The 0.21 kb PCR product was purified, cut with *Nhe*I and *Xho*I and cloned into the PCis vector [22] which was modified with the signal peptide [5] to yield the expression vector pCMVγ2III4. Residues Ser^{526} and Ser^{528} encoded in this expression vector were mutated to Asn and Val, respectively, by a strategy applied previously [11]. Two products were generated by PCR using primers 4 and 6 and primers 3 and 5 and contained the mutations and a common overlapping neutral *Bam*HI site for identification of the correct clones. The purified products were fused by one cycle of denaturation, annealing and extension. The correct fusion product (0.4 kb) was amplified by PCR with primers 5 and 6, cut with *Hin*dIII and *Xho*I to 0.3 kb and cloned into corresponding sites of the episomal expression vector pCEP4 (Invitrogen), which possesses the hygromycin B resistance gene. The correct structure of the inserts was verified by DNA sequencing and restriction mapping.

Vector pCMVγ2III4 and plasmid pSV_2pac were used to cotransfect the embryonic kidney cell line 293 (American Type Culture Collection) and to produce stably transfected clones and serum-free medium for purification of γ2III4 [23]. Human kidney cells which constitutively express the EBNA-1 protein from Epstein–Barr virus (293-EBNA cells; Invitrogen) were grown according to the manufacturer's protocol. Transfection of the cells with pCEP4 plasmids [23] was followed by selection of resistant cells in the presence of 300 μg/ml medium of hygromycin B (Calbiochem). These cells were then grown to confluency and used to obtain serum-free culture medium.

2.3. Binding assays and immunological methods

A radioligand assay consisting of soluble recombinant nidogen and ^{125}I-labelled laminin-1 fragment P1 was used in competition tests for the determination of relative affinities based on IC_{50} (inhibitory capacity, 50%) values [3, 9, 14]. Direct binding was analyzed in solid-phase assays using one ligand in plastic-immobilized form and an antibody detection system for the soluble ligand [24]. A rabbit was immunized with three 0.2 mg doses of γ2III4 by subcutaneous injections with complete Freunds' adjuvant at intervals of 3–4 weeks. ELISA and immunoblotting followed routine protocols.

3. Results

A radiological competition assay involving nidogen and ^{125}I-labelled fragment P1 from mouse laminin-1 (chain composition α1β1γ1) was used to examine nidogen binding affinity of two novel laminin isoforms (Fig. 1). The interaction of nidogen and fragment P1 could be completely blocked by fragment P1 and its recombinant subfragment γ1III4, to which the entire activity has been attributed [5], with IC_{50} values in the range 0.06–0.1nM. Human laminin-7 (α3β2γ1) had a similar activity (IC_{50} = 0.15 nM) while laminin-5 (α3β3γ2) had an activity too low to be measured (IC_{50} > 20 nM). A more than 100-fold difference between laminin-5 and fragment P1 was also observed in a direct nidogen binding assay [3, 24] using both laminins in the immobilized form (data not shown).

Domain III of the γ2 chain of laminin-5 possesses an EGF-like motif γ2III4, with a high sequence identity (77%) to the nidogen-binding motif γ1III4 of the γ1 chain [20, 21]. There are, however, several substitutions in a heptapeptide sequence of disulfide-bonded loop a (Fig. 2) which has been shown to be crucial for binding [11]. We therefore prepared γ2III4 in recombinant form in an eukaryotic expression system which was previously shown to yield correctly folded EGF-like domains [5]. The purified fragment showed an electrophoretic mobility similar to that of γ1III4 indicating a molecular mass of about 7 kDa (Fig. 3A). With an IC_{50} = 13 μM, however, its affinity for nidogen was 100,000-fold lower than γ1III4 (Fig. 1). This affinity was even distinctly lower than that of synthetic heptapeptide NIDPNAV (IC_{50} = 0.4 μM) designed according to a γ1III4 sequence [11]. We therfore prepared a γ2III4 double mutant where Ser^{526} and Ser^{528} within the heptapeptide binding region were converted to Asn and Val, respectively, corresponding to the substitutions found in γ1III4 (Fig. 2). The recombinant mutant showed a comparable electrophoretic mobility to γ2III4 (Fig. 3B) but had a much higher nidogen affinity (IC_{50} = 0.4 nM), approaching that of γ1III4 (Fig. 1). Thus a very restricted change in the sequence is responsible for a considerable improvement in binding.

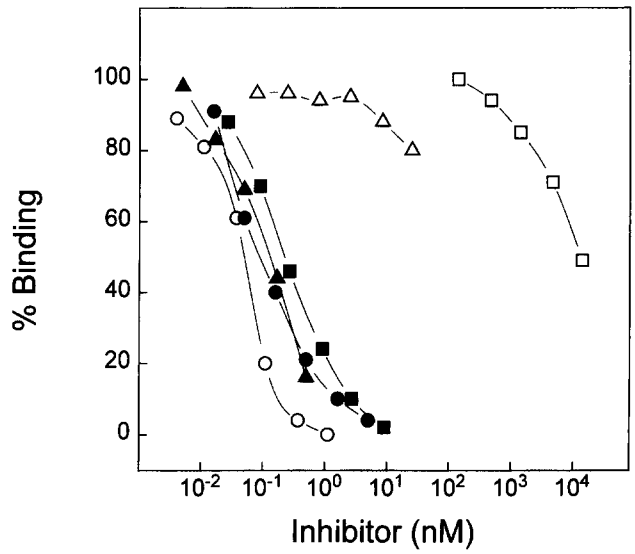

Fig. 1. Inhibition of radioligand binding between nidogen and laminin-1 fragment P1 by human laminins and recombinant EGF-like motifs. Inhibitors used were mouse fragment P1 (○) and its γ1III4 motif (●), human laminin-5 (△) and laminin-7 (▲), the γ2III4 motif of human laminin γ2 chain (□) and mutant γ2III4 (■) with Ser^{526} to Asn and Ser^{528} to Val substitutions.

It was also of interest to compare immunological epitopes of γ2III4 with those of γ1III4. An antiserum raised against γ2III4, showed a strong reaction with γ2III4 in ELISA but a 500-fold lower reaction with laminin fragment P1 or a recombinant fragment possessing the γ1III4 motif. Furthermore, in Western blots the antiserum bound primarily to a 105 kDa band of reduced laminin-5 and more weakly to a 155 kDa band (Fig. 3B). Since these two bands correspond to the precursor and processed forms of the γ2 chain [17], this demonstrates lack of cross-reaction with the α3 and β3 chains of laminin-5.

4. Discussion

The obvious importance of the laminin-nidogen interaction for basement membrane assembly, as indicated from in vitro and in vivo studies [1, 6], has raised the intriguing question of whether all laminin isoforms known so far [2, 12, 13] possess the same binding potential. A single binding site of high affinity

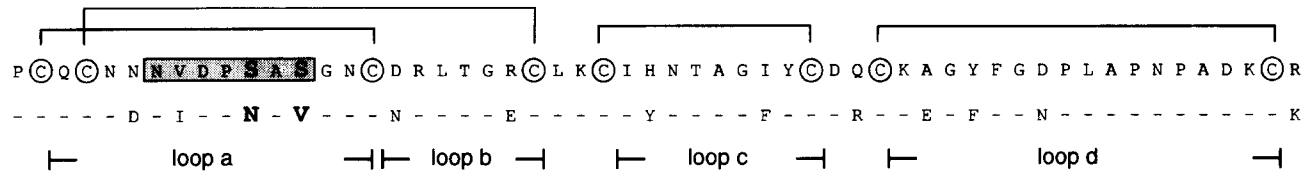

Fig. 2. Amino acid sequence of human laminin EGF-like motif γ2III4 (top) and comparison with the homologous nidogen binding motif γ1III4 (bottom) of mouse laminin. Only the substitutions are shown for γ1III4. The sequences correspond to positions 516–571 of the γ2 chain ([21]; including signal peptide) and to positions 792–847 of the γ1 chain [25]. The recombinant γ2III4 product possesses an additional APLA sequence derived from the signal peptide [5]. Loops a to d are indicated underneath the sequence. The shaded area in γ2III4 loop a indicates a related heptapeptide sequence involved in nidogen binding of γ1III4 [11]. Two substitutions in this region which are crucial for binding are shown in bold letters.

(K_d = 0.5 nM) has been localized to the short arm structure of laminin-1 (α1β1γ1)contributed by the γ1 chain [5, 9]. The same high affinity was previously shown for laminin-2 and laminin-4 [15] and in the present study for laminin-7. These laminins share the γ1 chain but differ in their α and/or β chains, indicating that chain composition is unimportant for nidogen binding, in agreement with the topological separation of the binding site from the other chains as shown by electron microscopy [26]. Additionally, the data predict that laminin-3 (α1β2γ1) and laminin-6 (α3β1γ1), which have not yet been studied, will also have a high affinity for nidogen.

Only a very low nidogen binding activity could be demonstrated for laminin-5 (α3β3γ2), apparently due to the replacement of the γ1 by the γ2 chain. It is very likely caused by the change of the nidogen binding EGF-like motif γ1III4 [5] to the homologous motif γ2III4, which show 77% sequence identity (Fig. 2). This identity is still remarkably higher than the 52–55% identity observed between other EGF-like motifs in domains III and V of both γ chains [20], suggesting a common ancestral motif for γ1III4 and γ2III4, which considerably changed its functional properties upon further divergence. Laminin-5 is a major component of anchoring filaments in squamous epithelium [19] and mutations in the β3 and γ2 chains are associated with blistering skin diseases [27–29]. Further data showed the formation of a disulfide-bonded complex between laminin-5 and laminin-6 [19], which could explain how such structures are connected to the lamina densa of basement membranes, for example by nidogen-mediated binding to laminin-6.

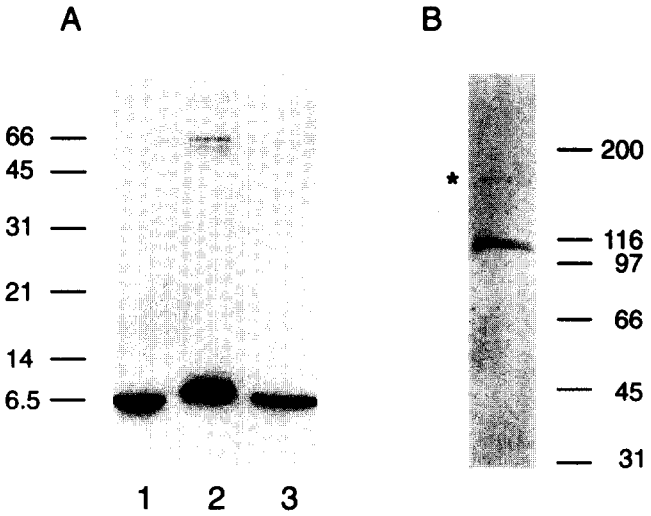

Fig. 3. SDS-gel electrophoresis of purified recombinant EGF-like motifs (A) and immunoblot reaction with an antiserum against γ2III4 (B). Samples in A were γ1III4 (lane 1), γ2III4 (lane 2) and γ2III4 mutant (lane 3). The blotted protein in B was laminin-5. The asterisk marks an additional faint reaction at about 155 kDa. All samples were reduced with 2-mercaptoethanol. Positions of molecular mass markers (in kDa) are indicated in the margins.

The nidogen binding site of mouse γ1III4 has been previously mapped to two restricted regions in the disulfide-bonded loops a and c by chimeric recombinant products, site-directed mutagenesis and synthetic peptides [11]. Cooperation between the two regions is required to result in high binding affinity although a heptapeptide sequence in loop a (see Fig. 2) makes the most critical contribution. Human γ2III4 differs in the heptapeptide region by three substitutions and in studies with synthetic γ1III4 variants, two of them, Asn to Ser and Val to Ser, were shown to inactivate binding [11]. This has now been clearly demonstrated by simultaneous site-directed mutation of both Ser in γ2III4 back to the γ1 chain substitutions, producing a γ2III4 mutant almost as active as γ1III4. Other residues crucial for binding are conserved in the heptapeptide region of γ2III4 (Asp, Ala) and in loop c where the important first Tyr residue is changed to the functionally equivalent His [11]. Therefore, only a few nucleotide substitutions give rise to a dramatic change of γ1 and γ2 chain function. The sequence of mouse laminin γ2 chain has recently available [30] and shows conservation of the last Ser in the heptapeptide region, while the first one is replaced by Asn as in γ1III4, which generates a novel N-glycosylation site, Occupation of this site by oligosaccharide may cause an inactive binding site, as presumably does the single Ser alone, as indicated from synthetic heptapeptide studies [11], although this has not yet been studied.

Acknowledgements: We would like to thank Mrs. Christa Wendt, Mrs. Mischa Reiter, Mrs. Heidi Alberty, Mrs. Christine Matzner and Mrs. Vera van Delden for expert technical assistance and Dr. C. Gorman for providing the pCis expression vector. The study was supported by grants from the Deutsche Forschungsgemeinschaft and USPHS Grant AR35689 (R.E.B.).

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